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Review

Photobiophysics and photobiochemistry of the heterogeneous phytochrome system

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Contents

Abbreviations: BR, bilirubin; BRh, bacteriorhodopsin; ΔA, difference absorption; ΔF, variable fluorescence; $\Delta\lambda$, halfband width; ϵ, absorption coefficient; E_a, activation energy; ϕ_F, fluorescence quantum yield; FR, far-red light; γ_1, γ_2, extent of the Pr phototransformation into lumi-R and Pfr at low and ambient temperatures, respectively; λ_a, λ_e, λ_m, wavelength of actinic, exciting and measured light; λ_{max}, position of maximum in spectrum; lumi-FR, lumi-R (I700, Lr), and meta-FR, meta-R (I_{bl}, P_{bl}), photoproducts and dark intermediates of the Pfr and Pr phototransformation, respectively; Lr_f, Lr_s, Lr_{vf}, subpopulations of Lr; P, phytochrome; P1 and P2, Type 1 and Type 2 P; phyA, phyB, phyC, phytochromes A, B and C; *phyA*, *phyB*, *phyC*, genes encoding for phyA, B, C; PHYA, PHYB, PHYC, apoproteins of phyA, B, C; PEC, phycoerythrocyanin; Pfr, far-red absorbing P form; Pr, red absorbing P form; Pr′, Pr″ and Pr_f, Pr_s, Pr_{vf}, Pr_{vs}, subpopulations of Pr; prelumi-R, unstable photoproduct, preceding lumi-R; R, red light; Rh, rhodopsin; τ_0, radiative lifetime; τ_F, fluorescence lifetime; UV, ultraviolet; VLFR, LFR, HIR, very low and low fluence responses and high irradiance responses.

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1. Introduction

Plants possess a highly elaborate and specialized apparatus for light perception independent of photosynthetic processes. It enables them to perceive quantity and quality of light, direction of its propagation and polarization in a more than 8 decade range of fluence rate and in a wide spectral region from UV-B to infra-red, the ability comparable with the light perception in animals. The apparatus comprises at least three photoinformation systems which are characterized by the interval of maximum sensitivity: UV-B, UV-A/blue and red/far-red. In the first two, photoprocesses are mediated by cryptochromes – flavins and probably pterins [4,94,123,267–270], in the last, by phytochrome (P), a chromoprotein whose prosthetic group is open tetrapyrrole.

The function of the P system is based on the operation of the photocycle converting the initial dark-adapted form of the pigment into the physiologically active one. The latter initiates a series of transduction steps leading eventually to a great variety of the photoresponsive biochemical events which in their integrity determine the strategy of plant development in light conditions, i.e., photomorphogenesis. The relationship between the P photoconversions and physiological responses can be illustrated by the scheme:

$$Pr \underset{730\,nm}{\overset{666\,nm}{\Leftrightarrow}} Pfr \rightarrow \text{processes of photomorphogenesis and photoregulation}$$

Since the discovery of P by the Beltsville group more than 40 years ago, there had been a dogma that one type of pigment molecule initiated all of the variety of the photomorphogenic and photoregulation responses. However, it is now becoming increasingly evident that it can be connected with the existence of multiple P's which differ in the structure of the apoprotein. This is the major issue in the current P research. We are trying to approach the problem mainly from the chromophoric side, which is also indicative of the heterogeneity of P species and which has not yet been discussed in detail. Detection of different P types, on the other hand, promises deeper insight into the nature of the initial photoprocesses and poses the question of the possible functional implication of the P heterogeneity already at the level of its photoconversion. This is another aspect of the present review. And finally, an effort to comparatively consider basic properties of the excited states and photoprocesses in P's and rhodopsins has been undertaken. The analogy of the two major types of photochemical pigment is obvious in many respects and their analyses could provide better understanding of the fundamental regularities of the photoprocesses in the two photoreceptor systems and of their evolution.

These considerations justify compilation of yet another review. However, due to the lack of space, the problem cannot be comprehensively dealt with in all its aspects and the choice of the material and the scope of the analysis of the recent literature is undoubtedly influenced by the author's interest and preferences. In order to enable those outside the field to enter into specialized discussions, I have incorporated in the text brief basic information and terminology on P. The reader is also referred to the relevant reviews [20,90,91,138,145–147,156,222,228,240,243,244,251,252,306,313,314,320,321,347,360,361,368].

2. The phytochrome system

2.1. Phytochrome: discovery and major aspects in its investigation

2.1.1. Detection of phytochrome. The fact that the perception of light quality (and also quantity) is connected with the photochromicity of P, i.e., the ability to exist in two photointerconvertible forms – a red-absorbing form (Pr) and a far-red-absorbing form (Pfr) – was recognized by its discoverers more than 40 years ago [17–19] during the first, photophysiological period of the investigations. It was found that the action spectra of a number of photoresponses in plants (inhibition of flowering, stimulation of germination) had a maximum in the red region around 660 nm and that these responses could be reverted by the far-red illumination at 730 nm given after the red. These crucial observations led to a prediction that there is a photochromic pigment which is synthesized and remains in the dark in Pr and upon illumination is transformed into physiologically active Pfr form and back into initial state by far-red illumination.

This prediction was successfully realised in the discovery of reversible photoinduced absorption changes with the bands at 660 nm (negative upon red illumination) and at 730 nm (positive) and using this method of P assay the pigment was then extracted and characterized as a chromoprotein [31]. These findings predetermined the future success of research in the two major interrelated directions – investigation of (i) physicochemical properties of the extracted pigment (see below in detail) and (ii) of different photophysiological responses.

2.1.2. Different types of photoresponse in plants

These were classified by the quantity of light and concentration of Pfr [Pfr] which are necessary to initiate the reaction [145,275]. They are (i) very low fluence responses (VLFR) (fluences on the order 10^{-10}–10^{-7} mol m^{-2} producing a relative Pfr concentration of 10^{-6}–10^{-3}), (ii) low fluence responses (LFR) (10^{-6}–10^{-3} mol m^{-2} and 10^{-2}–10^{-1}) and (iii) high irradiance responses (HIR) which are believed to be mediated by the rate of cycling between Pr and Pfr at different light intensities. HIR are distinguished also by the quality of sensitizing light: red- and far-red-light-mediated, R-HIR and FR-HIR. Among the three types, only LFR shows the classical red–far-red reversibility matching the photochromicity of P. They are also distinguished by the time which is needed for their development: short-term reactions in the seconds time-scale and those which require hours and days. The complex phenomenon of the photoresponses is also complicated by the existence of non-photochemical reactions which are involved in the determination of [Pfr]: synthesis of Pr which is down-regulated by the appearance of Pfr, destruction of Pfr, and dark reversion of Pfr to Pr. Response modes are different in etiolated and de-etiolated plants [184,314].

2.1.3. Phytochrome action: mechanisms of transduction

The transduction chain between the appearance of the physiologically active P form and the final responses remains the most elusive part of the light perception in plants. It is believed that the short-term phenomena develop on the biophysical and biochemical levels, while the long-term events involve the mechanism of gene expression (the membrane [185] and gene [191] hypotheses). In agreement with the membrane hypothesis, P, upon its transformation into Pfr, modifies membrane properties. There is evidence for the participation of G-proteins, Ca^{2+}, calmodulin, protein kinases and inositol lipids in this process [37,67,104,233,234,236,237,360]. These components, known as second messengers, of the animal transduction mechanism are also likely to act in the photocontrol of gene expression. According to the recent data [203], signal transduction in tomato cells initially involves G-protein activation and then there are two different pathways – one pathway requires calcium and activated calmodulin, and can stimulate expression of photoregulated genes participating in the synthesis of some photosynthetic complexes. The other pathway, controlling, in particular, anthocyanin biosynthesis, does not require calcium. Involvement of more than one G-protein is suggested. Participation of cyclic GMP in the phytochrome-mediated signal transduction pathway was also recently reported [204]: cyclic GMP alone was able to trigger the production of antocyanins and in combination with calcium could induce the development of fully mature chloroplasts in the cell of a phytochrome-deficient etiolated tomato mutant. It was demonstrated that cGMP and calcium act primarily by modulating gene expression. Control of light-regulated genes by P, which is the essence of the gene hypothesis, is likely to involve the binding of activators or repressors (protein factors, *trans*-acting elements) activated by P, to regulatory elements of the gene (*cis*-acting DNA sequence elements) [20,58,222,261]. It has been suggested recently (see [102] and the literature cited therein) that phosphorylation/dephosphorylation can play a role in the direct regulation of transcription via phosphorylation cascade activated by photoconversion of P as proposed in [304]. This theory is supported by two groups of facts: first, protein kinase activity has been found to be associated with purified P (probably, residing in a polypeptide that is tightly bound to P) and second, observation was made of fast photoinduced phosphorylation/dephosphorylation events in subcellular preparations (see [13,81,102] and the literature cited therein).

2.2. Multiple types of phytochrome

Heterogeneity of the pigment in the cell is shown by several lines of experiment.

2.2.1. Light-labile and light-stable pools

The kinetics of light-induced P destruction reveals two P pools which differ by the rate constant of dark destruction upon phototransformation of Pr into Pfr (light-labile and light-stable). P concentration which was measured with the use of difference absorption spectroscopy reaches its maximum in etiolated seedlings and drops down to approx. 1–3% of the initial level upon prolonged illumination [225]. The kinetics of the destruction is biexponential – the first part reflects the destruction of the bulk of the pigment (90–95%) with the half-time of approx. 20–40 min; the second, slow decrease of the minor part of the pigment with the half-time of several hours [27,108]. The light-labile species is abundant in etiolated tissues, while the light-stable type dominates in light-grown plants. The explanation for this difference could be sought either in different localization of the pigment in the cell or in different structures of the apoprotein (the chromophore is believed to be the same in all P's [222]).

2.2.2. Immunologically distinguishable phytochromes

The most important recent event in the P research is the discovery of the diverse P pools which are characterized by different structures of the apoprotein. The differences in the composition of the apoproteins were revealed with the use of immunochemical methods and molecular biology technique (see below). The existence of different P's (P1 or Type 1 and P2 or Type 2, predominant in etiolated and light-grown seedlings, respectively) was shown with the aid of antibodies [1,214,215,274,342]. Recent investigations [363–365] reveal an even more complex picture: three P's are distinguished in oats which have monomeric masses of 124 kDa ('etiolated' P) and 125 and 123 kDa ('green' P's). In addition to the molecular mass, there are other differences in the physicochemical characteristics of the extracted Type 1 and Type 2 P's (see for review [90,91,222] and below).

2.2.3. Soluble and bound phytochrome pools

The problem of P heterogeneity with respect to its localization in the cell is controversial (see [138] for a review). Immunovisualization experiments suggest that P is soluble and localized in cytoplasm [215,216]. However, physiological studies support the hypothesis that there is a membrane-bound fraction of P [103,105,185,236,237]. Biochemical experiments which reveal a certain amount, up to 15%, of P in association with membrane proteins (see [164,336] and the literature cited therein) support this conclusion. It is tempting to assume that the protein which binds to P might be a receptor protein. Recently, Sommer and Song [317] suggested that G-protein can be associated with P and protein kinase activity of P is believed to be connected with a protein bound to P (see [13] and above).

In the works of Pratt and coworkers (see [215,216] for review) it was shown that the bulk soluble P in the Pr form is homogeneously distributed in the cytoplasm. However, its distribution changes within seconds from diffuse to discretely localized upon transformation of Pr into Pfr. Sequestered P is connected with unidentified 200–400 nm subcellular particles and it was believed that the photoinduced association was physiologically significant and connected either with the light perception or destruction of the pigment. Recent investigations [48] of P sequestering in soybean, which requires a few minutes instead of seconds in oat, i.e., much longer than the time of fast photoresponses, put, however, at question the hypothesis that this process is a primary step in the mode of action of P (at least, in the fast responses).

2.2.4. Different phytochrome gene products

Molecular biology experiments revealed different genes encoding for different P's in a number of plant species (see [222,223] for review). They include pea [1], *Arabidopsis* [272], rice [60], oat [219], potato [106], *Nicotiana tabacum* [149]. In particular, in *Arabidopsis*, five P genes were detected, *phyA* through *phyE* [46,272] (for phytochrome nomenclature, see [224]). With the two novel *Arabidopsis* phytochrome genes, *phyD* and *phyE*, isolated and described in [46], the primary structure of the complete phytochrome family of this plant is known. *PhyA* encodes the etiolated-tissue Type 1 P and it is shown also that *phyA* is down regulated by P upon illumination while *phyB* (and *phyC*) are constitutively expressed irrespective of light treatment [60,149,272]. It is believed in this context that the products of different genes can be correlated with the labile and stable P pools. The picture, however, seems to be more complex: one cannot exclude the possibility that the light-labile P (Type 1) and light-stable P (Type 2) could be also the result of the posttranslational pigment modification because it is shown that phyA is present and functional in the light-grown plants, too (see [1,132,315] and below).

2.2.5. Spectroscopically and photochemically distinguishable phytochrome species

Two P's (Pr′ and Pr″) were detected in the cell with the use of fluorescence spectroscopy and photochemistry [280,299,302,303]. They were correlated with the light-labile bulk and light-stable minor pools of P [295]. Recent data [280,281,288] show that Pr′ and Pr″ reside within phyA and could be attributed to the soluble and membrane-associated P species. Moreover, Pr′ itself was found to be heterogeneous: the observed subspecies were attributed to different conformations of the Pr′ chromophore [282,303]. The properties of the heterogeneous phyA populations will be discussed in more detail below.

Thus, we can conclude using the words of H. Smith [306] that: "phytochrome is not a single photoreceptor, as had been assumed since its discovery in 1959, but a family of photoreceptors... This realization marks a watershed in

phytochrome research... We can now speculate that the many different modes of phytochrome action, distinguishable on physiological criteria, may be reconciled on the basis that each phytochrome has a different physiological function."

2.3. Functional role of different phytochromes

A predominant tendency in the recent works is an attempt to determine the physiological roles of different P's with the use of P mutants and transgenic plants. It is obvious that the most direct approach to their assessment is to obtain the mutants defective by one or another P and to analyze their phenotype. This approach has shown that different phytochromes have distinct functions.

2.3.1. Photomorphogenic mutants

In the recent years a number of photomorphogenic mutants were found, i.e., of the mutants with anomalous responses to the light stimulus (see reviews [147,150,228, 347]).

Phytochrome mutants. The P mutants, however, comprise only part of them. Moreover, within the P mutants there is a group deficient in the chromophore biosynthesis and chromophore incorporation into the apoprotein and thus deficient in all P's (chromophore mutants) and a group of mutants defective in one or another gene encoding for different apoproteins (apoprotein mutants).

The chromophore mutants with etiolated phenotype were isolated in tomato – (*au*) mutant [2,208,271,337]; in *Arabidopsis thaliana* – *hy1*, *hy2*, *hy6* mutants [44,151,210]; in tobacco – mutant of *Nicotiana plumbaginifolia*, *eti 1* [152].

The apoprotein mutants are selected by the immunochemical or molecular biology analysis along with the phenomenological responses. The following are the mutants which are believed to be deficient in phyA: *fhy2* [370,374], *fre* [197,226] and *hy8* [209] mutants of *Arabidopsis* and *eti2* mutant of *Nicotiana plumbaginifolia* [152]. The cucumber *lh* [3,151], *Arabidopsis hy3* [194,315] and *Brassica rapa* [63,64] mutants and *ma3R* strain in sorghum [42] are defective in phyB protein.

The transduction chain mutants. The second type of the photomorphogenic mutants is due to the abnormalities in the transduction chain. These are the mutants (i) which display de-etiolation in darkness resulting in short hypocotyls, expanded leaves and accumulation of anthocyanin (*det* [32,45] and constitutively photomorphogenic *cop* [61,62,366] mutants in *Arabidopsis* and *lip* mutant in pea [88]) and (ii) which have etiolated phenotype when grown in light (*lv* mutant of pea [198]).

2.3.2. Transgenic plants

Two approaches prove to be also very useful for elucidation of functions of different P's: first, modification of the level of synthesis of a particular constitutive P species [115,342] and second, overexpression of cloned genes in transgenic plant [21–23,143,228]. The latter promises to be useful especially in studying the biochemistry of the pigment (see below the functions of different domains).

2.3.3. Phenomenology of the photoresponses

Two phenomena are currently being intensively investigated in which different P's play different roles: de-etiolation and shade avoidance syndromes. In the de-etiolation and seedling development, the function of P is to perceive the light independently of its spectral composition (VLFR) while ecologically important perception of the proximity of surrounding plants inducing shade avoidance syndrome is mediated by P through recognition of the light quality in the red and far-red regions (R/FR ratio perception) (LFR) [308,310].

Analysis of the growing number of publications on mutants and transgenic plants reveals a complex picture of action and interaction of the two (and even more) P's clearly showing that different P's play different roles in photoregulation in plants [59,101,187,188,194,209,229, 307,315,352,371–374]. In short, the P functions can be summarized as follows. PhyA promotes germination in FR and plays a major role in de-etiolation [22,144,176,195, 272]. It has only minor influence on the R/FR ratio perception [312,374] and in general makes a higher contribution to VLFR than to LFR [34,35]. According to [372] phyA appears to be is dispensable in white-light-grown plants although there are data that phyA is also functional in light-grown plants even at its low levels found under these conditions [106,132,133] and can activate transduction pathway(s) involved in flowering [6,132]. PhyB promotes germination in the dark, but inhibits germination in FR, regulates gravitropism [175], plays a role in cell elongation in response to light and chlorophyll accumulation [43]. It is necessary for the R/FR ratio perception and shade avoidance and for the end-of-day FR response [187,229–231,312,374]. PhyB is involved in the flowering response [6,43,101,369]. PhyA and phyB appear to have complementary functions for some processes (germination, seedling development, flowering), they act synergistically or in the opposite directions via different signal pathways and could be localized in different tissues [43,196,212,226,227].

3. The phytochrome molecule

Unique photochemical properties of phytochrome are due to the specific state of the chromophore within the chromophore locus of the apoprotein.

3.1. The chromophore

From two experimental lines – the 'classical' analytical micromethod, which includes analysis of the chromophore

degradation products and its complete synthesis from the end products of degradation – it could be concluded that the chromophore is an open linear tetrapyrrole with the system of the conjugated double bonds (Fig. 1), see for review [238,239,243,244]. In [29] isomerization was considered as a possible mechanism of the photoreaction in the chromophore and later on it was shown using ^{1}H-NMR spectroscopy [245,340] and later on Raman spectroscopy [83,85] and difference infrared spectroscopy [277] that the differences between the chromophores in the Pr and Pfr forms can be considered as a result of isomerization about C15 double bond between rings C and D: 15*Z* and 15*E* configurations, respectively. It is believed that the configuration of the other two bonds (between the rings A and B and B and C) is the same in the both forms (5*Z* and 10*Z*) [238], although there is no definitive proof for this [243]. Moreover, Tokutomi et al. [226] have recently developed a chromophore model based on the absorption and linear dichroism experiments on 114 kDa pea Pr, which has C_5-*Z,syn*, C_{10}-*E,anti* and C_{15}-*Z,syn* configuration.

Phytochromobilin is synthesized by reduction of biliverdin IX-α in vivo in the process of formation and degradation of tetrapyrroles (heme) [28,79,80]. With the use of the *E. coli* and yeast experimental systems to express and assemble photoactive holophytochrome, it has been shown that the chromophore binds to the apoprotein autocatalytically [51,52,55–57,79,157,159,162,167,338, 339,362] and it is accepted that the chromophore is the

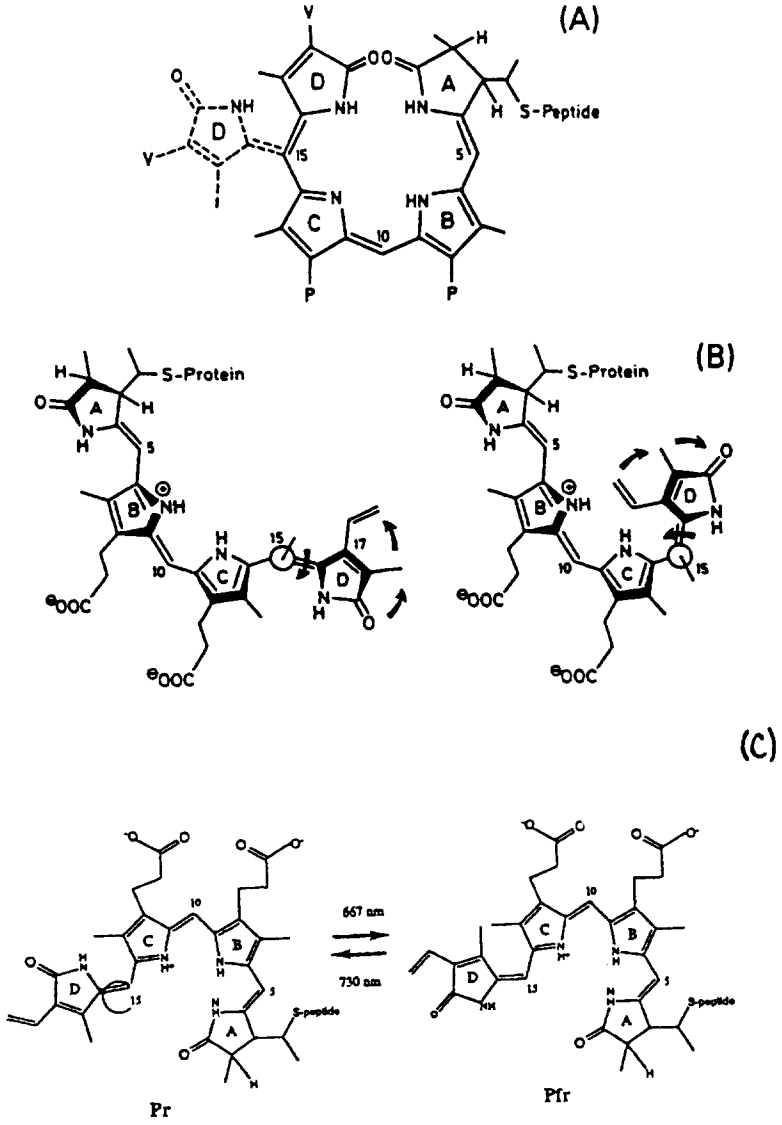


Fig. 1. Structure of the phytochrome chromophore in chromopeptides and native protein. (A) Cyclic-helical conformation in the peptides; solid lines: Pr form; dashed lines: Pfr form (V at C18 stands for the vinyl group, CH = CH_2; in C-phycocyanin, in this position is the ethyl group, CH_2–CH_3). (B) Extended comformation in the native protein, left: Pr form, right: Pfr form. The arrows indicate the movement of ring D and of the methine bridge for conrotation of bonds C14–C15 and C15–C16. (After [241,243]). The mechanism of the photoreaction (C) suggesting overall conservation of the semi-extended conformation of the chromophore has been recently presented in [321] based on the molecular modeling of phytochrome [205].

same in different P's [222,368]. Kunkel et al. [155] have shown that the light-stable tobacco P apoprotein (PHYB) expressed in yeast, can incorporate phycocyanobilin to give a photoreversible adduct. Similarity of the rate constants for the reconstitution of phyB and of light-labile phyA from oat [167] suggests that the mechanism of chromophore attachment is the same for PHYA and PHYB.

3.2. The protein

3.2.1. Primary structure

Primary structure was determined by analyzing the sequence of the amino acid residues of the apoprotein and of the nucleotides of the corresponding genes for more than 10 plant species. Molecular characterization of the polypeptides encoded by phyB and phyC was carried out on the basis of the analysis of respective cDNAs (see [222] for a review). PhyA, B and C include a polypeptide of about 1100 amino acid residues (molecular mass of about 124 kDa). The sequences of the three P's differ considerably, homology between them for one and the same species (*Arabidopsis*) is approx. 50%, while higher degree of homology is observed for the same P's in different plant species (65–80% identity for PHYA and 75% for PHYB [222] or even higher as revealed in [149]: PHYB identity of tobacco to PHYB of potato, 92.3%. Two novel *Arabidopsis* phytochrome genes, *phyD* and *phyE*, have been isolated and described recently [46]. The *phyD*- and *phyE*-encoded proteins are similar in size to the other phytochrome apoproteins and show similarity along their entire lengths. The PHYD and PHYE proteins are more closely related to phyB than to phyA or phyC and phyB and phyD show greater than 80% amino acid sequence identity. The recent data obtained on tomato and sorghum [218] indicate that the phy gene family in angiosperms might not be highly conserved, and that it might be more complex in some cases than that described for *Arabidopsis*: there is evidence for at least seven phy genes in tomato.

The distribution of the hydrophilic and hydrophobic residues in the polypeptide chain, where hydrophilic charged residues dominate, suggests that the pigment is water-soluble cytosolic and not an integral membrane holoprotein. This fact, however, does not exclude peripheral interaction of P with membranes. In the C-terminal part of the pigment, there is a highly amphiphilic region between residues 1081 and 1120 [138,211] and it is also shown that the N-terminal chain forms amphiphilic α-helix [92,206,207]. It is suggested [321] that either or both of the amphiphilic terminal segments could participate in the interaction of phytochrome with membrane.

3.2.2. Secondary structure

In holoprotein, secondary structure includes α-helices (approx. 50%), random coils (aperiodic) (25–30%), 20–25% β-turns and low content of β-sheets [36,316,320]. This is consistent with the results on UV resonance Raman spectroscopy [190,348], which show very small amount of β-sheet structure. However, these data are at variance with the secondary structure predictions by the Chou-Fasman and Garnier-Osguthorpe-Robson methods, by which a substantial proportion of β-sheets is obtained [211,320]. In these works, the failure of the two theoretical methods is considered to be the result of moderate success of the predictions in general and interactions between the chromophore and apoprotein modifying the apoprotein structure: there is evidence that apoprotein contains much higher proportion of β-sheet than holoprotein [93,316].

3.2.3. Tertiary structure

Biochemical and immunochemical investigations suggest that the P molecule in vitro is a dimer consisting of two identical subunits. Each subunit in its turn comprises two main domains [135]: the globular N-terminus domain of 70 kDa and the C-terminus domain of 55 kDa containing, respectively, the chromophore binding site and the dimerization site [69]. The two domains are connected by the segment sensitive to the proteolytic enzymes. Within the N-terminus domain, there is a subdomain with molecular mass of 10 kDa, which itself contains the sites which are sensitive to the proteolytic attacks [162]. Sequential cleavage of the 6 kDa and 4 kDa fragments from the N-terminus of the native 124 kDa P gives 118 and 114 kDa 'large' P's and the following cleavage of the 55 kDa from the C termius, 60 kDa 'small' P [137,353]. Several separable carboxy-terminal domains were identified by [39]; see below. Structural content of the P domains, which is important for the tertiary structure formation, was estimated by [232] on the basis of the amino acid sequences of different P's.

3.2.4. Quaternary structure

The X-ray crystallography data on P is absent and the structure was visualized with the use of electron microscopy (rotary-shadowed preparations) [134] and X-ray scattering under small angles [343]. The model for the intact dimer of pea P was constructed in [199], see Fig. 2a. It has an 'Y' shape with joint C-terminal segments and separated N-terminal segments and a two-fold symmetry axis. A model including noncovalent interactions was proposed for possible dimer contact area in [232]. Tokutomi et al. [346] put forward a model based on linear dichroism experiments [344] for the structure and orientation of Pr chromophores in the dimeric molecular model of Pr as shown in Fig. 2b. Amino acid sequences of phyA, phyB and phyC reveal a general identity of the distribution of the hydrophilic and hydrophobic residues what suggests a similar three-dimensional structure of their molecules [222,272]. In addition to the above biochemical data, indirect physiological evidence is obtained in vivo [26,350,351] also suggesting the existence of phytochrome as a dimer.

3.3. Chromophore binding site and chromophore-apoprotein interaction

A single bilin chromophore of the P is covalently bound with cysteine-323 (in *Avena)* in the hydrophobic pocket within the N-terminal part of the peptide. The segment of the peptide, where the chromophore is located, is between two strongly hydrophobic regions and it is relatively inaccessible to the external hydrophilic medium [74,100,318,330,358]. The chromophore interacts with the N-terminus peptide chain and the 6–10 kDa subdomain is important for stabilization of the chromophore in the apoprotein [137,355,356]. Denaturing of the pigment leads to the loss of the photochemical activity and substantial changes of its spectroscopic properties [239,243].

Rüdiger [240] deduces three major factors, which determine the spectroscopic properties and photoactivity of the native P, from (i) spectroscopic data (see below), (ii) the amino acid sequence adjacent to the P chromophore and (iii) the analogy with phycocyanin, which contains almost

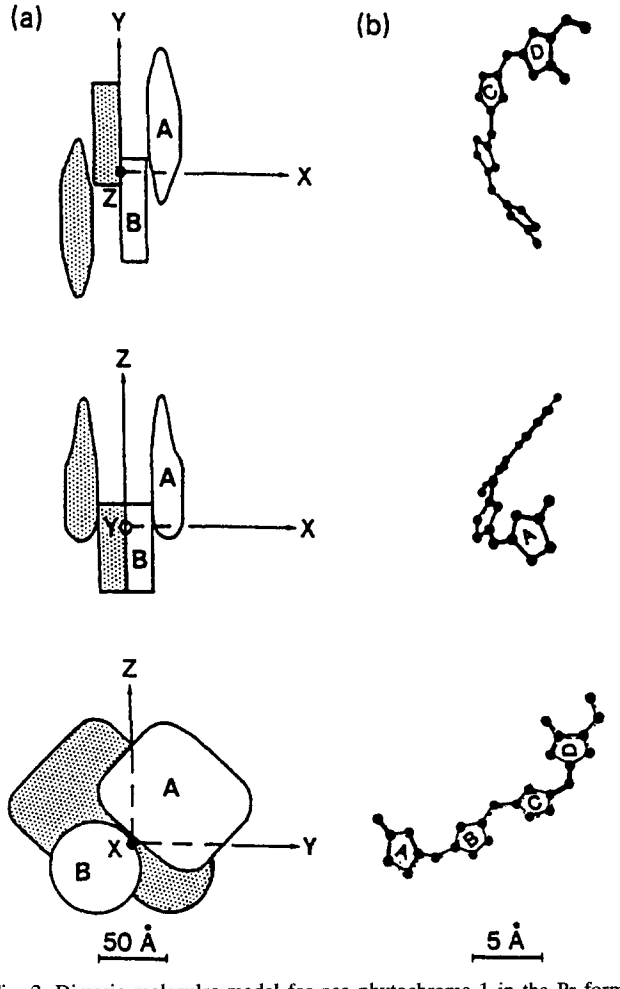


Fig. 2. Dimeric molecular model for pea phytochrome 1 in the Pr form [199] (a) and images of the Pr chromophore [346] (b). A and B in (a) indicate the N-terminal 59 kDa chromophoric and the C-terminal 55 kDa domains; A through D in (b) indicate the four pyrrole rings in the chromophore of phytochrome. (From [346]).

the same chromophore (ethyl group in phycocyanobilin and vinyl in phytochromobilin at C18) and for which the data on crystallography and X-ray scattering were obtained. They are: (1) stretching the chromophore; (2) protonation in the Pr form; and (3) charged groups on the protein residing nearby the chromophore. The stretched conformation is to be provided by the interaction between the tetrapyrrole and the amino acid residues. The covalent bond of the apoprotein with the ring A and ionic interaction between the arginine and propionic side chain of the ring B can be considered as the points of fixation for the rings A and B. The positive charge of the protonated phytochromobilin at ring B (at least in the Pr form) could be balanced by the negatively charged aspartate or glutamate. At this point the analogy between P and phycocyanin is likely to come to its end because rings C and D are fixed in phycocyanin while in P there is room and freedom for their movement to allow photoinduced conrotation about the C14–C15 and C15–C16 bonds. A model of the phytochrome chromophore binding pocket also based on the X-ray crystal structure of C-phycocyanin has been produced recently by Parker et al. [205]. It is suggested, using the numbering of the *Avena* sequence with chromophore bound to Cys-323, that Arg-318, Ala-319, the methylene of Ser-322, Leu-325, and Tyr-327 form a pocket in which one side of the chromophore is anchored hydrophobically, covalently, and electrostatically from several directions. The other side of the chromophore lacks hydrogen-bond donors and is involved only in van der Waals contact with the peptide segment.

3.4. Functional role and biological activity of the protein domains

Structure–function relationship of the P molecule is being effectively investigated by the analysis of the properties of gene engineered P and transgenic plants expressing mutated P (see for review [41,92]).

3.4.1. The N-terminal domain

Deforce et al. [57] have shown using in vitro assembly of apophytochrome and apophytochrome deletion mutants expressed in yeast with phycocyanobilin that truncation of the N-terminal tail to residue 46 is not critical to bilin attachment, but a deletion mutant lacking 222 amino acids from N terminus failed to yield holophytochrome. At the same time, deletion of the entire C terminus (to residue 548) did not interfere with bilin incorporation and red–far-red photoreversibility. Experiments on single amino acid replacements of conserved residues in the vicinity of chromophore-binding Cys-323 have shown that all mutants incorporated the chromophore with varying efficiencies [55]. It was concluded that residues substituted at Asp-309, Arg-318, His-321, and His-326 were indirectly involved in the catalysis of chromophore ligation. However, the His-324 mutants were severely affected in their chromophore

binding efficiency and in the photoreversibility of the reconstituted proteins suggesting an intimate involvement of this residue in the catalitic and photochemical processes [55,321]. Experiments on transgenic tobacco expressing a number of truncated oat P's A have also shown [39] that the N-terminal residues between the residues 70 and 398 are necessary for chromophore attachment and residues between 399 and 652, for spectral and photochemical integrity. Overexpression of the 68 kDa N-terminal domain containing chromophore leads to the accumulation of the polypeptide, which retains photochemical activity, but does not lead to the biological action – to the appearance of the phenotype with the shortened hypocotyl in *Arabidopsis* [20,23]. At the same time it is believed that certain segments of the N-terminal domain participate in the photobiological act. It was shown that the deletion of the 6 kDa segment in the overexpressed oat P, which mediates the light induced conformation changes, resulted in the loss of the physiological activity in the transgenic tobacco plants [40]. On the other hand, rice phyA in which 10 serine residues in the N-terminal domain were substituted for alanine residues had spectroscopic and photochemical properties indistinguishable from the wild-type pigment, but at the same time higher biological activity when expressed in transgenic tobacco plants [333].

3.4.2. The C-terminal domain

The fact that the N-terminal domain alone is not physiologically functional [20,23] suggests that missing segments of the C-terminal dimerization domain are needed for the correct photobiological activity. It was shown that the sequences between 919 and 1093 are essential for dimerization and 35 residues at the C-terminus, for biological activity [39]. Edgerton and Jones [70] have demonstrated that subunit dimerization interactions in the C-terminal domain of P involves sequences between amino acids 599 and 683 and a subset of this dimerization region, 623–673, was also shown. Dehesh et al. [117] have found on *hy8* long hypocotyl mutants of *Arabidopsis* defective in responsiveness to prolonged far-red light (FR-HIR) and selectively deficient in functional P A that glycine-727 in the C-terminal domain, which is invariant in all sequenced P's, has a function important to the regulatory activity of P A, but not to photoperception.

Recently, Boylan et al. [21] have identified three spatially discrete regulatory domains in P which coordinate the photoregulatory activity of phyA in *Arabidopsis* and which are belived to be necessary for different functions: (i) the chromophore-bearing N-terminus domain between residues 533 and 616, for the light-induced interaction, but not for the completion of productive interactions with transduction chain components; (ii) the C-terminal domain between residues 617 and 1129, for completion of productive interactions under all irradiation conditions; and (iii) N-terminal 52 amino acids, for completion of productive interactions only under far-red high-irradiance conditions. Thus, multiple sites in the P A molecule are essential for its physiological functions and its different parts could be responsible for different phenomenological responses.

4. Excited states of the chromophore and photoprocesses in phytochrome

In this section, spectroscopic properties of phytochrome and the processes of generation and relaxation of the excited states will be considered.

4.1. Light absorption

P in its two forms is characterized by high efficiency of the absorption in the red and near-infrared region of the spectrum and relatively low absorption in the region shorter than 500 nm. Registration and interpretation of the absorption spectra of the native P in the cell is hampered by its very low concentration, high lability, high light scattering of the plant tissues and the presence in the tissue of other pigment which effectively absorb (and fluoresce) in the red region, especially of (proto)chlorophyll(ide). Photochromicity of the pigment allows, however, registration of the in vivo absorption spectra in the difference regime.

4.1.1. Etiolated Type 1 phytochrome

The pigment which is the closest, by its *absorption spectrum*, to the native P in the cell is evidently the full length 124 kDa species [36,40,162,201,354–357]. This is shown by the similarity of the difference absorption spectra obtained for the in vivo P and of the extracted pigment. The absorption spectra of P in mono- and dicots have their maximum in the Pr form at 665–666 nm, a shoulder at 608 nm and a small maximum in the Soret band at 379 nm. The maxima in the Pfr form are, respectively, at 730 nm and 400 nm and the band at 673 nm is likely to belong to Pr because in the absolute absorption spectrum of Pfr, which is obtained by the subtraction of the spectrum of Pr, the shoulder at 673 nm is absent or very weak (see Fig. 3) [162,355–357]. Proteolytic cleavage of the 6 kDa N-terminus segment leads to a small short wavelength shift of the absorption spectra (by 3–4 nm): the red maximum is at 662 nm and 726 nm in the Pr and Pfr forms, respectively [40,213,248,341,355–357]. Further cleavage of the molecule does not change the spectrum, the maxima in the 'small' P (definition of 'small' and 'large' P, see above) are at 662–665 and 724–726 nm [31]. Absorption coefficients in the red band obtained by different groups [162,213,214,236,341,355–357] vary within approx. 70–130 and 40–50 $mM^{-1}cm^{-1}$ for Pr and Pfr, respectively. There is practically no difference in the coefficients for native 124 kDa and 118/114 kDa P [355–357]. *Oscillator strength* (f) of the different absorption bands determined on 'large' rye P at room temperature was 0.33 (main band at 660 nm), 0.25 (606 nm) and 0.47 (380 nm) for Pr and

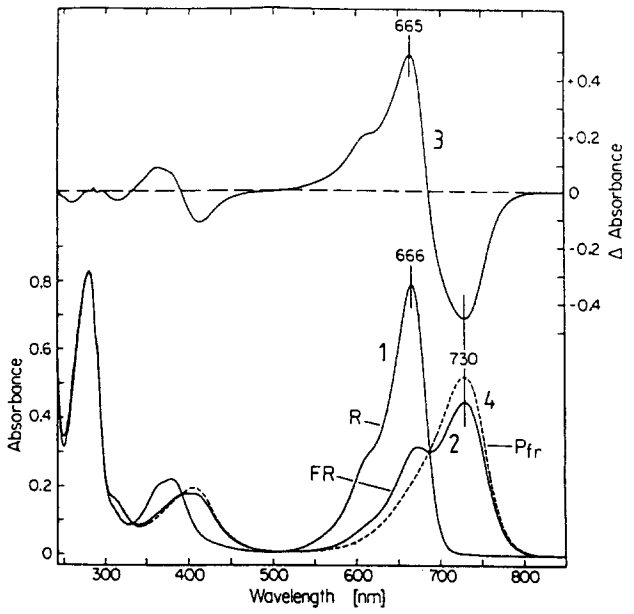


Fig. 3. Initial (1, 2), difference (3) and absolute (1, 4) absorption spectra of 124 kDa *Avena* phytochrome in the Pr (1) and Pfr (4) forms. Initial spectra were measured after far-red (1) and red (2) illumination. Difference spectrum (3) is spectrum (1) minus spectrum (2). Initial spectrum (1) represents absolute spectrum of Pr because practically all phytochrome is in the Pr form after far-red illumination; the absolute Pfr spectrum (4) was calculated from spectrum (2) after correction for incomplete photoconversion of Pr to Pfr assuming relative Pfr content of 0.86 at photoequilibrium. (The spectra from [356,357] are presented as in [238].)

0.20 (720 nm), 0.22 (660 nm) and 0.44 (390 nm) for Pfr [324]. The oscillator strength ratio of the red and Soret bands, which is approx. unity, suggests an extended or semiextended conformation of the chromophore in the pigment. Freezing of the sample (to 80–110 K), as shown on the 114 kDa P of pea [248], leads to a bathochromic shift of the band (from 665 to 671 nm in Pr and from 725 to 731 in Pfr), to a small increase of the absorption coefficient and to the narrowing of the bands, so that the oscillator strength changes insignificantly: it increases 1.06-times upon lowering the temperature from 20 to −190° C. In the cells of etiolated plants, freezing causes a 10 nm shift from 660 nm to 670 nm and from 730 to 740 nm [331]. No gross differences in the absorption spectra were found between the Pr forms in D_2O or H_2O [247].

Linear dichroism spectra. For Pr of 114 kDa pea P negative linear dichroism was revealed with the use of the method of compressed gel while Pfr showed positive signal [344,346]. Magnitude of the reduced dichroism (RD), which was measured as $(A_{par} - A_{perp})/A_{iso}$ ($_{par}$ and $_{perp}$ stand for parallel and perpendicular polarization with respect to the stretching axis, $_{iso}$, for unstretched gel and unpolarized light), linearly depended on the extent of stretching and was below 0.17 for Pr and 0.10 for Pfr. The two absorption spectra in the Pr form for the two directions of the polarization were identical by their form to the spectrum measured in unpolarized light in the region from 500 to 700 nm, suggesting that the band at 608 nm is a vibrational satellite of the main red maximum in the Pr form. This conclusion is, however, at variance with the view [324] accepted in the literature that the band at 608 nm belongs to the second electronic transition, Q_x (see below). *The spectra of circular dichroism* in the chromophore absorption region have a strong negative band for Pr and much weaker positive signal for Pfr. Optical activity is interpreted as the result of the chromophore chirality and/or the induced optical activity due to the tight binding of the chromophore to the apoprotein [311,324] (see below in more detail).

4.1.2. Type 2 phytochrome

The data on green-tissue Type 2 P are rather controversial. Absorption spectrum of the Type 2 P obtained from the light grown pea seedlings has the maximum at 667 nm for Pr and at 724 nm for Pfr [1]. In the difference absorption spectrum the extrema are at 665 and 726 nm and the ratio of the bands ($\Delta Ar/\Delta Afr$) is 1.04. These parameters are close to those observed for the Type 1 P with M.M. 114/118 kDa ('large' P). On the other hand, Tokuhisa et al. [342] have obtained a different absorption spectrum of P in green plant tissues of oats. The red maximum was shifted to 652 nm in the difference spectrum with an isosbestic point at 681 nm, i.e., 14 and 8 nm to the blue as compared with those in the difference spectra of P in etiolated oats [355]. The blue shift of the λ_{max} was detected in the light-grown seedlings [97,129,131]. Shimazaki and Pratt [274] have also shown on etiolated and green seedlings that the two pools of P differ not only by the immunochemical properties, but also by their absorption spectra.

4.2. Fluorescence

4.2.1. Detection of phytochrome fluorescence

Hendricks et al., [109] have reported the observation of P fluorescence in leaves of etiolated bean seedlings and in extracts shortly after the discovery of the pigment itself. In the low-temperature excitation spectrum for the emission beyond 690 nm, a small maximum at 670 nm was measured which was attributed to P. The authors did not, however, present the evidence to this assignment what was especially necessary because of a very low abundance of P in the cell and existence of the low-temperature fluorescence of etiolated leaves in the region beyond 650 nm belonging to protochlorophyll (PChl) and its aggregated longer wavelength species ($PChl_{682/669}$, $PChl_{692/673}$, where the numbers are positions of emission/absorption maxima) and to the intermediates of chlorophyll biosynthesis [179–182,305]. This complexity of the red emission of etiolated tissues probably explains the fact that until recently there were no reliable data on P fluorescence in the cell.

At the same time, investigations of P fluorescence were extensively carried out on extracted and purified P: on

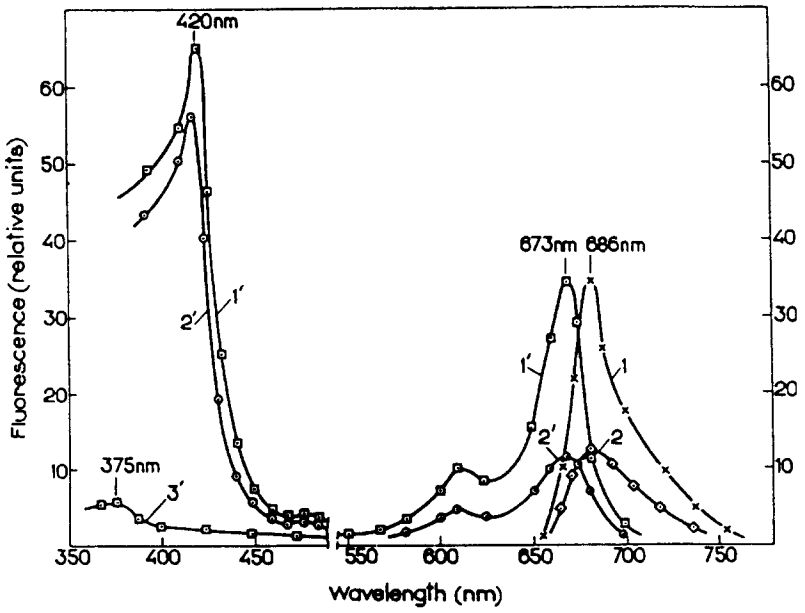


Fig. 4. Fluorescence of phytochrome in the Pr form in stems of etiolated pea seedlings. Emission spectra under monochromatic excitation at $\lambda_e = 630$ nm (1, 2): (1) 77 K; (2) 200 K. Excitation spectra, emission wavelength monitored, $\lambda_m = 700$ nm (1′–3′): (1′) 77 K; (2′) 200 K; (3′) spectrum (1′) calculated in the region 350–500 nm with due consideration of green background fluorescence with excitation maximum at 420 nm, calculation procedure based on different temperature dependence of the bands at 420 and 670 nm see in [297]. (From [297,302]).

'small' [53,109], 'large' [110,113,247,322–326,367] and native pigment [25,121,126,173,192,329]. Emission [25,53,110,113,173,193,247,289,325] and excitation [109,110,289,325] spectra of Pr, quantum yields [25,113,289,323–325,367] and lifetimes [25,113,121,173, 193,247,326,329,367] were determined. Dependence of the fluorescence characteristics on such factors as molecular weight of the pigment [326,367], temperature [323,326], viscosity of the medium [329], deuteration [25,193,247], the wavelength of fluorescence measurement and excitation [110] were followed. However, despite considerable experimental efforts in this direction, results obtained by various research groups differ in many ways. One of the reasons may be modification of the pigment during isolation and purification. In this context, the necessity arises for investigation of P in vivo without violation of the integrity of the cell.

Fluorescence of P in vivo was detected in the author's group in etiolated seedlings of monocots and dicots [297–299]. We have tackled the problem from two sides. First, the yield of the fluorescence of the pigments in the cell was increased by deep freezing (to 77 K), while the input of the background fluorescence of (proto)chlorophyll(-ide) was minimized by (i) using those parts of etiolated plant tissues which contain very low amount of background pigments – coleoptiles, stems, roots (instead of leaves) – and (ii) by exciting and monitoring fluorescence in the region of preferential absorbance and emission of P. Second, experimental efforts have been undertaken to identify the observed red fluorescence and to prove that it indeed belongs to P. From these experiments, the method of fluorescence investigation of P in intact cells has been developed [296,300], whose sensitivity is very high, better than 1 ng, which is comparable with the sensitivity of the immunochemical method [214] and much higher than that of the conventional difference absorption technique.

The attribution of the red emission to Pr (Fig. 4) has been shown in [289,297–299,302] by: (1) close similarity of the fluorescence characteristics of the P in the etiolated seedlings and of the native 124 kDa pigment extracted from them (Fig. 5); (2) close correlation of the fluorescence intensity and the P content determined with the use of difference absorption spectroscopy and the absence of such correlation with (proto)chlorophyll contents as shown

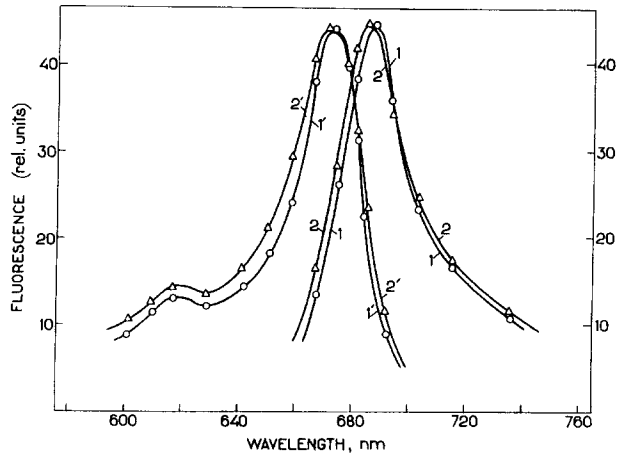


Fig. 5. Low-temperature (85 K) emission (1,2, $\lambda_e = 630$ nm) and excitation (1′,2′, $\lambda_m = 720$ nm) fluorescence spectra of the red phytochrome form in the cells of coleoptiles of etiolated oat seedlings (1,1′) and 124 kDa phytochrome isolated from them (2,2′) [289].

Table 1
Fluorescence characteristics of Pr in vitro

Phytochrome size (kDa), plant, conditions	Fluorescence						Ref.
	stationary			time-resolved			
	emiss. λ_{max}. (nm)	excit. λ_{max}. (nm)	quant. yield ($\times 10^3$)	lifetime ($\times 10^3$)	amplitude ($\times 10^3$)	emission λ_{max} ($\times 10^3$)	
Small, bean, 77 K	690	670					[109]
Small, rye, Tr	672						[53]
Small, rye, 298 K				800			[322]
199 K				1500			
Large 298 K				< 200			
199 K				800			
Small, rye, 298 K				780–860			[324]
199 K				1520			
Large 298 K				< 160–200			
199 K				770–830			
Small, oat, 275 K			1.5	45 ± 10			[367]
Large 275 K			2.0	45 ± 10			
Large, rye, Tr			$< 10^{-1}$	1 [a]			[323,325]
200–14 K	674.6		40	430 [a]			
Large, 277 K H_2O	692 [b]						[247]
Oat D_2O	692						
77 K H_2O		677	4 [c]				
D_2O		678					
Large, rye, Tr			400 [d]				[113]
Large, rye, 293 K	680	665	1.4 ± 0.2				[110]
		372					
124, oat, 275–298 K			2.9	48 ± 3	90–92		[121]
				190 ± 30	7–9		
				1040 ± 60	1		
124, oat, Tr			increas.	increas.			[193]
H_2OvsD_2O			in D_2O	in D_2O			
124 phosph. buff.				50–70	strongly		[329]
Oat				1100–1200	viscous		
Tr phosph.buff.				50–70	dependent		
+ glycerol				1100–1200			
124 H_2O			3.7 ± 0.4	40	65 [e]	689	[25]
Oat				180	20	686	
273–				1000	15	683	
293 K D_2O			3.4 ± 0.2	40	58		
				180	19		
				1000	19		
Large				45	58		[173]
Rye				165	38		
Tr				1100	4		
124 0% glycerol				37.9	88.9	685	[328]
Oat				151.7	10.2		
Tr				1112.0	0.9	675	
67% glycerol				53.6	77.6	685	
				244.5	15.6		
				1187	8.2	675	
121 0% glycerol				39	92.7	685	
Pea				239.9	5.5		
Tr				1331	0.7	675	
67% glycerol				53.1	82.3	685	
				258.6	14.5		
				1227	3.2	675	
Pea Tr							
114 0% glycerol				33.8	96.8	685	
Pea				244	2.8		
Tr				1878	0.4	660	
40% glycerol				37.8	94.5		
				227.5	4.9		
				1509	0.8		
124, oat, 275 K			2.3 ± 0.3				[47]

Table 1 (continued)

Phytochrome size (kDa), plant, conditions	Fluorescence						Ref.
	stationary			time-resolved			
	emiss. λ_{max}. (nm)	excit. λ_{max}. (nm)	quant. yield ($\times 10^3$)	lifetime ($\times 10^3$)	amplitude ($\times 10^3$)	emission λ_{max} ($\times 10^3$)	
Rye, H_2O				14			[111]
118,				44			
298 K				157			
				690			
D_2O				13			
				40			
				144			
				988			
Oat, 124, 85 K	684	672	0.2				[289]
Oat, H_2O				4–16	> 80	690	[120]
124,				45	16	< 690	
Tr				180	1.4	< 690	
				> 800	0.1	< 690	
D_2O				no deuterium effect			
Maize, soluble	683						[288]
124, membrane-							
85 K bound	680						

[a] Calculated from τ_0 and ϕ_F; [b] determined from the presented spectra; [c] ratio of fluorescence intensities in D_2O and H_2O; [d] decay not single exponential; [e] relative quantum yields of the kinetic components.

by the use of chlorophyll-less mutants; (3) reversible changes of the fluorescence following the phototransformation of Pr into Pfr at ambient temperatures and into lumi-R at low temperatures (Fig. 6.) and their similarity to the photoinduced changes of P fluorescence in vitro.

Fluorescence of the initial photoproduct on the route of the phototransformation from Pr into Pfr (designated lumi-R, I700 or Lr) was registered both in vitro [126] and in vivo [297–299], see (Fig. 6) and below. Pfr does not fluoresce according to the common view neither in vitro nor in vivo. Fluorescence of other intermediates in the Pr ⇔ Pfr phototransformations was not investigated.

4.2.2. Stationary fluorescence

In the *emission spectra* of Pr in vitro at room temperature, the maximum is located in the region from 672 nm to 693 nm (see Table 1), in particular, at 680 nm in 124 kDa oat P, as one can judge from the figures in [120,121]. At low temperatures (77–85 K), it is at 686 nm in 124 kDa rye P [126] and at 684–685 nm in 124 kDa oat P [289] (Fig. 5). Fluorescence maxima of soluble and membrane-bound 124 kDa P of maize were at 683 and 680 nm, respectively [288]. At liquid helium temperature, 1.5–4 K, the maximum of oat 124 kDa P was at 682.5 nm upon excitation at 610 and 650 nm (Sineshchekov, Deeg, Rüdiger, unpublished data). In vivo, in the emission spectra of monocots (oat, barley, maize, wheat, rice) and dicots (pea, bean, cress, tomato, potato, *Arabidopsis*), the maxima at low temperatures (85 K) are in the region of 682–687 nm [280,282,288,289,295,297–299,302,303] (Figs. 4–6). Their position depends on plant species and tissues used and on environmental and developmental factors (see below). Upon temperature increase to 0° C a 2–3 nm hypsochromic shift was observed. There was practically no dependence of the spectral shape on the wavelength of excitation in the red (600–650 nm) in vivo [299] and in vitro [110].

Excitation spectra of the extracted P have the maximum at 670–677 nm (see Table 1 and Fig. 5). In the cells, the maximum in the low-temperature spectra was at 670–673 nm depending on the sample used and experimental conditions and a satellite was well pronounced at 608–610 nm [289,297–299,302] (Figs. 4 and 5). The shape of the excitation spectrum in the red region does not depend on the wavelength of registration in vivo [299] and in vitro [110]. The blue region of the P excitation spectra is more complex for identification, it has an admixture belonging to 'blue' fluorescence attributed in vitro to the derivatives of the chromophore (bilirubin-type pigments) [47,251]. The band at 375 nm is close to that in the absorption spectrum only in well purified samples. In etiolated pea stems there is a band at 420 nm in the experimental excitation spectrum (Fig. 4) which also does not belong to P [297,302]. The real excitation spectrum of P was calculated in [297] to have a small band at 375–380 nm which is very close to the absorption spectrum of P in vitro [241]. This similarity suggests [299] that the spectrum belongs to Pr and that there is no energy migration from the centers absorbing in the blue region to P. The functional implication of this observation is that photoresponses in this region are not likely to be due to energy migration to P as proposed by [327] from model experiments. This conclusion concerns the P species whose fluorescence is detected in etiolated tissues (probably, the soluble and membrane-

bound fractions of phyA, see below). However, one cannot exclude such a possibility for the minor P's operating in green plants. In this connection it should be mentioned that phyA is shown not to be involved in the coaction with the blue/UV-A photoreceptors as revealed from investigations of stem growth responses in light-grown wild-type and *au*-mutant seedlings of tomato [33].

Comparison of the fluorescence emission and excitation spectra of oat P in vivo and in vitro (124 kDa) have shown [289] their identity except 1–2 nm shift of the in vitro spectra to the blue (Fig. 5). This similarity suggests, in particular, that the absorption spectra of Pr in vivo, which cannot be obtained by direct measurements due to high light scattering, can be estimated from the in vivo Pr fluorescence excitation spectra. The fluorescence of Pr is characterized by violation of the mirror symmetry rule: the emission spectrum is more diffuse than the excitation (absorption) spectrum with the well resolved band at 608–610 nm. The half-bandwidths of the excitation and emission maxima at low temperatures are 22–27 nm and 35 nm, respectively. The Stokes shift of native P in vivo and in vitro at low temperatures is approx. 14 nm and 0–0 transition in Pr at low temperatures is at 678.5 nm, i.e., corresponding to an energy of 1.83 eV [289,297–299,302].

Quantum yield of the fluorescence (ϕ_F). Fluorescence intensity of Pr in vitro at room temperature is very low, ϕ_F is from $1.4 \cdot 10^{-3}$ [110] to $3.7 \cdot 10^{-3}$ [25], see Table 1. Our estimations show that ϕ_F is approx. $5 \cdot 10^{-3}$ both for oat 124 kDa phytochrome and P in vivo [289]. Small variations comparable with an experimental error are observed in ϕ_F depending on the way it was obtained (ϕ_F measured, corrected for impurities and calculated from the fluorescence lifetimes) [25,121,251,367]. The yield does not depend on the size of the extracted pigment [47,367]. According to [113] ϕ_F increased with the shift of the excitation wavelength to the red and in [47] different values for ϕ_F were observed upon excitation in the blue and red regions. Controversial data were obtained on the effect of deuteration on ϕ_F: according to [193,247] ϕ_F increases in D_2O up to 4 times while in [25] the substitution practically had no effect – ϕ_F is $3.7 \cdot 10^{-3}$ in H_2O and $3.4 \cdot 10^{-3}$ in D_2O. Lowering of the temperature from 298 to 14 K increases the yield more than 100 times for 'large' P [323] and 30–40 times when temperature decreases to 85 K for P in oat and pea seedlings [289,297–299,302] and for 124 kDa oat P [289], ϕ_F at 85 K being 0.3 in vivo and 0.2 in vitro. The temperature curve for the fluorescence intensity was complex [299]. Presentation in the Arrhenius coordinates according to the procedure suggested in [349] gave two linear parts with two values of activation energy (E_a) for the fluorescence decay – 20–35 kJ mol^{-1} in the interval above 200–240 K and 3.5–5.0 kJ mol^{-1} below 200 K [289,303]. Activation parameters were similar for oat P in vivo and in vitro, although small variations were observed [289]. Existence of the two temperature – dependent processes with different E_a was interpreted as a manifestation of the heterogeneity of the emitting centers of Pr (see below in more detail).

Fluorescence polarization. A high and constant degree of polarization ($P = 0.5$) in the red region and its drop to

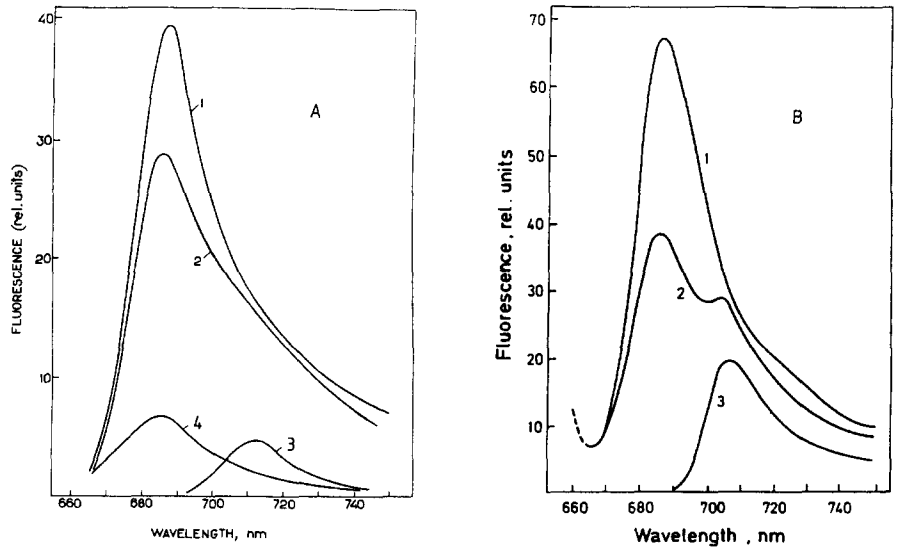


Fig. 6. Low-temperature (85 K) fluorescence emission spectra of Pr in the cells of 5–7-day-old etiolated pea seedlings ($\lambda_e = 650$ nm, $I = 0.1$ W m^{-2}) obtained in [303] (a) and [282] (b). (1) Etiolated sample frozen to 85 K in darkness; (2) the same as (1), but after illumination with saturating red light ($\lambda_a = 670$ nm) at 85 K establishing a photoequilibrium between Pr and the first stable photoproduct, lumi-R; (3) difference spectrum: spectrum 2 normalized to spectrum 1 in the maximum (not presented in the figure) minus spectrum 1; (4) the same as (2), but after thawing to 293 K, illumination with saturating red light ($\lambda_a = 650$ nm) transforming Pr into Pfr and freezing in darkness to 85 K. Note the different extent of the Pr → lumi-R phototransformation (γ_1), 0.27 in (a) and 0.43 in (b) which is measured as a ratio of the variable fluorescence intensity (a difference in the maximum of curves 1 and 2) to the intensity in the maximum of curve 1 ($\gamma_1 = \Delta F_1/F_0$). It was shown [280] that γ_1 depends on physiological conditions of the plant and on plant organ and tissue used for the experiment (see Section 5.2 for discussion).

0.25 in the broad band at 550 nm and to 0.1 at 400 nm were reported in the fluorescence polarization (excitation) spectrum of 'small' rye P recorded at 200 K [324]. Emission of Pr in etiolated pea stems was also almost completely polarized, $P \approx 0.4$, viz. the direction of the transition moment was the same for absorption and emission [299,302]. According to [324] the decrease in the degree of polarization in the Pr fluorescence excitation spectrum suggests that the band at 608 nm belongs to the second electronic transition, Q_x, and it was estimated that the Q_y and Q_x transition moments form an angle of 36°. High degree of polarization suggests that the chromophores in the P dimer are spatially separated and there is no interaction and homogeneous energy transfer between them or that they are oriented parallel to each other [299].

4.2.3. Time-resolved fluorescence of phytochrome

The fact that the fluorescence of P is quenched at room temperature agrees well with the short lifetime (τ_F) of the P excited states (see Table 1). Evaluations based on the radiative lifetime, τ_0, (14 ns [324], 20 ns [113]) and the quantum yields give τ_F on the order of tens ps.

Lifetimes of the Pr fluorescence. The picture of the fluorescence decay kinetics becomes increasingly complex along with the improvement of the picosecond technique. At least four exponential kinetic components in the interval from several ps to 1 ns are necessary to approximate the experimental curves [111,120] (see Table 1). The fact which attracts attention is that τ_F of the components practically does not depend on the degree of intactness of the P molecule [111,120,121,328,367] (see Table 1). Thus, the properties of the excited states of Pr are primarily governed by the immediate surroundings of the chromophore in its protein pocket. This is also evidenced by the effect of microviscosity on the kinetic parameters of P fluorescence [328]: glycerol, which penetrates into the chromophore pocket, slowed down the process of the degradation of the excited states, while Ficoll, which changes macroviscosity but has no access to the chromophore cavity, had no effect on the kinetic parameters. Finally, it was found that the short-lived (ps) decay kinetics showed only a minor deuterium isotope effect, or none at all [25,111,120], suggesting that the proton translocation is not likely to play an important role in the initial photoprocesses. The multicomponent kinetics of the Pr fluorescence decay was interpreted in [120,172] as a formation of the heterogeneous Pr excited states with different τ_F from the homogeneous ground state population.

Time-resolved Pr fluorescence emission spectra. These have been obtained in [25,120,328] with position of maximum varying from 660 nm to 690 nm, see Table 1. In all works, one could follow a certain regularity: the shortest-lived components (4–45 ps) had the longer-wavelength position of the maximum – 690 nm [25,120] and 685 nm [328] and for the longer-lived components (approx. 1 ns), the maxima were hypsochromically shifted by more than 10 nm. The spectra in the picosecond time interval did not depend on the size of the molecule, while for the longer-lived component with the blue-shifted spectrum the shift was more pronounced in the truncated than in the full-length P. In general, the kinetics data revealed certain differences in the conformational flexibility (or in the heterogeneity) of P preparations from monocots vs. dicots and intact vs. the amino-terminus-truncated P [328]. Kinetic measurements of the P fluorescence decay in vivo have not yet been performed; however, evaluations of τ_F from ϕ_F in vivo and τ_0 gave the value of approx. 100 ps [289].

4.3. Photoreaction

The quantum yield is within 0.07–0.21 for the forward reaction and 0.06–0.17 for the reverse, depending on the size of pigment [112,158,213,356]. Judging by the fluorescence lifetime measurements the Pr → Pfr photoreaction takes place within tens or hundreds of picoseconds and for its investigation the process is to be slowed down and stopped at the stage of the formation of the initial photoproduct by cooling or to follow it with the use of kinetics methods in the picosecond time interval.

4.3.1. Flash photolysis investigations of the phototransformations Pr ⇔ lumi-R

Photoreaction Pr → lumi-R. Linschits et al. [171] have observed a decrease in the absorption at 664 nm and the appearance of the band at 695 nm which belongs to lumi-R at registration time of 0.2 ms. The laser flash-photolysis investigations [24] suggest that lumi-R already exists 10 ns after the flash and recent ps absorption measurements on isolated 'large' rye P [173] reveal a decay of Pr* within 40 ps, while the lumi-R appearance within 100 ps. This difference was interpreted as an indication that lumi-R is formed via an unstable intermediate: Pr(665 nm) → Pr* (approx. 500 nm, 660 nm)–^{40ps}→ prelumi-R(?)–^{60ps}→ lumi-R(690 nm). A ground state species preceding lumi-R, which equilibrates with Pr*, has been proposed by Song and coworkers [329] to explain the viscosity dependence of the Pr fluorescence lifetimes. It is reported [251], however, that 99% of the thermal reverse reaction of pre-I700 to Pr proceeds directly rather than via Pr*.

In the work by Kandori et al. [142] existence of prelumi-R (pre-I700) was, however, questioned. The authors did not observe the delay in the lumi-R formation from the Pr* depopulation (both processes proceeded within 24 ps) on the 'large' Type 1 P from pea at 275 K. It should be noted, however, that the experimental picture was complicated by the superposition of the absorption and Pr fluorescence decay signals. Qualitatively similar results to those of Kandori et al. were obtained with 1–2 ps resolution by Savikhin et al. [250] on native etiolated oat P. Formation of the lumi-R absorption spectrum followed the decay of stimulated emission (with τ_F of 16 and 50–60 ps and the

spectra maximizing at 690–695 nm), i.e., lumi-R was formed directly from Pr* without intermediate (prelumi-R) stage. Time-resolved anisotropy studies indicate that the orientations of the Pr and lumi-R absorption transition moments are nearly parallel.

Results of Lipitsch et al. [172] present a much more complicated picture of the phototransformation Pr → lumi-R in 118 kDa rye P. It was concluded that (i) several spectroscopically distinguishable intermediates were involved in the picosecond phototransformation and (ii) absorption changes cannot be described by a simple kinetic model involving sequential or parallel pathways with first-order transformation kinetics of intermediates. The authors dissect the transients into different stages (A–G) (see Fig. 7). At stage A (0–15 ps) excited state absorption appears with the bands at 420 nm and 660 nm. The excited state absorption decays within 15 ps, which agrees with the Pr fluorescence decay [111]. Stage B (15–25 ps) is characterized by the full appearance of ground state bleaching. Stage C (25–45 ps): absorption rises steeply with the spectrum similar to the initial Pr (as in stage A). In stage D (40–120 ps) a new absorption arises around 690 nm, which is attributed to lumi-R, parallel to 660 nm absorption decay with a time constant of about 40 ps. This matches the second fluorescence decay with $\tau_F = 44$ ps [111]. In stage E (120–150 ps) only transient absorbance at 690 nm is present. During stage F (150–170 ps) and G (over 180 ps) the absorption at 660 nm increases again and another increase is observed at 690 nm suggesting formation of lumi-R along another path. The authors believe that the transients in the stages A–C reflect processes in excited Pr and excited lumi-R, and that the first ground state intermediate in the phototransformation of Pr appears only after 157 ps and is the second species of lumi-R (see the scheme in Fig. 7). Lumi-R at stages E and F represents what was initially termed prelumi-R by the same authors in [173].

Several questions arise with respect to the interpretation of the data. First, fluorescence decay kinetics of Pr are reasonably well described by the sum of exponentials [111,120] and this comes into contradiction with the observation that kinetic curves of the Pr absorption transients are neither exponential nor monotonous. Second, it is assumed in the scheme that the transition from Pr into lumi-R takes place in the excited state along the route without energy barrier. However, this is at variance with the data suggesting that the photoreaction is an activation process in the excited state and that up to 140 kJ mol^{-1} is accumulated in lumi-R, see below. The latter implies the existence of the energy gap of the same magnitude between the excited states of Pr and lumi-R. This gap cannot be breached even at ambient temperatures although it is known that lumi-R is easily formed at low temperatures. This also contradicts the assumption made by the authors that the chromophore phototransformation is only to trigger changes in the protein without providing energy for

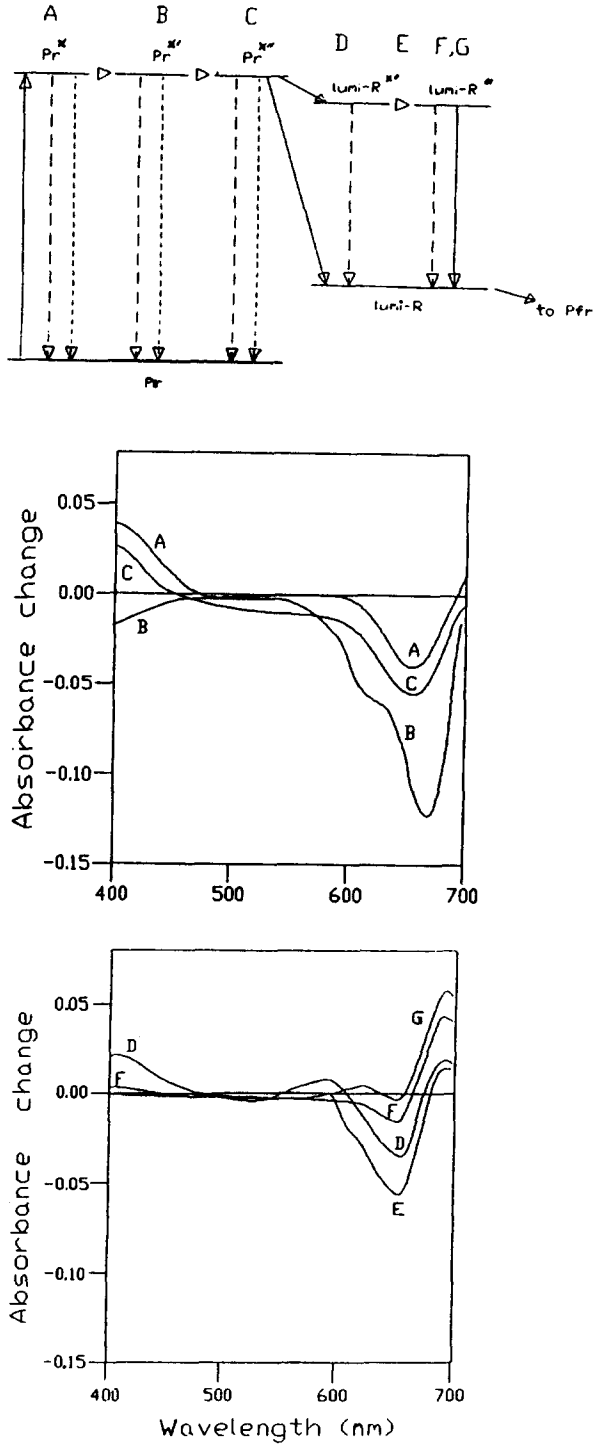


Fig. 7. Transient absorption changes in Pr at different times (ps) after excitation: (A) 10, (B) 20, (C) 35, (D) 50, (E) 130, (F) 170 and (G) 270 ps (upper panel). The lettering corresponds to the stages of the photoprocesses discussed in the text and presented in the kinetic model (lower panel) for the transformation of Pr to lumi-R, based on the time-resolved absorption [173] and fluorescence [111] measurements. Long-dashed arrows: fluorescence; short-dashed arrows: internal conversion; full arrows: photoconversion. Pr*: excited state Pr; Pr*′: orthogonal Pr*; Pr*″: fully rotated Pr*; lumi-R*′: orthogonal excited-state lumi-R; lumi-R*: excited state lumi-R. (From [173]).

them. Third, the authors suggest that both excited states of Pr and of lumi-R are populated upon excitation of only the Pr ground state and attribute the fluorescence component with $\tau_F = 157$ ps to lumi-R. This can be argued on the basis that no fluorescence of lumi-R is observed in etiolated tissues at low temperatures where P is present in the Pr form and the lumi-R fluorescence appears only upon transformation of Pr into lumi-R. Fourth, the longer lifetime components have shorter wavelength positions (see above), i.e., the 157 ps component has its fluorescence maximum at approx. 680 nm while that of lumi-R emits beyond 700 nm. The scheme of the initial photoprocesses suggested in [172] and [250] is similar in some respect to the scheme of Holzwarth et al. [120] explaining the heterogeneity of the Pr fluorescence decay by the existence of a Pr excited-state potential energy surface exhibiting minima along the isomerization reaction coordinate.

Photoreaction lumi-R → Pr. Investigation of the reverse reaction Lr → Pr seems to be important for the understanding of the nature of the forward reaction, Pr → Lr, and laser-induced physiological photoresponses (see below). The Lr → Pr photoreaction can be observed at ambient temperatures with the use of the double-flash/double-color technique, when the pulse which converts Lr into Pr is given after the pulse converting Pr into Lr with delay times within the lifetime of Lr. Observations of this kind were performed in [128,220] on native oat P. It was found that the amount of Pfr which is formed by the first flash is reduced by the second flash, which is absorbed primarily by lumi-R, and absorption of Pr is restored. These data were confirmed in [265] and the absorption spectra of I700 was determined on the native 124 kDa oat P at physiological temperatures to have $\lambda_{max} = 680$ nm and $\epsilon = 149$ mM^{-1} cm^{-1}, i.e., 15 nm blue-shifted as compared with the position at low temperatures. Photoexcitation of lumi-R in 124 kDa oat P after its formation from Pr upon laser illumination 'instantaneously' (1–2 ps resolution) recreates fluorescing Pr* [250] implying the existence of the common excited state level for Pr and lumi-R. This contrasts with the results of the steady-state low-temperature experiments on the Pr ⇔ lumi-R phototransformations [280,282], in particular, it is difficult to explain from this position the photoequilibrium between Pr and lumi-R, the almost complete photoreversibility of the Pr → lumi-R photoreaction, and existence of the Pr and lumi-R fluorescence independent of each other.

4.3.2. Time-resolved laser-induced optoacoustic spectroscopy

The photoreaction Pr ⇔ I700 was followed at room temperature with the use of pulse-induced optoacoustic techniques on the 'small' 60 kDa [130] and native 124 kDa [107] oat P. The action spectrum of the heat emission differed from the absorption spectrum of Pr in the region of 610 and 695 nm. This fact was interpreted as a formation of a dynamic photoequilibrium between Pr and I700, which is established during the 15 ns laser flash. Jabben et al. [130] have calculated the energy accumulated in lumi-R to be 140 kJ mol^{-1} taking into consideration the quantum yield of the Pr → lumi-R photoreaction of 0.13 in the 'small' 60 kDa oat P [177]. Later on, Heihoff et al. [107] were more cautious giving the upper value, 180 kJ mol^{-1}. The sum of the constants of the initial photoprocesses of Pr was estimated to be $0.3 \cdot 10^{10}$ s^{-1} [130]. The quantum yield of the initial photoreaction has been reconsidered in [107] and estimated to be more than 0.5. Later on, however, it was concluded that this was an overestimation (Braslavski, S.E., personal communication) and the value of 0.17 [213,356] is to be accepted.

4.3.3. Absorption and fluorescence investigations of the phytochrome phototransformations in the 77–293 K interval. Kinetics and fluence time–response curves

Photoconversion Pr → Pfr. At physiological temperatures (0–20° C), absorption kinetics of the Pr phototransformation in the seconds and minutes time range are monoexponential both in vitro and in vivo. The controversial data suggesting deviation from the monoexponentiality in the case of P in etiolated tissues have been interpreted as a result of the existence of an actinic light gradient in the highly light scattering medium (see [264,332] and the literature cited therein).

Pr ⇔ lumi-R phototransformations. Investigations of the low-temperature *light-induced absorption changes* of Pr were carried out in a number of early works comprehensively reviewed in [148,311]. The results relevant to the present discussion can be summarized as follows: (1) At low temperatures the phototransformation Pr ⇔ Pfr is not possible. The photoreversibility is also stopped by dehydration of the samples. (2) The first photoproduct, lumi-R, was shown to be stable below 150–170 K and the Pr ⇔ lumi-R reaction is photoreversible.

The absorption maximum of lumi-R is clearly seen in the difference absorption spectra upon phototransformation of Pr into lumi-R at 77 K. The lumi-R spectrum calculated in vitro [241] is characterized by the main maximum at 698 nm, a satellite at 630 nm and a small Soret band, by the better resolved structure and higher oscillator strength than those of the initial Pr form. The extinction coefficient was estimated to be 178–198 $mM^{-1}cm^{-1}$ at 77 K [242]. These parameters did not change much with temperature: $\lambda_{max} = 693$ nm and $\epsilon = 190$ $mM^{-1}cm^{-1}$ at 133 K [75].

Absorption measurements of the fluence time–response curves for the Pr ⇔ lumi-R phototransformations were carried out at 119–155 K on 124 kDa oat P by Eilfeld et al. [72]. The use of buffer-glycerol medium (in H_2O and D_2O) frozen as a transparent glass excluded the influence of the light scattering on the kinetics of the phototransformations. In these conditions two kinetic components were found in the Pr ⇔ lumi-R photoreactions – less reactive and more reactive – a picture different from that observed at room temperatures. The quantum yields of the photore-

actions depended on temperature and E_a of the forward and reverse reactions were evaluated to be 3.6 ± 0.5 and 5.7 ± 0.7 kJ mol^{-1}, respectively. Lowering of the rate of the total Pr phototransformation was also due to the fact that the share of the slow component increased with the temperature decrease. Deuteration insignificantly changed the kinetic and activation parameters of the two components, although the quantum yield of the photoconversion of Pr in D_2O increased by 40% and the share of lumi-R at the photoequilibrium was lower than that in H_2O. The yield at room temperature did not change in D_2O. Temperature dependence and heterogeneity of the fluence time–response curves would agree, according to the authors' view, with the assumption of at least two populations of lumi-R. The dependence of the quantum yield of phototransformation of Pr and Pfr on temperature was followed in [248] on 114 kDa pea P. In the interval 293–193 K, it showed a potential barrier in the excited state which was 5.9 kJ mol^{-1} both for the Pr → Pfr and reverse photoreactions. It was suggested that E_a might represent the barrier to proton transfer in the excited state. The authors in [72] point out, however, that it is impossible to discriminate between the isomerization and proton translocation merely from the E_a values.

Pr photoconversion into lumi-R and back were followed also by the photoinduced Pr (and lumi-R) *fluorescence intensity changes*. They were observed in vitro on the native P from rye by Inoue et al. [126]: photoinduced changes at 686 nm amounted to 25% and were followed by the appearance of an emission maximum of lumi-R at 702 nm. Investigations in vivo were carried out on etiolated seedlings of monocots and dicots [280,282,289,297–299,301–303] along three experimental lines by measuring: (1) light-induced changes of the Pr fluorescence spectra in the range 77–150 K; (2) kinetics and fluence time–response curves of the light-induced Pr fluorescence intensity changes; (3) temperature dependence of the Pr fluorescence yield and of the quantum yield and extent of the Pr ⇔ lumi-R phototransformation. With the use of low-temperature fluorescence difference [282,297–299,302,303] and also derivative spectroscopy [299], the maxima of lumi-R were determined at 696 nm in the excitation spectrum, and at approx. 705 nm in the emission spectrum (see Fig. 6). ϕ_F of lumi-R was found to be close to that of Pr, 0.3 at 77 K [298]. A dependence of the position of the Pr emission spectrum within 683–689 nm on the extent of its phototransformation into lumi-R was established, suggesting heterogeneity of the Pr population [282]. This conclusion is supported by the fact that the temperature dependence of the variable Pr fluorescence (F_v) connected with the Pr → lumi-R phototransformation differs from that of Pr fluorescence in the etiolated samples frozen in the dark (F_0) [303].

The kinetics of the Pr ⇔ lumi-R phototransformation at low temperatures are complex [299,302,303]. Investigations of the fluence time–response curves for the Pr ⇔ lumi-R phototransformation at 85–150 K, which was shown to be almost fully reversible at 85 K upon prolonged FR illumination (3–5% is left in the Lr form), revealed three kinetic components operationally designated as 'slow' (s), 'fast' (f) and 'very fast' (vf) both in the forward and reverse reactions: $Pr_s \Leftrightarrow Lr_s$; $Pr_f \Leftrightarrow Lr_f$ and $Pr_{vf} \Leftrightarrow Lr_{vf}$ [282]. The proportion of the amplitudes of the variable fluorescence of the three P states were found to

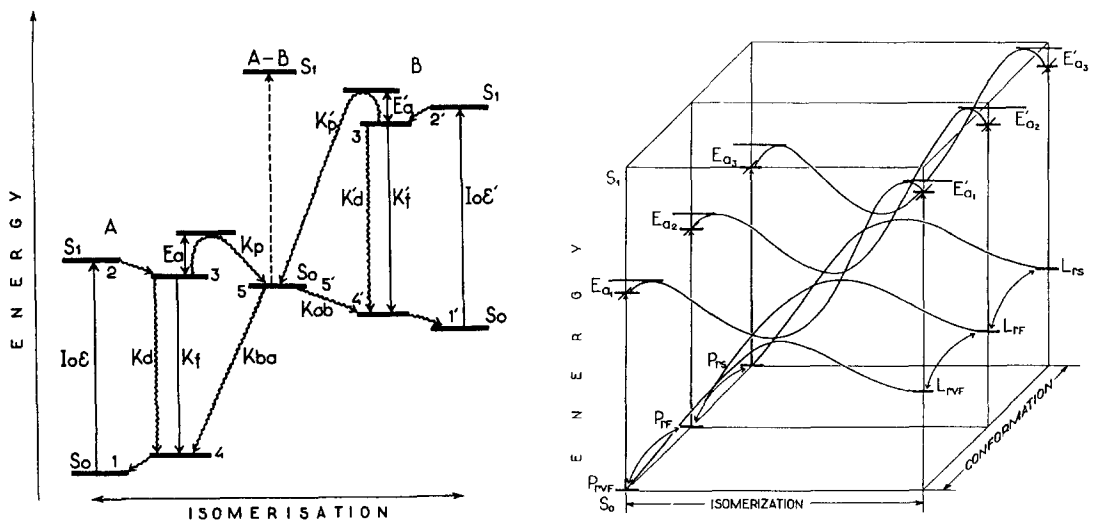


Fig. 8. Hypothetical scheme of energy levels and transitions between them in the molecules of pigments of photochromic nature including the Pr and lumi-R forms of phytochrome. A, initial state, Pr in the case of phytochrome; B, the first stable photoproduct, lumi-R; A-B, the shortliving unstable intermediate, prelumi-R; k, rate constants of fluorescence (k_f), temperature-independent degradation of excitation (k_d), primary photoreaction (k_p), phototransformation into the product (k_{ab}), and return to the initial state (k_{ba}); E_a and E'_a, activation energies of the temperature-dependent forward and reverse primary photoreactions (from [303]) (left). Third dimension is added to the scheme to incorporate the data [282] suggesting the existence of the conformers of the chromophore in Pr and lumi-R which differ by the energetic and kinetic parameters of the initial photoprocesses and whose relative ground state populations depend on temperature and state of isomerization of the pigment (right).

change with temperature and upon the Pr ⇔ Lr phototransformation. The quantum yields of the photoreactions for the three species differed one from the other by approx. a factor of 10 and those of forward and reverse conversions were close to each other. The latter agrees with the yields of the forward Pr → Lr and reverse Lr → Pr reactions at ambient temperatures estimated to be similar or differ by a factor of two (depending on the method of calculation) in the experiments on laser-induced germination of fern spores [260]. E_a for the forward and reverse $Pr_s \Leftrightarrow Lr_s$ photoreactions is 2.9 kJ mol^{-1} and 3.7 kJ mol^{-1}, respectively, and it should be lower for $Pr_f \Leftrightarrow Lr_f$ and $Pr_{vf} \Leftrightarrow Lr_{vf}$ [282]. From these data it was also concluded that the properties of the heterogeneous lumi-R states were very close to those of the Pr states.

4.4. Photophysical and photochemical heterogeneity of phytochrome and the scheme of the photoprocesses

The above-mentioned data point to the existence of the three Pr species with different quantum yields of photoreaction at low temperatures (Pr_{vf}, P_{rf}, Pr_s). The forth species, Pr_{vs}, which undergoes practically no photochemical transformations at low temperatures but reveals normal photoactivity at ambient temperatures, was detected [280,303] (see below in more detail). The difference in the quantum yield of the photoreaction at low temperatures is likely to be connected with the different activation barriers for the photoreaction in the excited state. This and other photochemical and fluorescence properties can be described in the framework of the scheme of the initial photoprocesses in Pr and lumi-R (see Fig. 8), which is characterized by the following features: (1) Participation of the singlet excited states of Pr and lumi-R in the photoprocesses with the energy (0–0 transition) of 1.83 eV and 1.77 eV, respectively. (2) Violation of the mirror-image relationship between the structured absorption and diffuse emission spectra. This fact can be taken either in support of the attribution of the two main red Pr absorption bands (see above) to different electronic transition, or, if they belong to one transition, this could point, in agreement with the theoretical considerations of Neporent [202] and comparison with stilbenes [84], to the electronic relaxation of the chromophore in the excited state into a sterically hindered less planar conformation. (3) The photochemical reaction is photoisomerization by its nature. (4) The photochemical reaction has an energy barrier in the excited state. (5) The photoreaction is the main (if not the only) temperature-dependent route for the deactivation of the excited state. This is suggested by the fact that there is a direct competition between fluorescence and photochemistry and that E_a for the fluorescence decay is very close to or coincides with E_a for the photoreaction. (6) Existence of the temperature-independent route for the deactivation of the excited states whose rate constant is on the order of magnitude of the constant of the fluorescence decay. (7) Existence of an unstable at low temperatures (orthogonal) intermediate (prelumi-R) with excess energy in the 'hot' ground state between Pr and lumi-R where branching of the photoreaction can take place – back to the initial ground state (uncompleted photoreaction) or to the photoproduct (completed photoreaction). (8) A symmetrical scheme of the photoconversion lumi-R → Pr is suggested based on the fact that the lumi-R states prove to be very close by their properties to the respective Pr states. However, the question of whether the reverse reaction goes via one and the same unstable intermediate state (prelumi-R) remains open.

In accordance with this scheme some assessments have been made [303] with respect to the yield and rates of the primary photoprocesses in the Pr states and their changes with temperature. The yields of fluorescence (3–4 transition) and of deactivation along the photochemical route (3–5), ϕ_P, are:

$$\phi_F = k_f / \left(k_f + k_d + k_{po} e^{-E_a/RT}\right) \quad (1)$$

$$\phi_P = \left(k_{po} e^{-E_a/RT}\right) / \left(k_f + k_d + k_{po} e^{-E_a/RT}\right) \quad (2)$$

The yield of the Pr → lumi-R phototransformation, ϕ_{Pr-Lr}, is equal to the product $\phi_P \times \phi_{ab}$, where ϕ_{ab} is the probability of the transformation from level 5 to the ground level of lumi-R ($\phi_{ab} = k_{ab}/(k_{ab} + k_{ba})$). A similar relationship should be true for the photoprocesses in lumi-R and the extent of the Pr → Lr phototransformation (γ_1) would depend on E_a and/or ϕ_{ab} for the forward and reverse photoreactions.

Theoretical curves of changes of ϕ_F and ϕ_P were constructed for the different Pr states with the activation parameters obtained in the experiment. For all the states ϕ_P approaches unity at temperatures near the physiological ones and the yield of the Pr phototransformation should be determined by ϕ_{ab}. ϕ_{ab} for different Pr species should be close to each other judging by the fact that the kinetics of the Pr phototransformation at room temperature in the seconds–minutes time range is monoexponential (see above) and that the extent of their Pr → Pfr phototransformation is approx. the same (75–85%) [303]. At low temperatures, < 150–170 K, where lumi-R is stable, the yields of the phototransformation of Pr and Lr and hence the extent of the Pr → lumiR phototransformation would depend primarily on ϕ_P since photoreaction at low temperatures is relatively slow and competes with the other deactivation processes.

The complex picosecond kinetics of the Pr phototransformation and fluorescence decay can be also interpreted in terms of the heterogeneity of the initial Pr state and the above-mentioned scheme of the initial photoprocesses. Estimations based on the activation parameters of the photoreaction and fluorescence decay for the three different Pr states with E_a of approx. 4.5–5, 10 and 30–35 kJ mol^{-1} obtained in [303] suggest that the lifetimes would be on the order of magnitude from approx. 1 ps to 1 ns, which is in

the range of the experimentally obtained lifetimes (see Table 1). It should be also mentioned that the components with different lifetimes are also characterized by different positions of the emission spectra, which vary in the range 680 – 688 nm (see Table 1). This is in line with the observation of the steady state emitting centers of Pr with different position of the fluorescence maxima.

Thus, we may conclude that there are two alternative

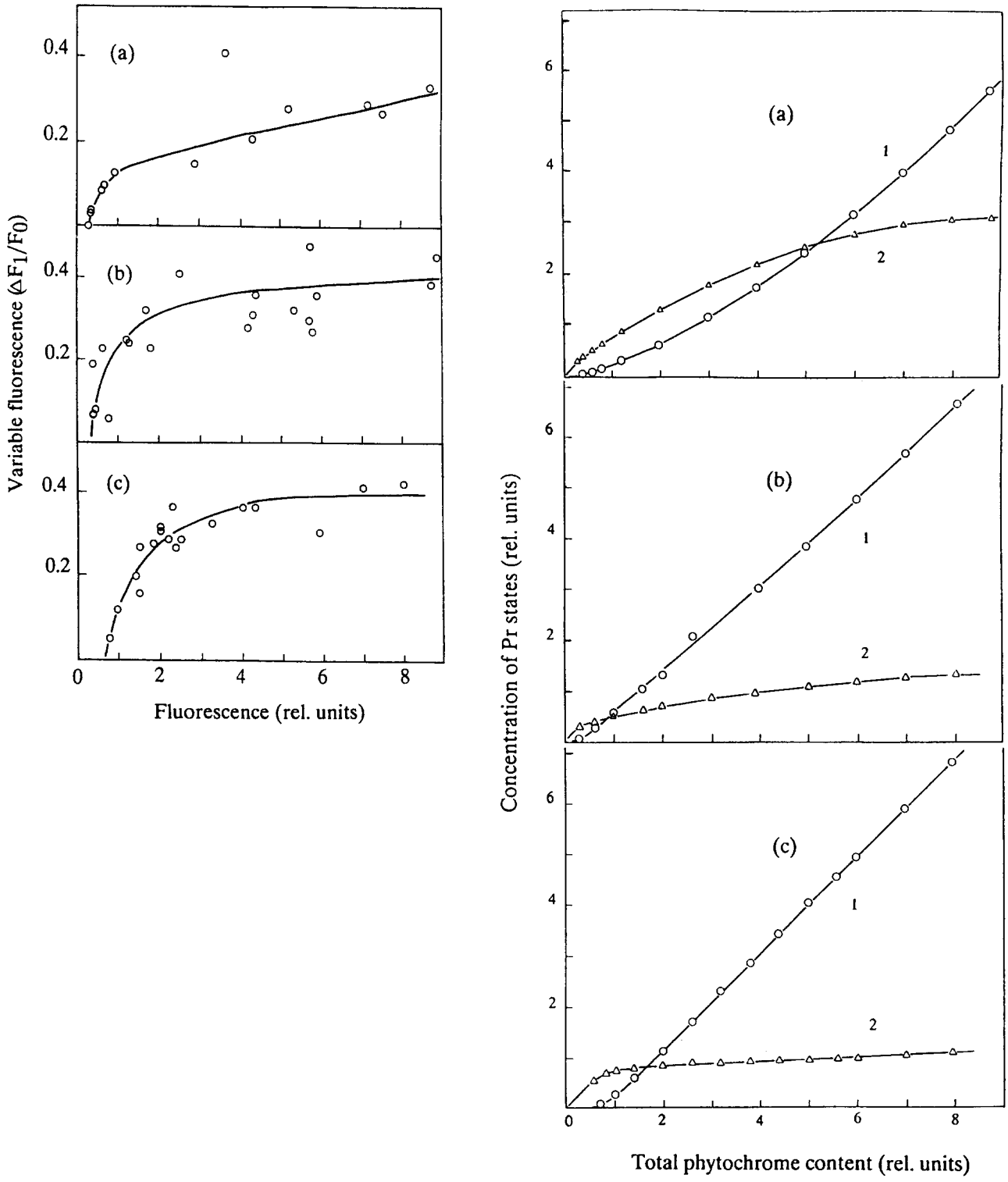


Fig. 9. Dependence of the extent of the Pr → lumi-R phototransformation at 85 K (γ_1) measured as shown in Fig. 6 (left panel) and of the concentration of the two Pr species calculated from the γ_1 values (right panel) on the total phytochrome content ([P_{tot}] proportional to F_0) in different parts of etiolated seedlings (shoots and roots) of maize (a), oat (b), and pea (c). (1) longer-wavelength Pr species, $Pr_{686/673}$, with high γ_1, Pr′, and (2) the shorter-wavelength species, $Pr_{682/669}$, with low γ_1, Pr″ [280].

explanations of the heterogeneity of the Pr states participating in the initial photoprocesses – heterogeneity of the excited state population of Pr while the ground state is assumed to be homogeneous [120,172] and heterogeneity of the ground state population of Pr which eventually yields heterogeneity of the excited states [282,303].

5. The nature of the heterogeneous red-absorbing phytochrome species

The data obtained allows at present to consider two possible causes of the observed ground state heterogeneity of the pigment: different conformations of the chromophore and different apoprotein moieties.

5.1. Different conformers of the chromophore

The fact that the proportion of the Pr centers which are characterized by different photoactivity at low temperatures (see above) changes with temperature and upon Pr transformation into lumi-R and back [72,282] was interpreted in [282] as an indication that they could be different conformers of the chromophore at thermoequilibrium with each other. The effect of glycerol, which changes the proportion of the components with different lifetimes [328], can be also considered in terms of different populations of the conformers whose equilibrium may depend on microviscosity in the chromophore pocket of the apoprotein or on solvent polarity. In Fig. 8 the relationship between the hypothetical process of the distribution of the conformers of the chromophore and their phototransformations is visualized with the three dimensional scheme where the x axis is the reaction (= isomerization) coordinate (configuration changes) and y axis is the coordinate along which the different chromophore species redistribute (conformation changes).

5.2. Different apoproteins

The assumption on the different Pr conformers cannot explain, however, all the data on P heterogeneity, in particular, the dependence of the fluorescence and photochemical parameters of Pr on plant species and tissues used and their physiological conditions. Investigations carried out on different parts of wild type shoots and roots of monocots (oat, maize, rice) and dicots (pea, cress, cucumber, potato) [280] and on phyB-less *lh* mutant of cucumber [281], transgenic potato with modified phyA levels (Sineshchekov and Gatz, unpublished data) and also on de-etiolated *lip* mutant of pea [287] have shown that the position and half-width of the emission bands vary from 682 to 687 nm and from 21–22 to 25 nm, respectively. For example, in cucumber and cress stems the emission maximum was at 687 and 684 nm, in stems and roots of pea, cucumber and other species, at 686 and 682–683 nm, respectively. There were variations in the profile of the temperature dependence curve of the Pr fluorescence intensity. The most sensitive parameter was, however, the extent of the Pr → lumi-R phototransformation, $\gamma_1 = \Delta F_1/F_0$ (see Fig. 6). It varied from less than 0.1 to approx. 0.5. At the same time the extent of the Pr phototransformation into Pfr, γ_2, was within 0.70–0.85. In general, these characteristics monotonously change as a function of the P content, $[P_{tot}]$: with its decrease to 3–5% of the maximal values, λ_{max} decreases from 686 nm to 682 nm, $\Delta\lambda$ rises from 22 nm to approx. 25 nm, γ_1 drops down from 0.45–0.50 to approx. 0.05–0.10 (Fig. 9) and γ_2, from 0.80–0.82 to approx. 0.70.

5.2.1. Phytochrome in different plant species and tissues, localization dependence

The data obtained were interpreted [280] in terms of two different P's whose relative concentration varied with $[P_{tot}]$: (i) a longer wavelength type (Pr′) with maximum at 686 nm, high individual extent of the Pr′ phototransformation to lumi-R, γ_1' (0.49 ± 0.03) and to Pfr, γ_2' (approx. 0.85), and low E_a of the photoreaction ($E_a < 3$–4 kJ mol^{-1}); (ii) a shorter wavelength type (Pr″) with λ_{max} = 682 nm, $\gamma_1'' < 0.1$, $\gamma_2'' \approx 0.70$, and higher E_a, ≤ 25–35 kJ mol^{-1}, (Pr″). Pr′ is the bulk P and [Pr′] widely varies in different parts of the seedlings (up to 100 times) and Pr′ dominates when $[P_{tot}]$ is high. Pr″ is a minor species and [Pr″] is much more constant (variations, < 10 times) and it becomes the major one when $[P_{tot}]$ drops down (Fig. 9). Estimations show that the plant species investigated make the following succession with respect to the relative Pr″ content in decreasing order: cress, maize, potato, pea, oat, cucumber. Thus, the first conclusion is that Pr′ is the bulk and variable component, while Pr″ is the minor, saturable and conserved one which is much more uniformly distributed in plant tissues.

5.2.2. Effect of preillumination of phytochrome – light-labile and light-stable pools

Now the question arises regarding the relation of the Pr species to the Type 1 (light-labile) and Type 2 (light-stable) P pools. The data on pea and oat (Sineshchekov and Sineshchekov, unpublished data) show that Pr′ sharply drops within 2 h in the dark after R preillumination in parallel with $[P_{tot}]$, while [Pr″] remains relatively constant. On the other hand, it was observed [295] that the extent of the P destruction is different in stems and roots of cress and that the proportions of the labile and stable species are very close to those of the Pr′ and Pr″ in these organs. The light-stable species itself is likely to undergo slow light-induced destruction. This is suggested by the fact observed on wild-type barley and its chlorophyll-free mutant that the Pr fluorescence intensity reflecting P_{tot} decreases to approx. 3% of its initial value in etiolated tissues after prolonged (hours) illumination (Sineshchekov and Rüdiger, unpublished results). This time course of the Pr′ (tens

of minutes) and Pr″ (hours) destruction is reminiscent of that of total P observed earlier by the absorption technique [27,108] (see above). It should be also noted that the green-tissue (light-stable) P has a shorter wavelength position according to some authors (see Section 4.1), as is the case with Pr″. Thus, the second conclusion, which is closely related to the first one, is that Pr′ is the light-labile pool and Pr″ is the light-stable one. The short-term (min) R-FR preillumination, which transforms Pr into Pfr and back and which is supposed to change P distribution from diffuse to sequestered (see above), has no effect on either the spectroscopic and photochemical properties of the Pr′ and Pr″ species or their relative abundance in the sample (pea, oat, maize) [280]. This suggests that Pr′ and Pr″ are not likely to be connected with the sequestering phenomenon.

5.2.3. Relation to phyA and phyB

The most intriguing question is whether the two spectroscopic and photochemical Pr types could be related to different P's. Experiments on transgenic potato with increased and decreased levels of phyA production have shown (Sineshchekov and Gatz, unpublished data) that the proportion of Pr′ drops down along with $[P_{tot}]$, which decreases by a factor of 10 in the anti-sense species. This observation together with the fact that Pr′ is the labile bulk pigment strongly suggests that Pr′ belongs to phyA. Ambiguity remained, however, with respect to Pr″, which could be either phyB or a small fraction of modified phyA because its production is not completely blocked in the anti-sense potato. To solve this, experiments were carried out [280,281] with the long hypocotyl *lh* mutant of cucumber, which is believed to lack phyB [147,228]. It was shown that there was practically no difference in the low-temperature fluorescence spectra in stems and roots in the wild-type etiolated seedlings and its *lh* mutant, and that the $[P_{tot}]$ and γ_1 were slightly lower in the mutant. This implies that the *lh* mutant has the same or even higher concentration of Pr″ and that Pr″ is not likely to be phyB (Type 2), but rather a small posttranslationally modified fraction of phyA (Type 1). A possibility that modification of P can influence γ_1 was demonstrated by two lines of experiment: first, it was found that freezing and thawing of the sample increases the fraction of the species inactive at low temperature (spectroscopically and photochemically similar to Pr″) at the expense of Pr′ [303]. Second, in the preliminary experiments of Sineshchekov and Lamparter (unpublished data) it was shown that phosphorylation/dephosphorylation of P changes γ_1: it was 0.33 ± 0.01 and 0.23 ± 0.01, respectively.

5.2.4. Subpopulations of phytochrome A: soluble and membrane-bound phytochromes

Finally, a correlation between the Pr′ and Pr″ and the isolated soluble and membrane-associated fractions of P have been established in our recent experiments [288]. Comparative fluorescence and photochemical studies of phytochrome in etiolated seedlings of maize and of the soluble and membrane-bound fractions isolated from them have shown that λ_{max} and γ_1 reached maximum in the tips of coleoptiles and roots, 686 nm and 0.30–0.40, and the lowest values were observed in root base, down to 682 nm and ca 0.05, respectively. γ_2 did not differ much: 0.80–0.85 and 0.70–0.75 for the samples with the two extreme values of γ_1, respectively. These parameters correlated well with those obtained for the 124 kDa pigment in the soluble and membrane-bound fractions, the figures were 684 and 680 nm and 0.33 and 0.06, respectively. These variations of the in vivo parameters can be interpreted in agreement with [280,281] in terms of the two P A species – Pr′ and Pr″ (see above) and the observed correlation of the in vivo and in vitro parameters suggest that Pr′ and Pr″ are the soluble and membrane-bound fractions of P A. This possibility is indicated also by the correlation in the relative content of Pr″ [280] and of the membrane-associated P [164]: each of them increases in the succession: oat, pea, maize. The question to what extent this heterogeneity could be due to the existence of heterogeneous P protein isolated from etiolated seedlings [98,99] and of several iso-P genes of Type A [114] remains unknown. It should be mentioned that Wang et al. [365] did not find evidence that any of the three immunochemically detectable oat P's belonging to Type 1 and Type 2 is membrane-bound. I believe that their negative result can be accounted for by the fact that oat has, as it was mentioned above, relatively low content of the membrane-associated (Pr″) fraction [164,280]. The existence of the amphiphilic P sequences should be also mentioned which are exposed on the surface of the protein and which are potentially capable of interaction with other cellular macromolecules [211]. Considering this attribution of Pr′ and Pr″ to different phenomenological types of P A it is of interest to note two points: first, that P from plasma membranes [336] differs from the soluble one by the kinetics of light-induced destruction: the onset of the loss of the associated pigment was delayed and its final relative abundance was higher and second, the P membrane-binding was saturable. This suggests according to Terry et al. [336] that the plasma membrane-bound P represents a pool whose regulation is partially independent of the total Type 1 P. This description of the membrane-associated P is consistent with that of the Pr″ species.

Thus, $Pr'_{686/673}$ and $Pr''_{682/669}$ (designated by their emission/excitation (absorption) maxima) are the bulk, variable, light-labile, photoactive at low temperatures species with high γ_1 and the minor, conservative, light-stable, less photoactive at low temperatures species with low γ_1, respectively. Both of them belong to the Type 1 P (PhyA) (probably, soluble and membrane-bound species) and Pr′ itself is comprised by at least three subspecies, which are most likely different conformers of the chromophore.

Phytochrome B was shown in vitro to possess a

shorter-wavelength position of the absorption maximum and lower extent of the Pr → Pfr phototransformation (see above and [90]). Fluorescence and low-temperature photochemistry of phyB, as far as we know, were not investigated.

5.2.5. Phytochrome species in dormant and germinating seeds

The situation with the heterogeneous P species becomes even more complex if the dynamics of P content and photoactivity is followed in the germinating seeds. In [278] total P content, proportional to Pr fluorescence intensity at 77 K, and photoactivity at ambient temperatures (γ_2) were measured in dry seeds of pea and bean and in the early stages of their germination and in dark grown seedlings. Both parameters showed a complicated pattern of changes including at least four phases. They were interpreted in terms of relative concentration changes of two pools of P, only one of which (probably, a hydrated species [311]) can be phototransformed into Pfr with γ_2 of about 0.8. It was also suggested that the newly synthesized pigment was initially incapable of photoconversion into Pfr either because it was incompletely formed (needs a posttranslational modification) or it was synthesized in hydrophobic sites in the cell and only later was hydrated and thus acquired photochemical activity [278,302].

A complex picture has been revealed with respect to the P species in seeds with the use of immunochemical technique (see [363] and the literature cited therein). Three P apoproteins in seeds of *Avena sativa* L. with monomeric molecular masses 125, 124 and 123 kDa and relative abundance of 15, 18 and 67%. have been identified. At least one of them is photoactive and is believed to be responsible for photoregulation of seed germination and for regulation of the de novo synthesis of P during germination. The 124 kDa P in seeds is the same as the one predominating in the etiolated seedlings. It is tempting to assume that the two sides of the same complex picture are observed with the fluorescence and immunochemical methods; however, direct comparative investigations are needed to establish their relationships.

6. The far-red-absorbing phytochrome form

The properties of the excited states and photoprocesses in Pfr are much less known than in Pr. This is largely due to the lack of Pfr fluorescence. However, a similar scheme as shown in Fig. 8 can be used with respect to the Pfr → lumi-F phototransformation and explain some of the photophysical and photochemical properties of Pfr: (i) very low ϕ_F ($< 10^{-3}$–10^{-4}) in 124 oat phytochrome at liquid helium temperature (Sineshchekov, Deeg, Rüdiger, unpublished data); (ii) the fact that the Pfr → lumi-F phototransformation is an activated process with E_a close to that of the Pr → lumi-R photoreaction [248] and (iii) the quantum yield of the Pfr → Pr phototransformation is close to that of the forward photoprocess [112,158,213,356]. It is obvious that the sum of the constants of the radiationless relaxation, $k_d + k_p$, should be 2–3 orders of magnitude higher than k_f in Pfr, even at low temperatures, in contrast with Pr, where they are comparable. (k_f should be about the same in Pfr and Pr because the oscillator strength does not change much upon the Pr → Pfr phototransformation.) The relationship between the temperature-dependent and temperature-independent constants of the excitation deactivation, k_d and k_p, should be such that at ambient temperatures $k_p > k_d$, i.e., the main channel of deactivation is photoreaction, and at low temperatures the situation is reverse, $k_p < k_d$. The picture is similar to the one observed for the Pr → lumi-R phototransformation. The higher value of the relaxation constants in Pfr than in Pr is likely to be connected with a higher degree of kinetic flexibility in a less rigid surrounding of the chromophore in Pfr. This is suggested by the fact that the absorption spectrum of Pfr is more diffuse than that of Pr (there is no or only a very small vibrational satellite at 670 nm) and that the thermal relaxation from Pfr to Pr could take place in the dark (dark reversion). It should be also mentioned that neither phytochromobilin nor denatured P in the Pr form show any photochemistry, while those in Pfr do [243]. It should be noted, however, that in general the two observations – the absence of fluorescence and the existence of a potential barrier for the photoreaction in the excited state of Pfr – are of a conflicting nature: the absence of emission implies a very fast photochemical and other deactivating processes and this, in its turn, demands that the potential curve in the excited state between Pfr and lumi-F has a barrier-less profile.

7. Transformations of the intermediates of the phytochrome cycle

The initial photoreactions of Pr and Pfr lead to the formation of the first photoproducts unstable at ambient temperatures, which are characterized by excess energy and which are capable of initiating a succession of endergonic dark reactions leading eventually to the appearance of the respective end products, Pfr and Pr.

7.1. Kinetics of the transformation

In the early works on flash-photolysis in the time interval from 0.2 ms to several min and on steady state low-temperature absorption spectroscopy [24,170,171,217] it was established that: (1) The phototransformations of Pr and Pfr take place via several intermediate forms. (2) No intermediates on the two pathways are common. (3) Parallel reactions of phototransformation exist. (4) Small positive entropy in the dark reactions suggests minor changes in the protein conformations [217].

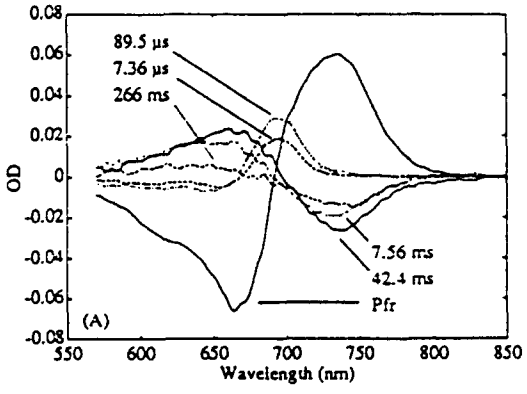
Kinetic measurements with microsecond time resolution including double-flash experiments on 'large' and native P [51,73,124,127,128,220,221,246,273] basically confirmed earlier observations held primarily on 'small' P. According to [128], the phototransformation from Pr to Pfr at physiological temperatures include four sequential intermediates: lumi-R, meta-Ra, meta-Rb′ (different from meta-Rb at low temperatures [75]) and meta-Rc. The decay process consists of three parallel reaction components, which differ by approx. one order of magnitude in their rate constants [127,221,273]. The pathways of the Pr → Pfr phototransformation in vivo are in principle the same as in vitro [125].

In the recent investigation, however, Zhang et al. [377] have challenged the validity of the earlier kinetic measurements on the grounds that they were carried out and analyzed separately at different wavelength and that this is not considered to be sufficient to determine the spectra of the intermediates and to resolve the mechanism of the phototransformation. They suspect that the transient spectra in [77,128,273] resulted from the absorption of multiple intermediates rather than a unique one. The authors obtained transient difference absorption spectra on the 124 kDa oat P after laser photolysis at 10° C. The global analysis of the data shows that the phototransformation from Pr to Pfr involves five kinetic intermediates with the lifetimes of 7.4 and 89.5 μs and 7.6, 42.4 and > 266 ms (lumi-R1, lumi-R2, meta-Ra1, meta-Ra2, and meta-Rc). The decay times of 7.4 and 89.5 μs were comparable with 11 and 102 μs in [246] and 14.5 and 217 μs in [51]. The data are compatible with the kinetic models involving two parallel pathways, or a sequential pathway with equilibria at certain stages. The formation of Pfr involves an unbranched reaction (see Fig. 10).

In [266] the first steps in the Pr → Pfr phototransformation in oat 124 kDa P were investigated by two laser/two color flash photolysis technique and analyzed using the global analysis algorithm. Three kinetic models, which might be applied to the observed multiexponential I700 decay, were considered: parallel, sequential and equilibrium. It was believed that the kinetic behavior of the system upon second flash could help to solve the problem. The experimental picture was, however, complicated by the appearance of a new intermediate(s) by the second laser excitation. Thus, at present there is no unequivocal answer to the problem. The authors, however, favor the parallel model based on the most probable spectrum for a particular transient species. It is also concluded that Pr is not a mixture of various chromoproteins differing significantly in their absorption spectra and transient decay kinetics.

7.2. Low temperature absorption spectroscopy of the intermediates

The picture obtained with the use of low-temperature technique (see for review [148,311]) was in many respects similar to that observed with the time-resolved method: appearance of the first stable intermediate, two other intermediates (with lower absorbance) were observed upon temperature increase, two intermediates were found in the reverse reaction, and the intermediate with lower extinction coefficient preceded the formation of Pr. In the recent studies the most elaborated characterization of the interme-



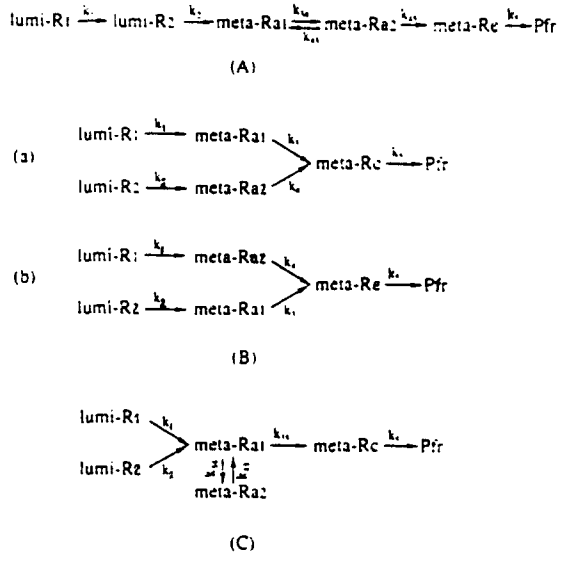


Fig. 10. Calculated transient difference absorption spectra of native oat phytochrome at 10° C following 7 ns laser photolysis of the red P form (left panel) and plausible kinetic models (A–C) for its transformation (right panel). The spectra were obtained from the experimental ones with the use of global analysis assuming five intermediates (lumi-R1, lumi-R2, meta-Ra1, meta-Ra2, and meta-Rc) with an apparent rate constants in a decreasing order as labeled. (Modified from [377].)

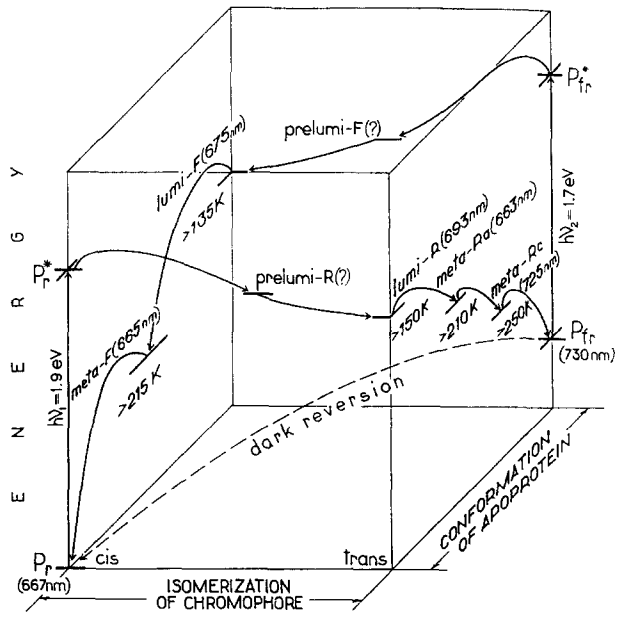


Fig. 11. Simplified energy level diagram of the phytochrome photocycle reflecting (i) changes of the chromophore configuration and energy storage in the photoreactions and (ii) dark relaxations of the photoproducts involving changes of the apoprotein conformation (based primarily on the data in [75,107,238,241]). Temperature presents approximate limits of stability of the intermediates, position of their absorption maxima are given in parentheses. The scheme is to be considered in the light of recent publications suggesting, in particular, formation of at least two lumi-R and, possibly, two meta-Ra states from homogeneous Pr pool and/or existence of heterogeneous Pr populations which initiate parallel cycles of Pr → Pfr phototransfomations (see for discussion Section 7).

diates was given by the Rüdiger's group, which in major details supports the scheme by Kendrick and Spruit [148] (see Fig. 11). According to [75] lumi-R is stable at temperatures below −120° C. The next intermediate, meta-Ra, hypsochromically shifted and with lower extinction coefficient, is formed by dark relaxation of lumi-R or pure meta-Ra is formed by irradiation of Pr at −85 to −65° C. Meta-Rb (P_{bl}) is formed in degraded P and it has a strongly bleached longer wavelength band. Native P yields another product, meta-Rc, with the maximum at 710–716 nm and which was observed also in vivo [148]. It is suggested [75,309] that meta-Rc can phototransform into meta-Rb, which slowly decays back to meta-Rc, and to Pfr, in the dark. In the reverse reaction, Rüdiger et al. [241] have found two intermediates called lumi-F and meta-F at −140° C and at −60° C with the maxima at 675 and 660 nm, respectively, in native and degraded P. Kendrick and Spruit [148] have detected two species of meta F, meta-Fa and meta-Fb, and recently two subspecies of meta-Fa were reported [360] in the Pfr phototransformation.

7.3. Circular dicrhroism measurements of the Pr → Pfr photoconversion

7.3.1. Red–far-red region

In the steady state CD measurements, the CD bands of Pr (positive) and Pfr (negative) practically coincide with the respective absorption band of the chromophore and are attributed primarily to the asymmetrical binding of the chromophore in the apoprotein and distortion of its conformation from that of free chromophore in solution due to protein constrains [15,148]. It is believed [15] that the CD of a protein-bound chromophore is often due to asymmetrical electric dipole interactions with nearby aromatic amino acid residues in the chromophore binding pocket. This type of interaction contributes predominantly to CD, in particular, in the case of visual pigments. CD investigations of the intermediates of the Pr → Pfr photoconversion have been carried out using the procedure of their entrapment at low temperatures [71] and by a time-resolved CD (TRCD) technique [15,38]. *Low temperature CD spectra* of the intermediates of the Pr → Pfr photoconversion have been measured by Eilfeld and Eilfeld [71]. The difference CD spectrum at 163 K with a maximum at 695 nm corresponding to the difference absorption spectrum at this wavelength was assigned to lumi-R. The latter intermediate species (meta-R) obtained upon the rise of the temperature and Pfr were characterised by decreased CD in their absorption bands as compared to the CD signal of Pr and lumi-R. Substantial technical progress has recently made possible *TRCD measurements* of native phytochrome at physiological temperatures in the visible [15] and UV [38] spectral region. Björling et al. [15] have recorded transient CD spectra of 124 kDa oat phytochrome in the 500 to 800 nm spectral region from 500 ns to 500 ms after its photolysis at 283 K. It was found that the CD intensity of Pr drops down upon generation of the species after photolysis. Moreover, a striking observation in view of the above low-temperature CD studies [71] was that the appearance of the lumi-R absorption band in the absorption transition spectrum at 695 nm was not followed by the appearance of the CD band. This fact was interpreted by the authors [15] in terms of the relaxed and unrelaxed conformations of the chromopohore in the apoprotein pocket. Assuming that the absorption and CD of the chromophore in P are due to different kinds of the chromophore–apoprotein interaction, i.e., absorption spectra of Pr, Pfr and the photointermediates are associated with the protonation status of the tetrapyrrole and its interaction with charged amino acid residue(s) while CD spectra, with aromatic residue(s), the authors suggest that in strained, unrelaxed lumi-R at low temperatures chromophore moves away primarily from charged residues after the photoisomerization while the dipole interaction between the chromophore and aromatic residue(s) remains significant. In the relaxed conformation of lumi-R the chromophore binding pocket undergoes a conformational relaxation to accommodate the new chromophore orientation and this results in a decrease of the chromophore interaction with aromatic residue(s). Weak interaction between the chromophore and the aromatic residue(s) are expected also in the latter intermediates and Pfr because they exhibit small CD in their chromophore absorption bands both in the time-resolved and low-tem-

perature studies. The authors also point out that the differences in the unrelaxed and relaxed lumi-R must lie in the apoprotein pocket and not at exposed sites of the protein since the first two steps of the Pr transformation are quite independent of macroscopic environmental conditions [77,246].

7.3.2. Far-UV region

Steady-state CD measurements detected a change in the ellipticity at 220 nm upon Pr → Pfr conversion, indicating a 3–5% increase of α-helix content in the protein [36,315,359]. Recently TRCD technique with the time resolution of approx. 900 ns was applied by Chen and coworkers [38] to probe Pr, Pfr and intermediates of native 124 kDa oat P in the UV region in the time interval from 100 μs to 1 s after illumination of Pr samples. The TRCD spectra also exhibited an increase in ellipticity at 220 nm in Pfr relative to Pr suggesting enhanced α-helical folding of the protein in Pfr (Fig. 12, left). Kinetics of the α-helical feature (Fig. 12, right) suggests that the increase in α-helical folding in Pfr is associated with decay of meta-Ra2 and/or meta-Rc with respective lifetimes of approx. 40 ms and > 266 ms as determined in [377]. The authors point to the fact that the structural changes are localized within the functionally important 6 kDa N-terminal domain, as suggested, in particular, by the absence of CD changes in the 'large' phytochrome [359], and also by suppression of the CD changes in native phytochrome by N-terminal-binding monoclonal antibodies [36]. Conformational changes of the N-terminal segment are also pointed at by proteolitic cleavage patterns [136], phosphorylation sites [375] and differential antibody binding [49]. Recently, the photoreversible conformational changes associated with the Pr → Pfr transformation have been probed by CD studies on dicot (pea) P [56]. It was shown that the secondary structure of pea P is very similar to the structure of monocot oat P determined in [38] and an increase in the α-helical folding of the apoprotein takes place when Pr is phototransformed to Pfr as with oat P. The authors believe this conformational change to be a general characteristic of all P's A.

7.4. Effect of environmental factors

The rate of the Pr → Pfr transformation is influenced by the surrounding medium of the P molecule. In the earlier work [148] it was reported that the accumulation of the intermediates and formation of Pfr depended on the redox state of the plant. Later [76,77] it has been shown that the formation of meta-Rc and Pfr was retarded by increasing microviscosity of the medium suggesting the involvement of the apoprotein rearrangement. These data support the above results of the kinetics of the far-UV CD [38] suggesting that the changes of the protein structure take place at the later stages of the Pr → Pfr conversion. Slight differences in kinetic parameters between native and in vivo P were brought about according to [124] by different micro-environment of P molecules, in particular by changes of pH. The latter was interpreted to mean that the proton uptake occurs in the final step from Pr to Pfr.

Lindemann et al. [169] investigated the effect of Ca^{2+}, Mg^{2+} and Ca^{2+} · calmodulin on the decay kinetics of the I700(1,2) intermediates of 124 kDa *Avena* P. It was shown that Ca^{2+} and Mg^{2+} selectively and sufficiently bind to P and that Ca^{2+} · calmodulin strongly decreased the Arrhenius parameters of I700(1,2) decay kinetics and blue shifted the I700 absorption maximum by 3–7 nm. The quantum yield of the overall Pr → Pfr conversion was reduced to almost 50% by 1 mM $CaCl_2$. The authors point to a possible specific site for Ca^{2+}-binding and modulation of the P action between the residues 20 and 60. However, they caution that the Ca^{2+} concentrations inducing the effects are much higher than those in a plant cell and that their findings do not necessarily lead to conclude that the Ca^{2+} binding to P is functionally relevant.

Bound monoclonal antibodies also alter the decay of the phototransformation intermediates I700(1,2) from native *Avena* P [168]. The strongest influence on these parameters was observed with OAT 8 (epitope between residues 624 and 686), which decreased by more than 50% the activation parameters of both components. This decrease was interpreted to result from an increased flexibility induced

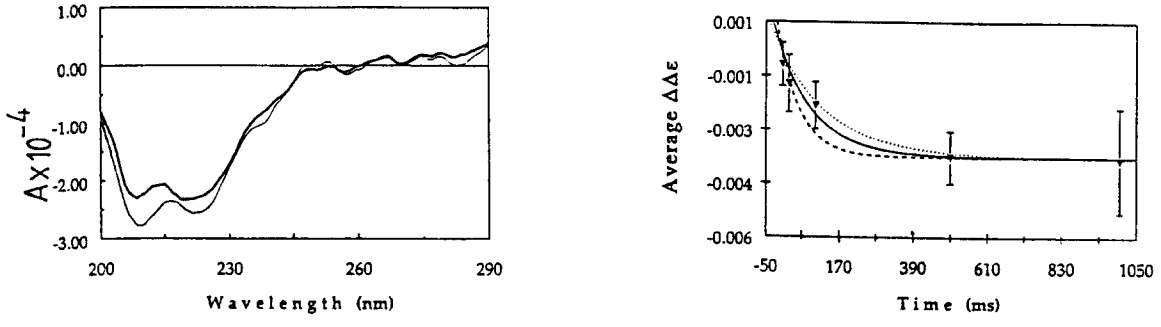


Fig. 12. Far-UV time resolved circular dichroism spectra of native oat Pr versus Pfr at 1 s after 7 ns 640 nm laser flash at 10° C (the Pfr spectrum exhibits an increase in ellipticity at 220 nm indicative of the enhanced α-helical folding in Pfr relative to the Pr (heavy line) spectrum) (left) and temporal behavior of the α-helical feature (comparison of the experimental data with single exponential functions with decay rates of 113 (—), 159 (· · · ·), and 67 ms (- - -)) (right) (From [38]).

by this antibody. The authors suggest that the OAT 8 epitope is functionally important during the decay of the I700(1,2) intermediates. For the case of I700(1), bound OAT 23 and OAT 25 (epitopes between residues 1 and 66) were shown to reduce the relatively small flexibility of these bonds in Pr. These data and the fact that the monomer molecular size has little effect on the initial stages of the Pr → Pfr phototransformation suggest that the photoprocess mainly depends on the chromophore domain of the apoprotein and on the flexibility of the P molecule [124,246].

7.5. Photoreactions of the intermediates and their functional implication

The problem of the photoactivity of the intermediates of the Pr ⇔ Pfr phototransformation cycle arises in connection with their possible accumulation under normal daylight, when up to 50% of P_{tot} could be accumulated in the state of the slow-transforming intermediates (see [309] and the literature cited therein). They themselves undergo photoconversions which change the operation of the cycle and concentration of the physiologically active Pfr. The situation is somewhat different in the case of the short-lived lumi-R state, which does not accumulate under natural light conditions but whose photoreaction can be induced with laser pulses [139,257–260,309]. Stimulation of seed and spore germination was greatly reduced upon irradiation in the region of maximal lumi-R absorption at 690 nm with respect to that obtained with laser irradiation at 620 nm [257]. This is interpreted in terms of lumi-R, which is formed during the laser pulse and is partially reverted to Pr at the expense of Pfr when a relatively long laser pulse (10 ns) is used. It is essential in the context of the present discussion that, by measuring the effect of nanosecond laser pulses reversing the Pr → lumi-R photoreaction on germination of fern spores, it was established [259,260] that the decay of the lumi-R (I700) species and formation of Pfr proceeds with various rate constants in agreement with the data of spectroscopic measurements (see above). The authors do not exclude, however, the possibility that different P pools could be responsible for the origin of the different rate constants. Physiological experiments on polarotropism of fern protonemata [140,141], in which effects of a polarized second flash on the suppression of the polarotropism were investigated, have also shown that dichroic orientation of I700 and Ibl are parallel to that of Pr and that the direction of the transition moment of P molecules should change at the last step in the Pr → Pfr transformation (see [361] for review on dichroic orientation of P).

8. Structural aspects of the phytochrome photoconversion

8.1. Vibrational spectroscopy

Resonance Raman (RR) spectra of the Pr form of 124 kDa oat P were obtained *with far-red excitation (752 nm)* at low (77 K) and ambient temperatures by Fodor et al. [85,86] (Fig. 13). The bands in the spectra were assigned by comparison with model compound spectra and vibrational calculations on protonated biliverdin dimethyl ester (BVDE) and by the characteristic shifts which occur upon deuteration of the pyrrole nitrogens. The lack of carbonyl intensity, the frequencies of the 1626 cm^{-1} which exhibited a downshift by 5 cm^{-1} in D_2O, a 1644 cm^{-1} C = C stretching mode, and an intense mode at 1326 cm^{-1} are all consistent, according to the authors view, with a protonated structure for the tetrapyrrole chromophore in Pr. Protonation of the pyrrole nitrogen is believed to be the cause of the approx. 50 nm red-shift of the protein-bound chromophore absorption compared to the chromophore in vitro.

Tokutomi et al. [345] have reported at 288 K *the*

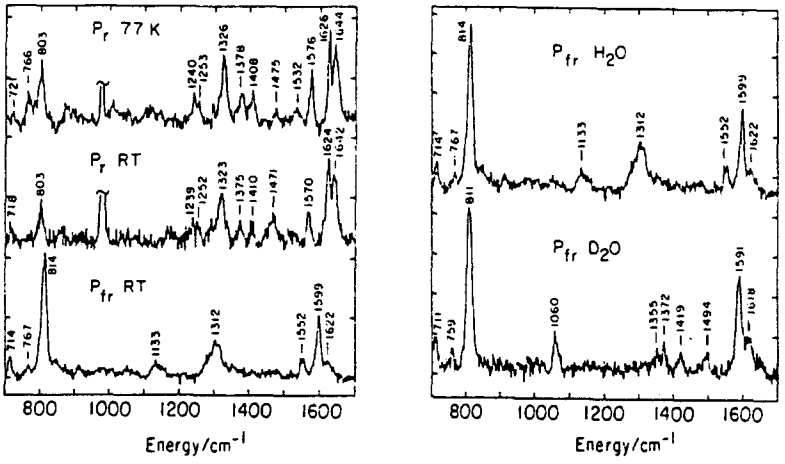


Fig. 13. Comparison of the resonance Raman spectra of the Pr and Pfr forms of 124 oat phytochrome (752 nm probe, RT stands for room temperature) (left) and of the Raman spectra of Pfr in H_2O and D_2O at room temperature (right) (from [86]).

blue-excited (at 406.7 and 363.8 nm) RR spectra of Pr and Pfr and also of P_{bl} at different pH on 'large' pea P in H_2O and D_2O. The sample was R-FR illuminated at different pH in a microspinning cell to obtain different populations of Pr, Pfr and P_{bl} estimated from the absorption spectra. The authors ascribe the two prominent bands at 1630 and 1590 cm^{-1} (and also the bands at 1546 and 1521 cm^{-1}) to Pfr. The 1625–1627 cm^{-1} bands are assigned to Pr in agreement with [85]. The 1625 cm^{-1} band, which shows a deuterium shift, presumably arises, according to the authors' view, from the C = NH or C = C stretching vibration of the pyrrole ring C. The deuteration shift of RR band (1590 cm^{-1}) was not recognized for Pfr (in contrast with 5 cm^{-1} shift of the 1626 cm^{-1} band in Pr), suggesting that the protonation state of ring C is the same in the Pfr and P_{bl} and differs from that of Pr. This is in agreement with proton release upon Pr → Pfr transformation [90], deuteration effect on phototransformation kinetics [5,25] and the character of the absorption spectra at low temperatures [241].

Surface-enhanced resonance Raman scattering (SERRS) spectroscopy was used [83,235] to probe structural differences between the Pr and Pfr forms of 124 kDa oat P and of low-temperature intermediates in the transformation of Pfr to Pr. The SERRS spectra clearly differentiated between the Pr and Pfr forms in the high and medium-to-low wavenumber regions, especially in the bands 1624, 1596, 1458, 1414, 1252, 1163 cm^{-1}, the greatest differences between Pr and Pfr being in the first two bands, which were assigned to the C15–C16 and C10–C11, respectively. The difference was rationalized in terms of the model involving a *Z Z Z* (= 4*Z*, 10*Z*, 15*Z*) to *Z Z E* photoisomerization about the C15 = C16 bond. The authors expect some subtle protein-controlled structural differences between the chromophores of the two forms because relative intensity differences rather than spectral shifts are observed. The overall chromophore conformations are believed to be conserved for the native Pr and Pfr and slight out-of-plane ring twisting, accompanying the Pr → Pfr photoisomerization, is thought to be responsible for the large difference in the spectroscopic properties of the native Pr and Pfr chromophores. The authors have observed the emergence of new bands and substantial relative intensity differences in the SERRS spectra of a single form of P with a change of only 7 nm in excitation wavelength (406.7 and 413.1 nm). This suggests the existence of at least two, near-degenerate electronic states in this spectral region.

Recently *Fourier Transform resonance Raman spectroscopy* (FT-RR) was applied to native oat P by Hildebrandt et al. [116] with near-infrared excitation (1064 nm). The FT-RR spectra of Pr and Pfr are in agreement with conventional RR spectra [85,86], although with better accuracy and slightly different peak frequencies. The authors presented a detailed vibrational assignment: (1) C = C stretching vibrations – the strongest bands in H_2O at 1639 and 1622 cm^{-1}, which reveal small isotopic shift upon H/D exchange, were assigned preferentially to C = C vibrations of the methine bridges adjacent to the rings A and D in Pr. (2) N–H vibrations – the bands at 1375, 1319, 1241 and 1176 cm^{-1} and also the broad and intense hump at 1100 cm^{-1} are attributed to the in plane (i.p.) bending and out of plane (o.o.p.) vibration. (3) C–H o.o.p. vibrations – the bands at approx. 800 cm^{-1} were attributed to the methine bridges. The authors expect from this analysis the non-coplanarity of ring D with respect to the other rings, which is in agreement with NMR studies of chromopeptides from Pr and Pfr [86,161,239,245,340]. It reveals the rotation around the double and single bonds and suggests that the *Z*, *Z*, *E* configuration is not relaxed. This may provide the driving force for the thermal reverse reaction of Pfr to Pr. Deprotonation of ring C in the Pfr form is suggested by the disappearance of the N–H o.o.p. vibration in Pfr. And finally, a slight distortion about the central C10 bridge is expected in both forms.

Ultraviolet resonance Raman (UVRR) spectra of the pigment in the Pr and Pfr forms have been recently obtained at ambient temperature by Toyama et al. [348] on 121 kDa pea P (excitation at 240 nm) and by Mizutani et al. [190] on intact, 'large' and 'small' pea P's (excitation at 244 nm). This technique provides information on conformational changes in the protein moiety accompanying the above-discussed primary photoprocesses of the chromophore. The results of these two pioneering works are in agreement with each other and can be summarized as follows: Major peaks of the UVRR spectra at 1621, 1553, 1358 and 1342 cm^{-1} are assigned to tryptophan (Trp) based on the comparison with aqueous Trp solution. The majority of Trp residues are located in non-aliphatic (hydrophilic) microenvironments in Pr. Upon phototransformation into Pfr (and I_{bl}), the microenvironment around some Trp residues becomes hydrophobic due to conformational changes and hydrophobicity increase occurs mainly in the chromophore domain. Interactions between Trp residues and the chromophore occur in Pr and the strength of these interactions diminishes in the Pfr form.

Fourier transform infrared difference spectroscopy have been applied to phytochrome by Siebert and coworkers [277]. Films of 124 kDa oat phytochrome hydrated with ^1H_2O and ^2H_2O were used to obtain the initial IR spectra in the Pr and Pfr forms and their difference spectra. Comparison of the latter with protonated and unprotonated model compounds (Et8-bilindion and H2Et8-bilindion) and the ^2H_2O effect on the difference spectrum suggested that phytochrome in the Pr form was protonated in agreement with the resonance Raman studies [85]. The authors came to the conclusion that the Pfr form was also protonated.

8.2. Structural differences between the red- and far-red-absorbing phytochrome forms

Thus the result of the photoreaction and following dark transformations of the intermediates is the formation of the

physiologically active Pfr form from the initial inactive Pr. Structural differences which are believed to be the cause of the physiological activity of Pfr can be summarized as follows.

Chromophore. (1) The Pfr chromophore is different by its structure and electronic state from that in Pr. This is evidenced by the absorption spectrum of Pfr, approx. 60 nm red-shifted as compared with the spectrum of Pr, its more diffuse character and especially by the complete quenching of fluorescence in the Pfr form. (2) Analytical methods and Raman, FT-difference infrared and ^{1}H-NMR spectroscopy suggest that the basis of their structural differences is to be found in the state of the isomerization of the chromophore. The reaction is likely to involve photoisomerization about C15 = C16 double bond and also simultaneous rotation about C14–C15 single bond [240]. This requires the existence of the excited state surface with an activation barrier (which was experimentally shown, see above). (3) The question of the involvement of proton transfer in the Pr → Pfr phototransformation is not yet settled [319]. The Raman spectroscopy studies support the photoreversible de-protonation of the chromophore in the late stages of the Pr → Pfr conversion. 114 and 62 kDa chromopeptides of pea P were found to first release and subsequently to take up protons during the Pr ⇔ Pfr photoconversions, although with native 124 kDa P significantly less proton release and uptake were observed. The proton transfer between P and the medium may be prevented by C-terminal and probably N-terminal fragments [90]. The appearance of 315 nm band in the Pr → Pfr transient spectra in the 124 kDa oat P is believed to be a result of deprotonation of tyrosine residue(s) [377]. The data by Siebert et al. [277] indicate, however, that the chromophore is probably protonated in Pfr (although it should be mentioned that the experiments in this work were carried out on a solid film of the pigment). (4) The chromophore moves within the protein during photoconversion from Pr to Pfr as revealed by physiological experiments (see [361] for review) and linear dichroism studies (transition dipole moment rotates by 31°) [78]. Differences in tryptophane and tyrosine phosphorescence between the Pr and Pfr forms of 124 kDa P are interpreted in terms of energy migration resulting from changes in the distance or/and reorientation between the residues and the chromophore [318]. Recent investigations with the use of a novel fluorescence energy transfer method using labeled antibody fragments [82] also show that the chromophore moves relative to the N-terminus upon the Pr → Pfr phototransformation so that the distance between them decreases by approx. 10–12 A.

Apoprotein. These changes in the state of the chromophore are intimately connected with the alterations in the apoprotein. They are: (1) Differential exposure of Pfr chromophore relative to Pr in 124 kDa oat P as shown by means of oxidizing or reducing reagents [316,319]. The movement of the chromophore from its binding pocket upon photoisomerization in the Pr → Pfr photoconversion is suggested also from the molecular modeling of phytochrome [205]. (2) Folding of the random coil structure at N-terminus into an α-helix (α-helical content increases by 3–5%) upon the Pr → Pfr transformation, as revealed by circular dichroism measurements [36,38,207,320,359]. The data of [277] on FTIR and of [190] on UVRR seem to be in contradiction with the above CD studies suggesting that the photoreversible changes in the α-helix content is very low or even opposite to the reported. It should be noted, however, that Mizutani et al. [190] admit the relatively low resolution of their UVRR spectra, which does not allow them to discuss the changes of the Pr conformation quantitatively, and Siebert et al. [277] have used phytochrome in solid film, i.e., under the conditions in which amphiphilic N-terminal chain could fold into α-helix already in the Pr form as suggested in [92,206]. 3. Changes in the apoprotein are suggested by different thermostability of the two forms, see [165,360] and the literature cited therein and Pfr has a larger (by several hundreds Da) molecular mass [135,160]. 4. Differential sensitivity to proteolysis [160,162,358] and binding studies with monoclonal antibodies, which show that a number of antibodies preferentially bind to Pr [50,117], reveal major regions of photoconversion-induced conformational changes in P A (summarized in [222]): within a 10 kDa subdomain at N-terminus; at the junction of the principal N- and C-terminal domains; and around glutamate-354. Recent limited proteolysis studies [200] on 121 kDa pea phytochrome have also shown different sites of cleavage in the two forms: three in Pfr (arginine-746 to lysine-752, around glutamate-877 and arginine-1010) and only one in Pr (glutamate-38–arginine-62), suggesting different patterns of the apoprotein surface exposure in both forms. The surface-exposed hydrophobic regions in Pfr are believed to be involved in the biological activity of the pigment. Some of the above differences between Pr and Pfr are illustrated by the scheme proposed in Song's group [38,235,318] (see Fig. 14). According to this, the first event is a chromophore rearrangement in the chromophore pocket connected with the lumi-R formation as the result of the tetrapyrrole isomerization. This leads to the decrease of the distance between the chromophore and the N-terminus. The *cis-trans* isomerization and subsequent movement of the chromophore may introduce to the protein a strain that is manifested in part as a helical change. The final configuration of Pfr is assumed after stepwise protein rearrangement which relieves the strain.

9. Photoprocesses in related compounds

It seems expedient for further elucidation of the photophysical and photochemical properties of P to touch upon other systems containing bile pigments as a chromophore [255].

9.1. *Phycobiliproteins: photoreaction versus energy transfer*

Phycobiliproteins by their functions fall into two groups of pigments: the first, quantitatively major one, is the group of the *light harvesting pigments* of photosynthesis in cyanobacteria and red algae (phycoerythrin, phycocyanin, phycoerythrocyanin, allophycocyanin). Efficient energy migration between these pigments proceeds by the inductive resonance mechanism from the shorter wavelength species to the longer wavelength ones in the ps time interval [95,256]. Distinct routes of the energy transfer in the complex system of pigment species of phycobilisomes were followed by time-resolved fluorescence spectroscopy [118,119], by fluorescence sensitization measurements ([286,294] and the literature cited therein) and by theoretical calculations ([249] and the literature cited therein). One critical parameter for the efficient energy donation is high fluorescence yield, which is characteristic for all light-harvesting biliproteins. With the flexible chromophore of the pigment – viz. a linear tetrapyrrole – this is achieved by the rigidity of the immediate protein surrounding of the chromophore restricting the freedom of its torsional motion (see Section 3.3 and [262,263]). According to [279,303] this may result in the increase of the activation barrier for the photoreaction in the excited state and slowing down the radiation-less deactivation processes. Our estimations of E_a for the fluorescence decay in phycoerythrin and phycocyanin gave values higher than 100 kJ mol^{-1} (Sineshchekov, unpublished data), which certainly cannot be overcome at ambient temperatures.

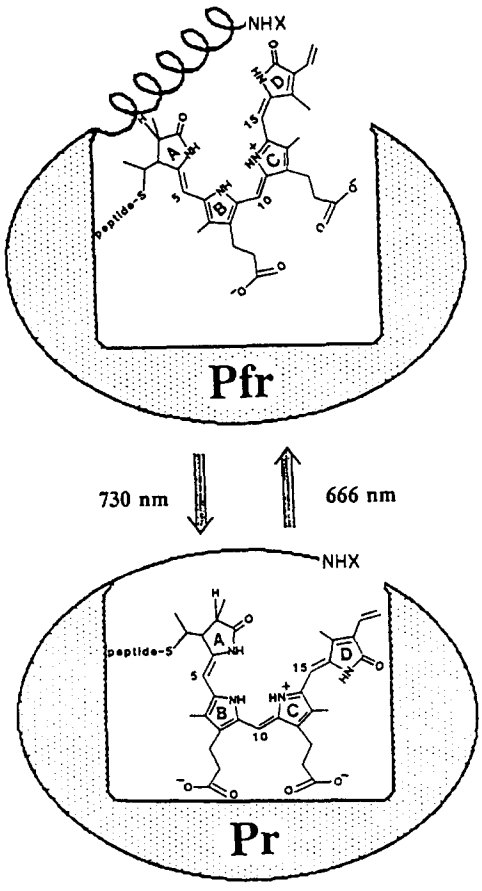


Fig. 14. Representation of the phototransformation of the Pr chromophore to the Pfr chromophore. The model incorporates: semi-extended conformation of the chromophore both in Pr and Pfr with conservation of the exocyclic dihedral angle at ring D by a chromophore–apoprotein interaction, protonation of ring C, reorientation and more exposed nature of the Pfr chromophore, increase in α-helical folding of the N-terminus. [235].

The second group are *photomorphochromes*, the functional pigments which have not yet been isolated, and *phycochromes*, which are isolated and which are capable of photochromic transformations but whose function is unknown [16]. The most investigated of the phycochromes is phycochrome b–α-subunit of phycoerythrocyanin (PEC). Its chromophore, phycoviolobilin, undergoes photochromic transformations under orange/green light similar to those observed for P [11,12,153,154,186,189,276]. Molecular mechanism of the PEC photoconversion includes *Z/E* photoisomerization of the double bond between rings C and D. Determination of the detailed molecular structure of PEC [66] allowed theoretical analysis of the molecular mechanism of its photoreversible photoconversion [253,254]. General conclusions from these works can be summarized as follows. First, in *the ground-state*, protein electrostatic and steric effects determine isomerization barrier (which is about 60 kJ mol^{-1} for thermal isomerization). Chromophore geometry, in particular, its Δ15 configuration, determines the active-site dynamics and the character of the torsional motion. The chromophore is fully protonated and its charged state is stabilized by a small set of strong local interactions (complex counter-ion – two carboxylate groups, one arginine and one tyrosine). The counter-ion network and the chromophore protonation site are only slightly affected by a ground state Δ15-*Z-E* isomerization of the chromophore, i.e., the protonation states are conserved. *The excited states* are localized on the chromophore and have an admixture of electron transfer states from nearby amino acids. The excited-state torsional potential surface shows a negative barrier for isomerization and trapping of an active complex. Strong coupling of excited states localized in the chromophore and charge transfer states from the surrounding polar residues provides favorable prerequisites for fast excited state crossing in competition with other deactivation processes. In other words, the excited state minima are located above the ground-state potential energy maxima along the photoisomerization coordinate and this provides a potential surface that is optimal for rapid and efficient photochemical isomerization.

Thus, competition between the photochemical and radiative routes of the excitation deactivation in phycobiliproteins depends on the state of the chromophore in the protein surrounding. This is evidenced also by the fact that loosening of the chromophore fixation in unfolding algal

biliproteins (as the result of increasing temperature or urea concentration) quenches its fluorescence and allows photoreaction (see [240] and the literature cited therein). In agreement with the above discussion and the advanced schemes [279,303], the chromophore–protein interaction determines the height of the energy barrier (E_a) of the photoreaction and, eventually, its rate constant. This, in its turn, determines the functional role of the pigment as a photosensitizer or light harvester.

9.2. The complex of bilirubin with human serum albumin

The above opinion gains support from the in vitro investigations of the photophysical and photochemical properties of the complex bilirubin (BR) (4*Z*,15*Z*-bilirubin-IX α') human serum albumin (BR/HSA) in which the chromophore is uncovalently incorporated in the protein. (It is not a biliprotein in the common sense and does not perform photobiological functions, but is involved in the transport of BR to the liver for processing and excretion.) The features of the photophysical and photochemical processes in BR/HSA (reviewed in [163]) show certain similarity to those of the photoprocesses described above for the P phototransformation: (i) ϕ_F is low at room temperature ($\phi_F = 0.003$), but increases enormously reaching 0.92 at $T < 120$ K; (ii) τ_F at room temperature is calculated to be approx. 10 ps; (iii) no phosphorescence has been observed at 77 K; (iv) the complex is photoactive and undergoes $Z \rightarrow E$ isomerization at 14,15 double bond with the yield of $\phi_P = 0.2$; (v) ϕ_P drops down practically to 0 along with the rise of ϕ_F upon lowering of temperature to $T < 120$ K. All the parameters and their changes with temperature were consistent according to [163] with operation of an activated (temperature-dependent) twisting about the 14,15 double bond in the excited singlet state. The activation barrier ($E_a = 23$ kJ mol^{-1}) was attributed to viscous drag in the protein environment. The twisting, which is very rapid (approx. 10 ps), competes with fluorescence, which is itself faster than other deactivation modes. In the twisted geometry, the excited state and ground state potential surfaces are brought together thus allowing very fast conversion from the excited to the ground state. And finally, efficiency in the $Z \rightarrow E$ isomerization is also due to partitioning of the twisted states: relaxation in the ground state returns the molecule to its original geometry or to the configurational ($Z \rightarrow E$) isomer. Thus, by all the above parameters this system can be considered up to a point as a physical model of the initial photoprocesses in Pr.

10. Comparative analysis of the photoprocesses in phytochromes and rhodopsins

The comparison of the two different classes of photochromic pigments – biliproteins and retinal proteins – is prompted by two major considerations. First, rhodopsins are the most important class of photobiological pigments which mediate the processes of vision in animals (for a review see [14,376]) and of phototaxis in unicellular motile algae *Chlamydomonas* [87,174] and in halobacteria [334]. The well investigated bacteriorhodopsin (BRh) also serves a photoenergetic function as a light-driven proton pump in *Halobacteria* [8,14,334]. Second, biliproteins and retinal proteins, in spite of their chemical differences, have much in common by the character of their molecular organization and by the nature of their photoprocesses (in particular, such features as flexible chromophores covalently bound to and specifically interacting with apoproteins, isomerization step in the initial photoreaction, stepwise transformation of the intermediates in the photocycles, existence of complex systems of signal transduction).

It is obvious that the excited states of the pigments and initial stages of the photoprocesses are to be considered first as a ground to establish common principles of their photochemistry. This becomes possible, in particular, because fluorescence spectroscopy has been also applied to the investigation of (bacterio)rhodopsin (see the reviews [68,96,166,293]) and here I will briefly summarize some of the results which are relevant to the current discussion.

10.1. Excited states, fluorescence and photoreactions of (bacterio)rhodopsin

In [290,291] it was shown that the emission of BRh is located beyond 550 nm, has a well resolved vibrational structure and low quantum yield even at low temperatures (77 K). The fluorescence proved to be heterogeneous. Three major components were distinguished by their fluorescence and photochemical properties: (1) photoactive and nonfluorescent even at low temperatures – *trans*-BRh and bathoproducts of *trans*-BRh and 13-*cis*-BRh ($\phi_F < 10^{-4}$); (2) photoactive and fluorescent – 13-*cis*-BRh and iso-BRh (the latter is formed from *trans*-BRh upon illumination at low temperatures and is very similar to it in all respects except higher yield of fluorescence) (ϕ_F is $4 \cdot 10^{-4}$ for 13-*cis*-BRh and 10^{-3} for iso-BRh) (Fig. 15) and (3) modified BRh species with relatively high quantum yield of fluorescence ($\phi_F > 10^{-2}$ at 77 K) and low photochemical activity [283,285,290,291]. This group includes also BRh incorporating artificially altered chromophores (e.g., acetylenic analog) with reduced flexibility [9,10].

A similar picture was observed with rhodopsin (Rh). Initially it was considered to be nonfluorescent [30,166]. It was shown, however, that the photoactive states of Rh in frog [284] and cattle [292] emit in the region 600–650 nm with quantum yields at 77 K of $(6 \pm 2) \cdot 10^{-4}$ and 10^{-3}, respectively (Fig. 15). Doucas et al. [65] have observed fluorescence of squid and cattle Rh in the region 620-640 nm with the yield 10^{-5} at room temperature. Fluorescence of the bathoproduct of Rh was not detected, even at low temperatures [284,292]. At the same time, fluorescence of

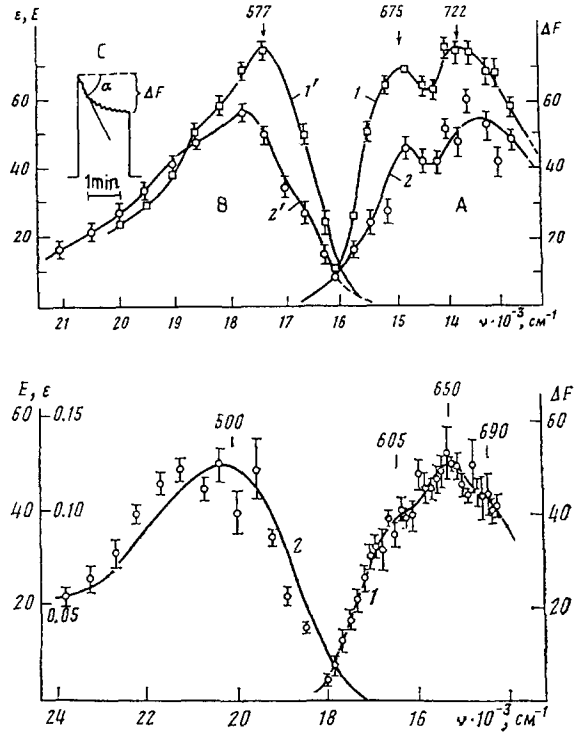


Fig. 15. Fluorescence emission and excitation spectra of bacteriorhodopsin (upper panel) and cattle rhodopsin (lower panel) at 77 K. Bacteriorhodopsin: emission (a) and excitation (b, points) spectra of *iso*-BRh which is formed from *trans*-BRh upon illumination at 77 K (1,1') and 13-*cis*-BRh (2,2'). (1') Absorption spectrum of light-adapted purple membrane (a mixture of *trans*-BRh and *iso*-BRh states); (2') absorption spectrum of 13-*cis*-BRh. The excitation spectra are constructed by the initial slope of the fluorescence intensity changes (α) in the photoreaction (c), fluorescence emission, by the amplitude of the variable fluorescence (ΔF) [283]. Rhodopsin: emission (1), excitation (2, points), and absorption (2, solid line) spectra [292]. Points are an average of 3–10 measurements, vertical bars, standard deviation, position of the maxima are given in nm.

crawfish meta-Rh was registered in [54] at room temperature with the maxima at 670 and 515 nm in the emission and excitation spectra and the yield of $(1.6 \pm 0.4) \cdot 10^{-3}$. Fluorescence of relatively high yield was observed in the modified Rh in which *cis-trans* isomerization was slowed down [96]. Analysis of the fluorescence characteristics of BRh and Rh allows certain generalization with respect to the properties of the excited states of the two pigments (see [293] for review).

Fluorescence quantum yield. This characteristic is the most important and, at the same time, the most variable for the different species. It drops down more than 10 fold by the temperature increase from 77 K to room temperature. E_a for the fluorescent BRh species, iso-BRh, was estimated [290,291] to be in the region 1.2–4.2 kJ mol^{-1} in the temperature interval where the bathoproduct is stable, 77–120 K. This agrees with the notion that the activation barrier for photoreaction of BRh is low. The yield of Rh fluorescence is also strongly dependent on temperature. The fluorescence yield of BRh and Rh does not depend on the wavelength of excitation. And finally, there is a reverse correlation of the quantum yield of the fluorescence and photochemical activity of the pigment species: the lowest quantum yield is in the photoactive species while the highest, in the modified photoinactive states.

Lifetimes of the excited states and the rate of photochemical reactions. Calculations of the real lifetimes give values in the range 0.7–70 ps. On the other hand, direct measurements of the lifetimes also give variable values and there is good correlation of the calculated and measured parameters. This complex picture reflects the existence of different species of BRh and Rh. There is a good correlation between the fluorescence lifetimes and the times of the photoreaction. This correlation points to the fact that the emission belongs to the allowed transition which competes with the photoreaction.

Fluorescence spectra. They are characterized by much more pronounced vibrational structure than that of the absorption (excitation) spectra, in other words, they do not follow the mirror image relationship (Fig. 15). The violation of the mirror image relationship suggests [202], that changes of the chromophore structure and of its immediate surrounding take place already in the excited state and that the emission proceeds from the state with altered molecular and vibronic structure. Because the emission spectra are more structured one can expect, by analogy with the spectra of stilbenes [84], that these changes of the structure in the excited state remove steric hindrance and the chromophore adopts a more planar conformation. These events might represent the first stage of light energy stabilization in the pigments, which precedes the formation of the ground state of the initial photoproduct.

Fluorescence polarization. The emission is highly polarized and its degree does not depend on the wavelength of emission and excitation. Directions of the transitions are the same both in absorption and emission. This suggests that structural changes in the excited state do not lead to substantial changes in the direction of the transitional moment in the chromophore.

These data point to the participation of the singlet excited states of the pigments in the photoreactions and the energies of 0–0 transitions are estimated to be about 1.7 eV for BRh and 1.9–2.0 eV for Rh.

In general we may conclude that the excited states and initial photoprocesses in P and (bacterio)rhodopsin have a number of fundamental common properties and that the scheme of the electronic levels of the red-absorbing P form and its initial photoproduct and transitions between them presented in Fig. 8 is applicable also to (bacterio)rhodopsin.

10.2. Photocycles

At ambient temperatures Rh and BRh (in its *trans*- and 13-*cis*-forms) undergo respectively the photobleaching se-

quence [376] and photocycle [9,178,183,335]. The primary event is photoisomerization – *cis-trans* in the case of Rh and 13-*cis*-BRh and *trans-cis* in the case of *trans*-BRh (see [14] for review). In spite of the different locations and directions of the isomerization, energy is stored in the photoreaction and all the subsequent reactions are thermal. In [291] we have postulated that isomerization serves a function of separation of the active groups in both pigments. A similar view was developed by Honig et al. [122] who consider isomerization as a means of intramolecular charge separation and as a universal mechanism applicable to other systems with flexible chromophores. Energy stabilization in the photoreaction and its spectral effects are explained in agreement with this model by the increase of the electrostatic interaction between the chromophore and the apoprotein. Recent models of the primary events in Rh and BRh are discussed in detail in [14]. Photoreactions are followed by restructuring of the apoprotein, which eventually leads to the photobiological effect. Rüdiger et al. [241] have applied the general considerations of Honig et al. to the cycle of P phototransformation (see above). And finally it should be noted that the heterogeneity of the photoactive BRh species implies the existence of several cycles of BRh [7,285], which is analogous to the existence of parallel cycles in P initiated by the subspecies of P A (see [302] and above).

10.3. Protein dynamics

Frauenfelder [89] advances several substantial points of general importance on the protein dynamics deduced from myoglobin investigations. First is the necessity of including friction in all electronically controlled reactions and the formation of intermediate states in the protein reactions. Second, complexity of the system is explained by the existence of conformational substates of the protein with slightly different tertiary structures, each with a somewhat different barrier height for the reactions. Protein dynamics includes two types of motion: first, transitions from substate to substate (equilibrium fluctuations) and nonequilibrium motions from one state to another (functionally important motions) (in the terminology of the present discussion the substates = states and states = intermediates). And finally, Frauenfelder introduces the concept of a proteinquake which takes place in the system under stress in which strain energy is stored when the stress is relieved. The system returns to equilibrium in a series of steps which can be followed by spectroscopic markers (chromophores in the case of pigments). The occurrence of several steps – functionally important motions – implies, according to the author, several tiers of conformational substates and suggests a hierarchical model for protein substates. This picture was suggested to be applicable to BRh. I believe from the above analysis that P, in spite of its higher complexity, also fits this picture by all the above criteria.

11. Conclusion

Two aspects in the problem of the heterogeneity of P deserve special attention. These are the existence (i) of several P's differing in the primary structure of the apoprotein and (ii) of different species within one P with one and the same 'chemical' or 'molecular' structure of the apoprotein. Considering the first aspect, it should be said that different P gene products, which vary by their physiological functions, were unequivocally shown to exist in the cell, although spectroscopic and photochemical properties are known primarily for P A. Recent advances in the genetics and photophysiology of P apoprotein mutants and development of the in vivo fluorescence spectroscopy of P promises rapid progress in our understanding of the photochemical properties of types other than P A and the role of the chromophore interaction with the apoprotein and individual residues by which the P's differ may thus be elucidated. Very promising in this context are the experiments with gene-engineered deletion and site-specific mutant P's.

Much less is known about the possibility of the existence of different P's within one gene product, which could be due to posttranslational modification of the pigment. Heterogeneity within one P pool (phyA) is currently connected with possible modification of the apoprotein, which could account for the appearance of membrane-bound fraction of the pigment. Preliminary data suggest that the membrane-bound fraction is present in the cell and is not an artefact of the extraction procedure. Further parallel investigations of fractionated and in vivo P with the use of immunochemistry and spectroscopy are needed (i) to prove the fact of the existence of P associated with other proteins, (ii) to understand the nature of the associates and (iii) to determine the functional significance of such an association. In the case of affirmative answers to these questions, the problem arises of the existence of posttranslationally modified species within the other pigment pools – phyB, phyC and others – which would substantially widen the number of potentially different functional states of P.

The second aspect, heterogeneity of P within each single chemical entity, seems to be important for the elucidation of the nature of the initial photochemical processes. In the literature, there are two points of view on the origin of this heterogeneity. According to the first one, the observed multiexponentiality of fluorescence decay and photoreaction kinetics in the ps time domain is a reflection of the heterogeneity of the excited states which originate from a homogeneous ground-state population. The other view is that the heterogeneity exists already at the level of the pigment ground state and is connected with the existence of different conformers of the chromophore which are in a thermodynamic equilibrium with each other. The conformers differ by the rate constant of the photoreaction although the extent of the Pr → Pfr phototransformation

remains practically the same. If the second point is correct then the problem of structure–function relationship arises with respect to the photochemical reaction. In other words, it is necessary to understand the nature of chromophore–apoprotein interactions which are optimized to adjust the barriers for photoisomerization. This would allow us to come closer to the elucidation of the catalytic action of the apoprotein towards its own chromophore. The question of the heterogeneity of the initial Pr state is closely related to the heterogeneity of the intermediates of the P cycle suggesting the existence of several cycles and, probably, of different states of the physiologically active Pfr form.

On the basis of a number of its principal photophysical and photochemical properties, P seems to be closely related to retinal proteins and substantially differ from the relative light-harvesting biliproteins. Such a similarity of the structurally dissimilar photochromic pigments and dissimilarity of structurally related pigments permits from the conceptual point of view the elucidation of the most essential features of the organization of the photochromic pigments which are necessary and sufficient for the effective photochemistry.

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