Recombinant Type A and B Phytochromes from Potato. Transient Absorption Spectroscopy†

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ABSTRACT: The cDNAs encoding full-length type A and B phytochromes (phyA and phyB, respectively) from potato were expressed in inducible yeast systems (*Saccharomyces cerevisiae* and *Pichia pastoris*). In addition, a deletion mutant of phyB (Δ1−74) was expressed. The apoproteins were reconstituted into chromoproteins by incorporation of the native chromophore, phytochromobilin (PΦB), and of phycocyanobilin (PCB). The incorporation of PΦB yielded chromoproteins with difference absorptions λ_{max} at 660 and 712 nm (P_r and P_{fr}, respectively) for phyA, and at 665 and 723 nm for phyB. All difference maxima of PCB phytochromes are blue-shifted by several nanometers with respect to those obtained with the PΦB chromophore. The deletion construct with PCB shows difference absorption maxima at 652 and 705 nm with the P_{fr} absorbance considerably reduced. Time-resolved kinetic analysis of a phyB-type phytochrome by nanosecond flash photolysis was performed for the first time. Recombinant full-length phyB afforded transient absorbance changes similar (but not identical) to those of phyA from *Avena*, whereas the kinetic behavior of these intermediates was very different. Contrary to phyA from *Avena*, the I_{700} intermediate from phyB reconstituted with either PCB or PΦB decayed following single exponential kinetics with a lifetime of 87 or 84 μs, respectively, at 10 °C. The formation of P_{fr} of PCB-containing recombinant phyB (phyB-PCB) could be fitted with three lifetimes of 9, 127, and 728 ms. The corresponding lifetimes of phyB-PΦB are 22.5, 343, and 2083 ms. Whereas for phyB-PCB all three millisecond lifetimes are related to the formation of P_{fr}, the 2 s component of phyB-PΦB is concomitant with a rapid recovery of P_r. For recombinant potato phyA and Δ1−74 phyB, no time-resolved data could be obtained due to the limited quantities available. As described for phytochromes of other dicotelydons, the P_{fr} forms of full-length phyA and phyB of potato underwent rapid dark conversion to P_r.

Phytochromes comprise a family of photosensory chromoproteins of plants (Quail, 1991; Vierstra, 1993; Whitelam & Harberd, 1994) which initiate and regulate a broad variety of photomorphogenic and physiologically important cellular processes as a function of light wavelength and intensity. Phytochromes can adopt two stable, spectrally distinct forms: P_r[1] with λ_{max} around 665 nm and P_{fr} with λ_{max} at 730 nm, which can be interconverted by irradiation with light of the appropriate wavelength. The phytochrome system, $P_r \rightleftarrows P_{fr}$, thus functions as a light-driven biological switch. Higher plants contain ensembles of phytochromes, which differ by *ca.* 50% of their amino acid sequences (Sharrock & Quail, 1989), and which are selectively responsible for sensing of the various light qualities, *e.g.*, end-of-day response, shade-avoidance, or high/low-irradiance-control. Neither the possibly different photochemical behavior nor the signal transduction pathways of the various phytochromes are fully characterized, and are therefore intensively studied.

Phytochromes consist of a protein moiety of *ca.* 125 kDa (about 1100 amino acid residues) to which the bilatriene phytochromobilin (PΦB) is covalently bound. A sulfhydryl group of a cysteine residue, located in the center of the N-terminal half of the protein, is condensed to the C-3 ethylidene double bond of the chromophore (see Figure 1). In the best studied phytochrome, phyA from oat, this position is at cysteine 321.

The most abundant information on the function of phytochromes has been derived from the study of phyA, the predominant species in etiolated plants. Physiological functions have been localized in various protein domains of phyA by the study of truncated peptides and deletion mutants, obtained either from proteolytic digestion or from recombinant DNA (Rüdiger & Thümmler, 1991; Cherry & Vierstra, 1994). The photoinduced reactions responsible for the $P_r \rightleftarrows P_{fr}$ interconversion are influenced selectively by the N-terminal half of the protein (Schaffner et al., 1991). Some of the physiologically important domains have been identified by the generation of transgenic plants which expressed only fragments of phytochromes, or by the characterization of specific phenotypes traced back to the loss of phytochrome-mediated functions. Expression of phytochrome fragments has allowed to identify the C-terminal part of the protein and a short stretch at the very beginning of the N-terminus (spanning amino acid residues 1 to *ca.* 65) as essential for

† Dedicated to Professor Horst Senger on the occasion of his 65th birthday.

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[1] Abbreviations: bp, base pair(s); DMSO, dimethyl sulfoxide; I_{700} and I_{bl}, intermediates of phytochrome photochemistry with maximal absorption around 700 nm (I_{700}) and with low absorption coefficient ("bleached" intermediate, I_{bl}), respectively; P_r and P_{fr}, red and far-red absorbing forms of phytochrome, respectively; PCB, phycocyanobilin; PΦB, phytochromobilin.

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Arg - Ala - Pro - His - *Gly* - **Cys** - His - *Ala* - Gln - Tyr - Met - *Ala* -.... phy B (potato)
Arg - Ala - Pro - His - *Tyr* - **Cys** - His - Leu - Gln - Tyr - Met - Glu - .... phy A (potato)
Arg - Ala - Pro - His - Ser - **Cys** - His - Leu - Gln - Tyr - Met - Glu - .... phy A (oat)

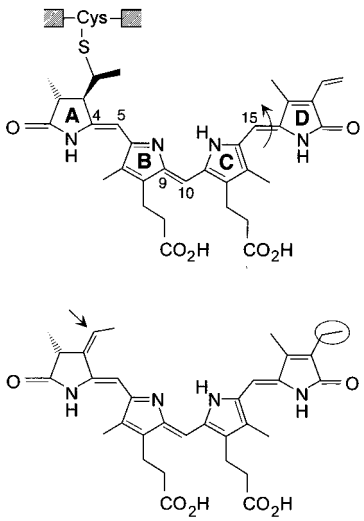

FIGURE 1: Structural formula of (top) the phytochrome chromophore phytochromobilin, PΦB, covalently attached to a cysteine residue of the protein *via* a thioether bond. The conformation of the chromophore (*anti*,*syn*,*anti*) has been chosen arbitrarily [*cf.* Schaffner et al., (1991) and Matysik et al., (1995)]. The only structural difference between PΦB and PCB (bottom) is the C-18 substitution in ring D (encircled). The 3′-position of the ethylidene substituent of the free chromophore which is covalently attached to the protein during the reconstitution is indicated by an arrow. The partial sequence in the vicinity of the chromophore-binding cysteine residue (given in boldface type) for potato phyB and the corresponding sequences for phyAs from potato and from oat are also shown for comparison. Amino acids given in italics refer to deviations from the consensus sequence.

phytochrome activity (Quail et al., 1995; Jordan et al., 1995). From studies with *Arabidopsis thaliana*, also a protein domain between positions 680 and 800 has recently been identified as being involved in signal transduction (Quail et al., 1995). For most of the proteolysis-derived studies and for extensive kinetic analyses of the $P_r \rightarrow P_{fr}$ phototransformation [see, *e.g.*, Zhang et al., (1992) and Scurlock et al., (1993b)], phytochromes from pea or from oat have been employed.

The primary reaction products of the phototransformation, I^i_{700}, are thermally unstable and convert with time constants of 8 and 80 μs (at 10 °C) into an intermediate (or a set of intermediates) possessing a low absorption coefficient (I_{bl}). The final product of the photoreaction, the thermally stable P_{fr} form with λ_{max} 730 nm, is generated with complex kinetics comprising components of *ca.* 8, 40, and 270 ms at 10 °C (Zhang et al., 1992). The presence of additional intermediates in the $P_r \rightarrow P_{fr}$ photoreaction and the detailed pathways followed by the intermediates are still subject to debate (Scurlock et al., 1993a,b).

The low abundance of phytochromes other than phyA, in both etiolated and de-etiolated plants, has prevented so far any kinetic analysis of photoinduced changes. Apart from $P_r - P_{fr}$ difference spectra for plant-extracted phyB (Abe et al., 1985; Tokuhisha et al., 1985; Wagner et al., 1991), no spectroscopic data have been reported for any non-phyA phytochromes. In fact, kinetic studies of phyB phototransformations must recourse to recombinant DNA technology. Recently, phyB from tobacco has been expressed in yeast and reconstituted into a functional (*i.e.*, photoreversible) chromoprotein by incubation with a chromophore analogue (phycocyanobilin, PCB). A $P_r - P_{fr}$ difference spectrum and an analysis of the thermal stability of the P_{fr} form have been presented (Kunkel et al., 1993, 1995). The P_r and the P_{fr} difference absorption maxima of this phyB-PCB phytochrome (at 658 and 712 nm, respectively) were very similar to those of recombinant phyA-PCB phytochromes (Wahleithner et al., 1991; Schmidt et al., 1996).

We here report on the cloning and expression of phyA and phyB apoproteins from potato in an inducible yeast expression system, the reconstitution of the apoproteins into photoreversible chromoproteins by incubation of each with the native chromophore PΦB and with PCB, and the time-resolved investigation of the photochemical reactivities. In addition to phyB full-length cDNA, also a deletion mutant, (Δ1−74), has been expressed and analyzed. This fragment was designed in analogy to large phyA phytochrome (Δ1−65), for which differences in physiological (Cherry et al., 1992) and spectroscopic (Cordonnier et al., 1985; Farrens et al., 1992) properties have been reported.

MATERIALS AND METHODS

Plasmid Preparation. phyB. PhyB was expressed in two inducible yeast systems. Full-length cDNA encoding phyB was cloned into the plasmid p2μG for expression in *Saccharomyces cerevisiae*, and into the plasmid pHIL-D2 for expression in *Pichia pastoris*. The cDNA encoding full-length phyB from potato was obtained from two originally cloned, overlapping cDNA fragments (Heyer & Gatz, 1992b) which encode the entire rear and most of the front part of phyB except the very first 15 base pairs (bp). Ligation of both cDNA fragments and the attachment of a PCR product completing the 5′-end resulted in full-length cDNA for phyB. Since cloning into the *S. cerevisiae* vector requires a *Bam*HI restriction site at the 5′-end of the cDNA, the same PCR was used to introduce such a site directly in front of the ATG-codon. This strategy allowed cloning into a *Bam*HI/*Kpn*I-restricted p2μG vector and induced expression.

The vector pHIL-D2 for expression in *P. pastoris* in its original form allows cloning only into a unique *Eco*RI site. Since *Eco*RI recognizes several sites within the phyB cDNA sequence, new restriction sites had to be introduced into the vector. pHIL-D2 was digested with *Eco*RI, and two complementary primers carrying a *Bam*HI and an *Xho*I site were ligated with the restricted pHIL-D2 vector. The sequences at both ends of the insertion primers were complementary to the *Eco*RI-derived ends of the vector and allowed ligation with it. However, both primers carried a mismatch at the last nucleotide of the *Eco*RI recognition motif (5′ end of front primer, AATT**G**...; 5′ end of rear primer, AATT**A**...) which upon successful ligation would result in the loss of the originally present *Eco*RI site. In order to avoid multiple insertion of the new cloning-site DNA, dephosphorylated oligonucleotides were used, and the phosphorylation was maintained at the ends of the linearized vector. Thus, vectors which were ligated without insert could be digested again with *Eco*RI, only leaving those vectors intact which had received the insert and the altered *Eco*RI site. This plasmid was named pHIL-MCS. phyB-cDNA was then cloned into pHIL-MCS *via* a *Bam*HI/*Xho*I restriction.

Δ1−74 phyB. The deletion of the first 74 codons from full-length cDNA (generation of the Δ1−74 mutant) was

performed by PCR. Primers were designed such that by the forward primer a *Bam*HI site was placed directly in front of an ATG codon which preceded the codon for glutamine-75. The reverse primer carried a restriction site for *Hpa*I according to the *Hpa*I site located 726 bp downstream of the ATG of the phyB gene. This strategy allowed the replacement of the original full-length *Bam*HI/*Hpa*I front part of phyB with the PCR product.

phyA. The cDNA encoding full-length phyA from potato was derived as a *Bam*HI restriction fragment from the clone for phyA, originally produced by Heyer and Gatz (1992a). The cDNA was cloned into the *Bam*HI site of p2μG, yielding the plasmid p2μGphyA. This cloning resulted in 27 additional bp in front of the ATG codon (*Bam*HI-site-$(N)_{27}$-ATG) which reduced the expression yield (see Results). A PCR was therefore performed with primers which carried a *Bam*HI site directly in front of the ATG codon and deleted these additional bp. The new plasmid was named p2μGphyA2.

Apoprotein Expression, Isolation, and Identification. Apoprotein expression was accomplished in two different inducible yeast systems. Work with *S. cerevisiae* followed a strategy outlined by Yamamoto and co-workers (Schena & Yamamoto, 1988; Picard et al., 1990). Under this regime, protein expression was induced by activating the co-transformed/co-expressed rat glucocorticoid receptor through the exogenous addition of glucocorticoid deoxycorticosterone (Sigma).

S. cerevisiae cells [strain BJ2168, phenotype *ura3 trp1* (Sorger & Pelham, 1987)] were made competent for electroporation (Ausubel et al., 1989), and then co-transformed with a plasmid carrying the cDNA for the mammalian glucocorticoid receptor [pG-N795 (Schena & Yamamoto, 1988)] and with the plasmid p2μG carrying the potato phytochrome-encoding cDNA (phyA or phyB, as required). The transformation was monitored by following the growth of transformants which were able to survive on minimal medium due to complementation [minimal medium: 5 g of $(NH_4)_2SO_4$, 1.7 g of yeast nitrogen base without amino acids and ammonium sulfate, 0.7% solution of casein hydrolysate, and 2% glucose per liter]. Routinely, 2 L of medium was inoculated with a culture of transformed cells, grown overnight, and then allowed to grow up to a cell density with an extinction of $\sim$0.7 at 600 nm, which took 5−6 h following inoculation. At this state of growth, the cells were induced by addition of deoxycorticosterone (dissolved in ethanol) to a final concentration of 5 μM. Under these conditions, the cells were allowed to grow for another 2.5 h. This time interval gave a maximal yield of expression. Cells were then harvested by centrifugation (5000*g*, 10 min, 4 °C).

In the alternative expression of phyB in the methylotrophic yeast *P. pastoris*, induction is achieved by cloning the cDNA of choice under the control of the alcohol oxidase promoter which becomes active when *Pichia* cells are grown in a minimal medium with methanol as the sole carbon source. The instructions of the manufacturer (Invitrogen) were essentially followed for transformation, cell growth, and induction.

The harvested fully grown cells were disrupted by either of two methods: with an Ultra-Turrax under liquid nitrogen as described recently (Gärtner et al., 1996) or by two passages through a French press (American Instruments) as described by Hill et al. (1994). The solutions were clarified by ultracentrifugation (100000*g*, 15 min, 4 °C) and concentrated by $(NH_4)_2SO_4$ precipitation, if required. This protein solution was used for the biochemical and kinetic experiments without further purification. The amount of recombinant phytochrome was determined after reconstitution from the absorption at 660 nm on the basis of an absorption coefficient of 125×10^3 M^{-1} cm^{-1} for full-length oat phyA (Lagarias et al., 1987). Routine yields were on the order of 20−25 μg/g of cell pellet.

An aliquot of the apoprotein mixture was loaded onto an SDS−polyacrylamide gel and separated by electrophoresis, followed by the identification of the proteins by Western blot analysis. As a primary antibody, either a polyclonal anti-phyA serum (Heyer et al., 1995) or a collection of monoclonal anti-phyB antibodies (Somers et al., 1991) was used. The antibody binding was visualized by a secondary antibody, coupled to alkaline phosphatase.

Phytochrome Formation (Reconstitution) and Recording of Absorption Difference Spectra. The final solution (typical volumes were between 2 and 5 mL) in phosphate buffer, 100 mM, pH 8.0, supplemented with 2 mM EDTA, 2 mM DTT, and 1 mM Pefabloc (Merck), was incubated in the dark with either PΦB or PCB at 4 °C for *ca.* 5 min. PΦB had been prepared according to Cornejo et al. (1992), and PCB according to Kufer and Scheer (1979). Both compounds were purified by HPLC and stored in dried form at −40 °C in the dark. For reconstitution, PΦB and PCB were dissolved in DMSO (10 μL) to concentrations of *ca.* 1 mM. Addition of either of these solutions to the apoprotein samples afforded a final chromophore concentration of *ca.* 2 μM. In order to ensure exclusively the presence of the P_r form, the reconstituted recombinant phytochrome samples were irradiated first with far-red light (RG9 cut-off filter T; $\lambda > 715$ nm), then with light of 658 nm (interference filter, bandwidth ±7 nm) for 2−5 min, and again with far-red light for another 2−5 min. Irradiation was always exhaustive, *i.e.*, until the sample had reached a photostationary state and no further absorbance change was observed upon additional irradiation. This irradiation protocol was repeated several times and monitored by absorption spectroscopy. The absorption spectra were always taken after complete termination of the thermal reactions.

Dark Conversion of P_{fr} Forms. Samples of potato phyA and phyB, reconstituted with either PCB or PΦB, were exhaustively irradiated with red light (IF 658 nm) at ambient temperature and placed into a spectrophotometer. Spectra were recorded after certain time intervals. The dark reversion into the P_r form was monitored for 170 (phyA) and 120 min (phyB), and a final spectrum was measured after 4 days in the dark at 4 °C. No photochemistry was induced from the recording of the absorption changes.

Flash Photolysis Apparatus, Transient Absorbance Measurements, and Data Processing. The samples for nanosecond flash photolysis exhibited ΔA 0.04−0.1 (peak-to-peak, P_r, P_{fr}) and were measured in 1 cm path length cuvettes. The flash photolysis apparatus was based on a setup described previously (Scurlock et al., 1993a). Due to the limited amount of recombinant phytochromes, very small absorbance changes had to be detected, which required changes of several devices of the formerly described instrumentation.

The apparatus had three light sources (for excitation, observation, and sample preparation) and a dual−beam detection system. Excitation pulses at 650 nm and 11 ns

full width half-maximum from a Nd:YAG-pumped dye laser (DCM) were employed. Energy and polarization (linear or circular) were controlled by optical elements. The pulse was shaped to a circular spot of 2.5 cm diameter. The absence of two-photon processes was verified by measuring the signal amplitudes as a function of excitation fluence. A deviation from linearity of less than 15% was selected as an upper limit for the excitation fluence (<3 mJ/pulse).

The analyzing light (perpendicular to excitation) was delivered by a 100 W cw tungsten halogen lamp (for detection in the millisecond-to-second range) and a 150 W pulsed xenon arc lamp (microsecond detection). Transient absorption measurements were performed under magic-angle conditions to avoid artifacts due to rotational diffusion. Filters, a shutter, and a monochromator in front of the sample reduced light intensity and exposure time in order to avoid secondary photochemistry. The bandwidth was set to 4 nm.

Sample preparation light to convert P_{fr} back to P_r was provided by a second 100 W cw tungsten halogen lamp and filtered by an 8 mm RG9 (λ >715 nm) and a water filter. The exposure time to this light was also controlled by a shutter. A dual-beam detection arrangement compensated fluctuations of the observation light intensity, especially critical for long-time measurements. Photomultiplier tubes (Hamamatsu R3896) served as detectors in the observation and reference pathways. While the observation pathway for the transient absorption change included a second monochromator in front of the photomultiplier, the reference pathway utilized only filters. The photocurrents of the photomultipliers were coupled to ground through resistors of variable values, depending on the time window analyzed. The voltages were recorded with a dual-channel digital storage oscilloscope (Tektronix TDS 520a) and transferred to a Vax station 3100 for signal handling. For each wavelength and time window, a data set composed of three types of measurements was produced, *i.e.*, the measurement of the transient signal, of the base line, and of a prompt light (laser scatter and fluorescence) trace taken after firing the laser only. From these measurements, the absorbance changes were calculated. For each time window and wavelength, the data set was generated by averaging 20−80 shots of every type of measurement. The low rate of the back-conversion required long far-red irradiation periods, resulting in a delay of *ca.* 1 min between two laser flashes.

Further data analysis was performed with a global fit program (Scurlock et al., 1993b) on a DEC-alpha workstation. All time windows and wavelengths were fitted simultaneously. The function used was

$$\Delta A^{\lambda}(t) = \sum_{i=1}^{n} \Delta A_i^{\lambda} e^{-t/\tau_i} + \text{const}^{\lambda}.$$

$\Delta A^{\lambda}(t)$ is the observed absorption change at time t and wavelength λ and ΔA_i^{λ} is the absorption change associated with wavelength λ and with lifetime τ_i (the τ_i values are assumed to be constant over the whole wavelength range). The constant (const) represents the absorption difference between a species of longer lifetime than the time window and the ground state.

RESULTS

Apoprotein Preparation. (A) Expression in S. cerevisiae. Fully grown *S. cerevisiae* cells, which had been transformed with either phyA- or phyB-encoding plasmids, produced the corresponding apoproteins of expected length after induction with deoxycorticosterone according to Picard et al., (1990). An optimal yield of apoprotein (from visual inspection of antibody binding) was obtained for a final concentration of 5 μM glucocorticoid. As a control, cultures of transformed cells were grown without glucocorticoid induction. No gene product was detected in these cells. The Western blots (Figure 2) show that gene products of expected size are obtained, although the apparent molecular weights are somewhat lower, when compared with the marker bands. The samples were of the same apparent molecular mass, irrespective whether a chromophore was incorporated or not, and independent of the yeast used for expression. The loss of ca. 7 kDa, due to the deletion Δ1−74, can readily be identified in Figure 2 by comparison with a full-length phyB reference. The identity of full-length potato phyA is evident in the lower part of Figure 2 which shows that phyA from oat and from potato migrate at identical positions and can be determined with the same set of antibodies.

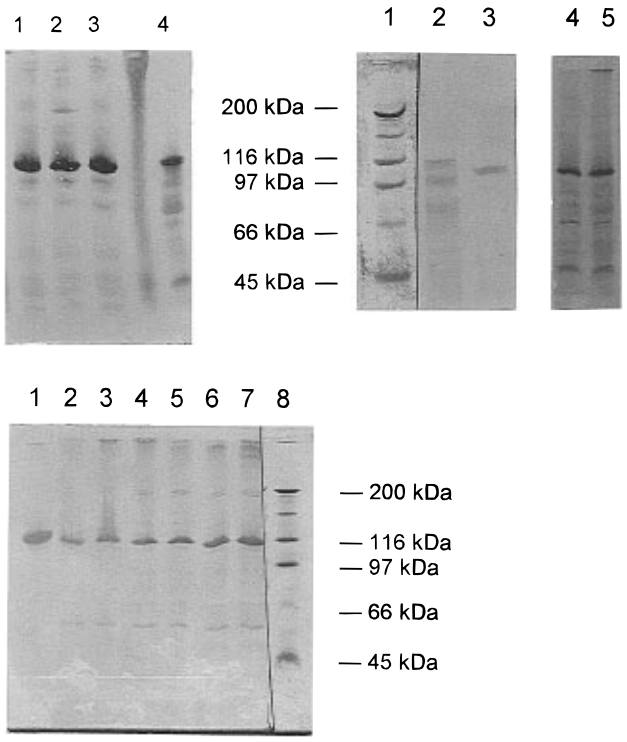

FIGURE 2: Western blot analysis of heterologously expressed phytochromes of potato. The protein solutions obtained from harvested and lysed cells were separated on SDS electrophoresis gels. The separated proteins were transferred onto PVDF membranes and visualized as described (see Materials and Methods). Top, left: Full-length phyB of potato; lane 1, apoprotein from expression in *S. cerevisiae*; lane 2, as lane 1, but after reconstitution with PΦB; lane 3, as lane 1, but after storage of the sample for 1 week at −20 °C; lane 4, full-length phyB from expression in *P. pastoris*. Top, right: Deletion mutant Δ1−74 of phyB of potato in comparison to full-length phyB; lane 1, molecular weight markers; lane 2, full-length phyB (exhibiting slight degradation); lane 3, Δ1−74 phyB-PCB from expression in *S. cerevisiae;* lanes 4 and 5, Δ1−74 phyB-PCB from expression in *P. pastoris*, two different preparations. Bottom: phyA of potato from expression in *S. cerevisiae* compared to phyA from oat; lane 1, phyA from etiolated oat seedlings; lane 2, phyA from vector p2μGPhyA-PCB; lanes 3 and 4, phyA from p2μGPhyA2-PCB; lane 5, as lane 3, but apoprotein; lane 6 as lane 3, but sample derived from a second expression; lane 7, phyA-PΦB; lane 8, molecular weight markers.

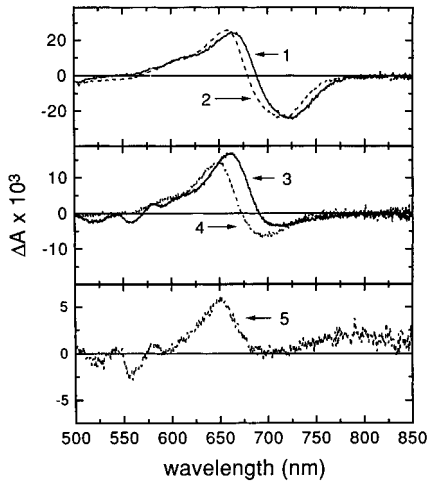

FIGURE 3: Absorption difference spectra ($P_r - P_{fr}$) of all potato-derived recombinant phytochromes with PCB (dashed curves) and PΦB (solid curves). Spectra 1 and 2, full-length phyB; spectra 3 and 4, full length phyA; spectrum 5, Δ1−74 phyB-PCB (note that the base line >750 nm is displaced). The spectra were recorded in a 1 cm path length cuvette. Since all samples were prepared from 1 L cell cultures, the different absorbance intensities reflect the various expression and reconstitution yields. For irradiation conditions, see Materials and Methods.

The yield of chromoprotein obtained by incubation with either PΦB or PCB was estimated from the $P_r - P_{fr}$ difference spectra (Figure 3 and Table 1). Routinely, incubation with either compound yielded volumes of ca. 2−5 mL with concentrations of *ca.* 0.3 μM.

(B) Expression in P. pastoris. Only full-length phyB was expressed in *Pichia* cells. Induced expression of proteins in the methylotropic yeast *P. pastoris* is accomplished only when a minimal medium containing methanol as a sole carbon source is supplied, since the cDNA is cloned under the control of the alcohol oxidase promoter. Cloning of phyB-cDNA into the expression vector pHIL-D2 afforded an alternative strategy since only a single *Eco*RI site can be used for insertion of the cDNA. Since *Eco*RI recognizes several sites in the phyB-cDNA, a new cloning site was constructed such that an oligonucleotide was designed which carried sites for *Bam*HI and *Xho*I and mismatches at the original *Eco*RI site (see Materials and Methods). This PCR product was inserted into an *Eco*RI-cut pHIL-D2 vector.

For both expression systems, the expression yields per gram of cell pellet were similar with respect to reconstitutible protein. However, since the cell density is higher for *Pichia* than for *Saccharomyces*, the overall amount of apoprotein per liter of cell culture was 2−3 times higher for *Pichia* cells. For absolute amounts, see Table 1. It should be mentioned that the amount of phytochrome from these expressions is below 1% of the total protein content in these samples. Still, measurements of time-resolved absorption changes can be performed with such solutions with high confidence, as has recently been demonstrated in two studies (Schmidt et al., 1996; Mozley et al., 1996). We have shown that (a) the kinetics of PΦB-containing recombinant phyA of oat are nearly identical to those determined for plant-extracted phytochrome, and that (b) the kinetics of an affinity-purified recombinant phytochrome are identical before and after the purification. From the latter study, one can exclude unspecific complex formation between recombinant phytochrome and protein from the expression host, and one can also estimate the extent of reconstitution which is on the order of ca. 50%.

Absorption Difference Spectra of Recombinant Potato Phytochromes. (A) Full-Length phyB. Incubation of apoproteins in the dark with either of the compounds PΦB or PCB yielded the P_r form of the corresponding recombinant phytochromes (phyB-PCB and phyB-PΦB). The reconstitutions using *ca.* 2 μM chromophore concentration were complete at ambient temperature within *ca.* 5 min. The $P_r - P_{fr}$ difference spectra exhibit maxima of 665 and 723 nm for P_r and P_{fr} of phyB-PΦB, respectively, and 658 and 714 nm for phyB-PCB (Figure 3 and Table 1). Identical λ_{max} values of the reconstituted phytochromes were obtained with the apoproteins derived from both expression systems. A recent report that *P. pastoris* does produce biosynthetically phytochromobilin in the dark (Wu & Lagarias, 1996) and forms holophytochrome when transformed with a phytochrome-encoding cDNA has led us to perform control experiments. In accordance to the observation cited, we only find chromophore biosynthesis by *P. pastoris* in the dark. Light-grown transformed cells (our routine conditions) did not exhibit photoreversible properties without the exogenous addition of the chromophore.

(B) N-Terminal Deletion Mutant Δ1−74 phyB-PCB. For expression, the cDNA construct was prepared in the same way as the full-length phyB-cDNA with respect to the distance between promoter and 5′-end of the coding sequence. Although from the inspection of the Western blots quite comparable amounts of expressed apoprotein seemed to be present (Figure 2), only a small amount of chromoprotein was obtained from reconstitution. Furthermore, the absorption difference spectrum of this construct shows a nearly depleted P_{fr} absorption (λ_{max} = 652 and *ca.* 705 nm for P_r and for P_{fr}, respectively; see Figure 3). The limited amount of material available did not permit a kinetic analysis to be performed.

(C) phyA from Potato. Full-length phyA from potato was also cloned and expressed in *S. cerevisiae*. Two cloning constructs, with respect to the distance between the promoter and the 5′-end of the DNA, were prepared. Making use of the restriction sites already present in the original construct, 27 bp were cloned upstream of the ATG sequence. The expression yield from this construct was very low and did not allow us to follow chromoprotein formation. Deletion by PCR of the 27 noncoding bp upstream of the ATG codon resulted in a remarkably improved apoprotein expression yield which now was ca. 40% of that of phyB expressions and thus allowed reconstitution with PCB and PΦB. Similarly to the Δ1−74 phyB deletion mutant, a difference spectrum with a strongly reduced P_{fr} absorption resulted after reconstitution with either chromophore. For phyA-PCB, the difference spectra maxima were 649 (P_r) and 700 nm (P_{fr}). The maxima of phyA-PΦB were red shifted by *ca.* 11 nm (Figure 3). The P_{fr} absorption maximum of the potato phyA is at a shorter wavelength than that of other phyA-type phytochromes. The intensity of the P_{fr} band of phyA-PCB is larger than that of phyA-PΦB (the intensity ratios P_r/P_{fr} are 2.1 for phyA-PCB and *ca.* 5 for phyA-PΦB). Here again, the amount of chromoprotein available was too low for any quantitative kinetic analysis.

Dark Conversion of P_{fr} Forms. Samples of potato phyA and phyB, reconstituted with either PCB or PΦB, were converted into the P_{fr} form and kept at constant temperature

Table 1: Expression Yields and Absorption Parameters of Recombinant Potato Phytochromes

sample	protein content, (μg/g of cell pellet)[a]	protein concn (nM) in final solution[b]	$\Delta\Delta A$ (10^3)[c]	λ_{max} (nm) of phyB-PCB, P_r, P_{fr}	λ_{max} (nm) of phyB-PΦB, P_r, P_{fr},
full-length phyB	22	320	40	658, 714	665, 723
Δ1−74	1	45	6	652, 705[d]	nd[e]
phyA[f]	5	113	11	649, 700	nd
phyA2[f]	9	152	20	649, 700	660, 712

[a] The amount of cells is higher in *P. pastoris* than in *S. cerevisiae* cell cultures, leading to a higher overall yield. [b] These values do not strictly correlate to those of the first column due to slightly different volumes of the final samples. [c] Determined from the $P_r - P_{fr}$ difference spectra for phy-PCB. [d] The P_{fr} absorption band of this construct is considerably weakened. [e] nd, not determined. [f] phyA and phyA2 differ by (phyA) the presence or (phyA2) the absence of 27 bp between promoter and ATG codon (see text for details).

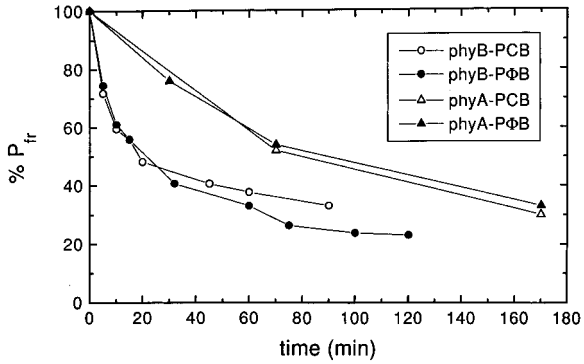

FIGURE 4: Dark reversion of the P_{fr} forms of potato (△, ▲) phyA and (○, ●) phyB. Open symbols refer to PCB- and closed symbols to PΦB-reconstituted samples. The samples were converted into the P_{fr} forms as described under Materials and Methods, and placed into the spectrophotometer. Spectra were recorded at the given time intervals (data points in the figure), and were normalized with respect to the amount of P_{fr} which was initially present (100%).

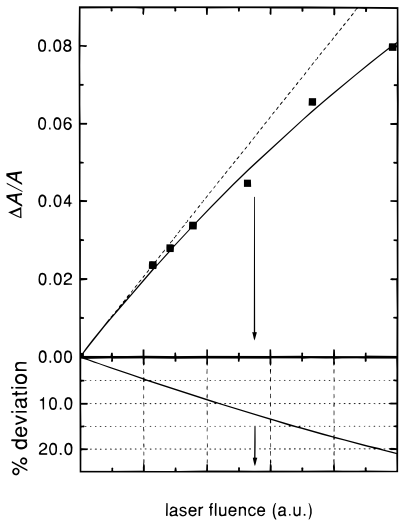

FIGURE 5: Top: Energy dependence of P_{fr} formation, presented as the fraction of molecules converted by a single laser flash ($\Delta A/A$); λ_{exc} = 650 nm, λ_{obs} = 720 nm. The nonlinear fitting (−) was performed with the function $f(F) = A[1 - \exp(F/F_0)]$. Bottom: Deviation from linearity. Arrow: Fluence used for the kinetic measurements.

in the dark. Spectra were recorded after certain time intervals (see data points in Figure 4) in order to determine thermal reversion into the P_r form. Both potato phytochromes underwent rapid reversion into the P_r species when compared to the behavior of monocotyledonous phytochromes which remain nearly stable for the observed time interval (2−3 h, data not shown). Within the limits of detection, nearly identical reaction rates were determined irrespective of which chromophore was used. After having reached values of ca. 30% of the initial P_{fr} concentration, the reaction became very slow, but led to complete conversion into the P_r forms after 4 days. For the long-time incubations, the samples were kept at 4 °C after the first 3 h of observation.

Flash Photolysis. Two-photon processes, which result from high photon densities, may produce erroneous data. Figure 5 shows the dependence of P_{fr} formation from the laser fluence in phyB-PCB. Kinetic traces were recorded at 720 nm for increasing excitation fluences, F, and then simultaneously fitted with the global analysis program. The data points at the top of Figure 5 refer to the laser-induced absorption change determined *ca.* 4 s after the laser flash (delay related to the "constant" term in the equation). It can be seen that they coincide with the values obtained from the fitting routine as a function of laser fluence, F, *i.e.*, $f[F] = A^*[1 - \exp(-F/F_0)]$. At the bottom of Figure 5, the percentage of deviation of the exponential function $f(F)$ from the linear function ($g[F]$) is plotted against the fluence of the laser pulse. The fluence used for all further experiments (indicated by the arrow) yields a nonlinearity of <15% at an overall $P_r \rightarrow P_{fr}$ conversion of 5% (see scaling of the ordinate of Figure 5). This presentation, together with the quantum efficiencydetermined for the phytochrome photoreaction [Φ = 0.15 (Kelly & Lagarias, 1985)], allows us to calculate that the ratio of photons to phytochrome molecules is ≤1. Results with phyB-PΦB were similar (not shown).

Transient absorbance changes of phyB-PCB and phyB-PΦB after laser excitation at 650 nm and 10 °C were monitored at selected wavelengths in the range 620−760 nm. The absorption changes in the microsecond time domain (I_{700} decay) were followed in the time window 0−800 μs with a resolution of 200 ns per data point. Absorption changes in the millisecond time domain (P_{fr} rise) were observed with two time windows, from 0−180 ms with 40 μs resolution per point and from 0 to 4 s with 1 ms resolution per point. The absorption differences thus determined were subjected to a global fitting (Scurlock et al., 1993b). Each of the resulting lifetime-associated difference spectra is correlated to a particular lifetime. For a mathematical description, refer to the equation given under Materials and Methods.

The lifetime-associated spectra of both recombinant phyBs are very similar (●), including the I_{700} decays (insets in Figure 6), which are monoexponential with a lifetime of *ca.* 85 μs (Figure 6). Although the low S/N ratio did not allow an unambiguous evaluation of the data, the statement of a monoexponential decay is supported from the observation that addition of a second decay component to either data set did not improve the χ^2 value. We have recently shown (Schmidt et al., 1996) that mono- and biexponential decays of recombinant phytochromes can readily be differentiated and analyzed under comparable experimental conditions.

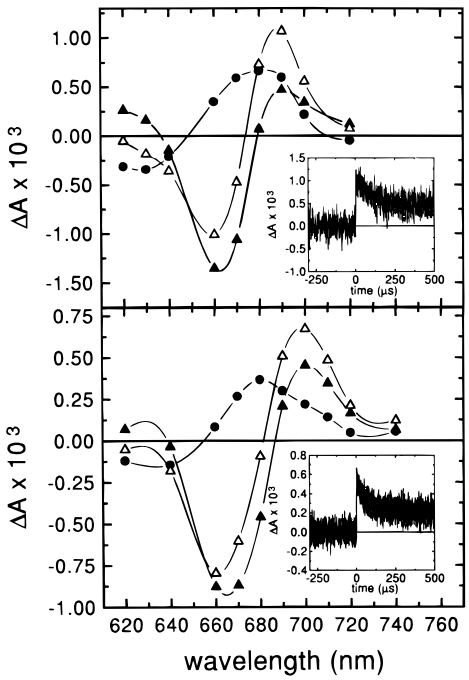

FIGURE 6: Lifetime-associated difference spectra (●) of recombinant potato phytochromes in the microsecond range (10 °C, λ_{exc} = 650 nm). (▲) Constant absorbance difference at the end of the experiment; (△) calculated difference spectrum (● + ▲) between I_{700} and P_r. Top, phyB-PCB; bottom, phyB-PΦB. The insets show single traces recorded at 690 nm.

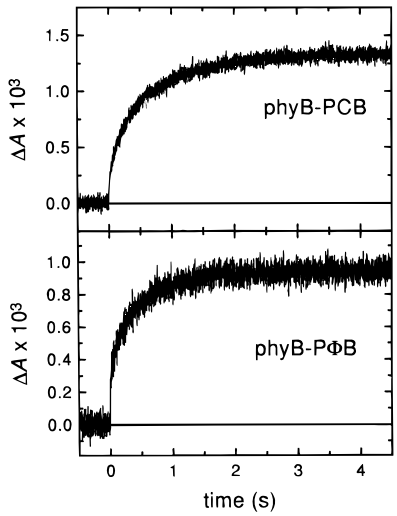

FIGURE 7: Time course of absorbance changes at 720 nm for recombinant potato phytochrome in the ms time range. Top, P_{fr} rise of phyB-PCB; bottom, P_{fr} rise of phyB-PΦB. For experimental conditions, see Figure 5.

Besides the lifetime-associated spectrum (●), also the absorbance differences which remain constant at the end of the experiment are shown (▲). This difference spectrum represents the constant term at the end of the monitoring time window, in this case to the $I_{bl} - P_r$ difference. It exhibits the same shape for both samples and a 12 nm blue shift for phyB-PCB. The addition of both traces yields the difference spectrum at time $t = 0$ after the flash (△) and represents the difference spectrum between I_{700} and P_r. Inspection of the traces from both chromophores reveals a slight blue shift for the I_{700} intermediate of PCB-phyB.

Figure 7 shows the time course of P_{fr} formation at 720 nm for phyB-PCB and for phyB-PΦB. It is already evident from the traces of the absorbance changes, and it becomes

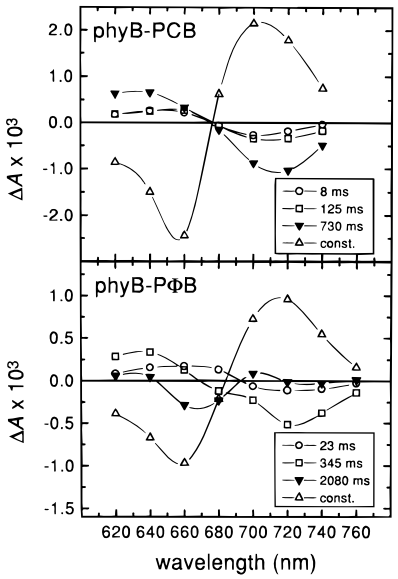

FIGURE 8: Lifetime-associated difference spectra for phyB-PCB (top) and phyB-PΦB (bottom) in the millisecond range.

even clearer from the detailed analysis of the result of the global fit procedure exemplified in Figure 8, that much slower kinetics are involved in phyB P_{fr} formation than in phyA. The corresponding kinetics for native phyA were recently determined as ≥266 ms (Zhang et al., 1992).

Contrary to the similarities in the I_{700} decay, the P_{fr} rise exhibits striking differences with respect to the chromophore used. These differences become evident only in the transient-associated spectra (Figure 8). The 8, 125, and 730 ms lifetimes of phyB-PCB all correlated with the formation of P_{fr} (negative absorbance changes in the wavelength range around 720 nm). The transient-associated spectra for the three components showed only slight differences in the position of the absorption maxima and exhibited the same isosbestic point (Figure 8, top).

A different picture emerges for phyB-PΦB (Figure 8, bottom). Again three lifetimes resulted from the fit. The 23 and 345 ms components correlate indeed with a rise at 730 nm and a concomitant decrease at 660 nm, whereas the third component of *ca.* 2 s (▼) exhibits a recovery at ca. 660 nm without a corresponding loss of absorbance around 730 nm (Figure 8, bottom).

DISCUSSION

For the first time, recombinant phytochromes of potato, phyA and phyB of various molecular sizes, and each with either of the PΦB and PCB chromophores, were prepared and investigated by flash photolysis.

In the case of full-length phyB-PCB, the $P_r - P_{fr}$ difference maxima (Figure 3 and Table 1: 658 and 714 nm for P_r and P_{fr}, respectively) are identical from potato (this work) and from tobacco which had also been expressed in yeast (Kunkel et al., 1993). Furthermore, they are quite similar also to the values of recombinant oat phyA-PCB (651 and 715 nm). The bathochromic absorption shifts of potato phyA-PΦBs are somewhat more different (Figure 3 and Table 1: 660 and 712 nm) from those reported for other recombinant phyA-PΦBs [*e.g.*, 666 and 730 nm (Cornejo et al., 1992), 663 and 728 nm (Schmidt et al., 1996)], and the amplitude ratios are very different.

The finding that the P_{fr} forms of both full-length recombinant potato phyA and phyB, irrespective of the chromophore, undergo rapid *dark* reversion to P_r to ca. 30% after 3 h of the initial P_{fr}, followed by continued slower reversion of the remainder within several days, concurs with analogous observations with tobacco phyB extracted both from tissue and with recombinant material (Mancinelli, 1994; Kunkel et al., 1995; Lamparter et al., 1995). The fact that also phyA from potato—in contrast to oat phyA—reverts to P_r in the dark is in line with the proposal that the $P_{fr} \rightarrow P_r$ dark reversion is an inherent property of dicotyledons and is independent of the phytochrome type (phyA *vs* phyB). We should note, however, that a perusual of the published data suggests that the capability of $P_{fr} \rightarrow P_r$ dark reversion is strongly influenced by the sample integrity and buffer conditions (Mumford & Jenner, 1971; Tokuhisha et al., 1985).

To date, the only studies of time-resolved absorbance changes of phytochromes have been performed with native phyA and phyA-derived smaller chromopeptides, and with recombinant full-length and truncated oat phyA expressed in *Escherichia coli* and in *P. pastoris* (Schmidt et al., 1996). The results obtained here for phyB from potato are the first time-resolved data reported for any phyB-type chromoprotein.

In earlier studies of oat phyA, we have attributed the microsecond biexponential decay of the I_{700} intermediate to two—probably parallel—first relaxation steps of the chromophore and the surrounding protein domain in this primary photoproduct (Heihoff et al., 1987). The kinetics are apparently more simple in the case of potato phyB-PΦB and phyB-PCB: Here, the I_{700} decay times of both phytochromes are identical and appear to be monoexponential (Figure 6). Although the quality of the kinetic traces suffered from a relatively poor S/N ratio, the inclusion of a second component into the fit procedure did not improve the quality of the fit, giving good evidence for the proposal of a monoexponential decay (one has to keep in mind that the lack of the second component does not exclude the presence of such a component with low amplitude or, alternatively, with a much faster time constant which would escape detection). This is quite in contrast to the situation encountered in the case of oat phyA. Here, the native and recombinant I_{700}-PΦB forms decay with biexponential kinetics (6−8 and *ca.* 90 μs), and only the recombinant I_{700}-PCB exhibits a monoexponential decay of *ca.* 90 μs (Schmidt et al., 1996). Evidently, the interactions between chromophore and protein regulating the I_{700} decay are different in oat phyA and in potato phyB.

Throughout the later steps of P_{fr} formation in the millisecond time range, the nature of the chromophore has an even better visible impact on the kinetics than in the microsecond events. Three rise terms were fitted to P_{fr} formation in potato phyB-PCB, with a time constant of 728 ms for the longest component. This is clearly longer than the corresponding value of *ca.* 270 ms in native phyA (Zhang et al., 1992), and in recombinant phyA (Schmidt et al., personal communication). Three millisecond-to-second components (*ca.* 20, 340, and 2000 ms) were also found for potato phyB-PΦB. However, the lifetime-associated spectra of this phytochrome reveal that the longest lifetime correlates with the formation of a species which is spectrally similar to P_r (see Figure 8). It is possible that already in the period, during which the flash photolysis is performed, a thermal reversion to P_r competes with P_{fr} formation. Moreover, the rise of the P_r-like absorption is not accompanied by a loss of absorption in the spectral range of P_{fr} [the lifetime-associated spectrum for these kinetics (▼) is nearly zero around 720 nm]. This result suggests that the P_r regeneration does not take place after the formation of P_{fr}, but starts from an intermediate. Such a reaction scheme would then indicate a kinetic competition between two pathways.

Sequence differences in the chromophore-binding protein region probably have a strong influence on the $P_{fr} \rightarrow P_r$ dark reversion. Thus, in phyB phytochromes, a glycine residue replaces either a serine or a tyrosine residue which precedes the chromophore-binding cysteine in most phyA phytochromes. In addition, a neutral alanine (or a serine in phyC of *Arabidopsis thaliana*) replaces the glutamic acid located six positions further toward the C terminal. These changes in the amino acid sequence may well contribute to a faster dark reversion in the dicotyledons than in the monocotyledons, and to a kinetic differentiation between phyB-PΦB and phyB-PCB due to different chromophore−protein interactions. Further differences between at least two phyB alleles with respect to four amino acid residues, N687T, K696N, R793K, and P838T (M. Herold, personal communication), in a region essential for signal transduction (Quail et al., 1995) may have an additional influence on the kinetics. It remains to be seen whether the kinetic differences between recombinant potato phyB (Figures 6−8) and oat phyA reflect inherent properties of the phytochromes from green plants or are due to their origin from either mono- or dicotyledons, as has been discussed also in connection with $P_{fr} \rightarrow P_r$ dark reversion (see above).

The recombinant Δ1−74 deletion mutant of phyB-PCB reveals an interesting facet of functionality of the N-terminal domain, the composition and length of which differ in phyA and phyB (an additional 15−20 amino acid residues are inserted into the phyB sequence compared to the phyA consensus sequence). Since in the recombinant mutant fragment this domain is missing, the photophysical properties of Δ1−74 phyB-PCB and 118-kDa phyA-PΦB (the so-called large phytochrome obtained by proteolytic removal of the N-terminal domain from native phyA) were expected to be similar. However, contrary to this expectation, the Δ1−74 chromoprotein exhibits a comparatively very weak P_{fr} absorption band. Furthermore, the apoprotein fragment on reconstitution merely afforded very small amounts of the Δ1−74 chromoprotein, although the apoprotein expression yield was comparable to that of full-length phyB (Figure 2). All this suggests that in phyB the N-terminal part is of even greater importance for protein conformation and interaction with the chromophore than in phyA, and probably the deletion leads to a less than optimal polypeptide folding of Δ1−74. Such an argument of impaired folding is supported by the observation that the "poor" difference spectrum of this phyB-PCB construct resembles the equally poor spectrum of recombinant 118-kDa phyA-PCB when expressed in *E. coli* in the absence of chaperonins, which occurs with imperfect folding (Hill et al., 1994).

At the time of this study, literature data on recombinant phyA kinetics had been restricted to information on oat phyA (Schmidt et al., 1996). In addition, a direct comparison of data sets from oat and potato faces the difficulty that it has to take into account also the differences between mono- and

dicotyledonous phytochromes. Recombinant potato phyA-PΦB and phyA-PCB were therefore prepared as well and characterized for reference purposes. The expression yields for both samples were quite low (Table 1). Moreover, the P_{fr} absorption bands are relatively poor and blue-shifted with respect to the P_{fr} spectra of other phyA phytochromes (see above). It is questionable whether the "uncharacteristic" P_{fr} data of potato phyA correlate with differences in the amino acid sequence. Thus, a simple sequence alignment of the potato and tobacco phyAs does not reveal readily any differences expected to exert a strong impact on structure or function. In particular, highly charged or polar domains are not involved, and there is a satisfactory N-terminal consensus sequence for potato phyA and all other phyA phytochromes.

CONCLUSION

Investigation of a phyB phytochrome by time-resolved absorption spectroscopy has been rendered possible for the first time by providing sufficient quantities of material in the form of recombinant potato phytochrome. The most noteworthy results are significant differences between the full-length phyA and phyB regarding the decay kinetics of the I_{700} intermediates, irrespective of which chromophore (PΦB and PCB) the two recombinant chromoproteins had been reconstituted with. Also, $P_{fr} \rightarrow P_r$ dark reversion is more pronounced in potato than in oat phytochrome. In conclusion, the photophysical and photochemical characteristics of phyB (potato), as they have emerged so far, appear in general to be qualitatively similar to those of phyA (oat). Differences are found only with regard to kinetic details, and in the thermal stability of the various P_{fr} forms. An eventual evaluation of these differences in terms of molecular connectivities will require considerably more detailed structural knowledge about the chromophore−protein complex than is available at present.

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