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Long-lived Intermediates in Phytochrome Transformation II: In Vitro and In Vivo Studies[1]

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Abstract. Conditions of illumination which cause phytochrome to cycle rapidly from P_R to P_{FR} and back lead to the accumulation *in vivo* of detectable amounts of long-lived intermediates on the P_R to P_{FR} pathway in oat coleoptile tissue. They appear to decay independently and in parallel to P_{FR}. Their behavior under different intensities of illumination and exposure time suggests that they are homologous with 2 similar intermediates previously observed *in vitro*. Available evidence favoring this suggestion is discussed. Equivalent illumination apparently causes far higher steady state levels of absorption by intermediates *in vivo* than *in vitro*, suggestion that native phytochrome is in a different physical state in the cell than it is in solution. A difference spectrum for the intermediates *in vitro* between 365 and 580 nm is presented. It has a maximum at 380 nm, a minimum at 418 nm, and crossover points at 398 and 485 nm. Glycerol in the phytochrome sample enhances the signal without otherwise changing the spectrum in any way. The difference spectrum represents the difference in absorption between the combined intermediates and P_{FR}.

In a preceding paper (3) we showed that long-lived phytochrome intermediates accumulate *in vitro* under continuous mixed red and far red illumination of high intensity. The evidence suggested that 2 kinetically distinguishable intermediates, decaying independently in parallel to P_{FR} could reach steady-state levels high enough to account for almost 10 % of the total phytochrome available in the sample. It seemed reasonable to identify the 2 intermediates as the 2 longest-lived forms of phytochrome on the P_R to P_{FR} pathway, as elucidated by the flash photolysis experiments of Linschitz *et al.* (8).

Two important questions remained unresolved, however. First, do the 2 intermediates seen *in vitro* represent parallel transformation of 2 distinct molecular species of P_R, or do they represent transformation of a single species *via* a pathway with alternate routes to a single species of P_{FR}? Though evidence from the literature would seem to favor the former alternative (7, 10), the question is still unresolved. Assuming the first alternative, however, the second question is considerably more important. Do the 2 species assumed *in vitro* also occur *in vivo*, or do they result from alteration of some but not all of the native phytochrome during the extraction procedure? There is excellent evidence that isolated phytochrome may show both alteration in absorption maxima and apparently independent alteration in molecular weight during isolation procedures (4). Could the 2 intermediates simply represent 2 different spectral forms arising during extraction and purification, or could they represent different molecular weight forms as well, also arising during extraction?

The present paper represents an attempt to resolve the problem by looking directly at oat phytochrome *in vivo*, and studying the intermediates under the same conditions as used in the previous *in vitro* study. It also presents spectral data on the intermediates in the blue and ultraviolet portion of the spectrum where they had not previously been observed. The data supplement difference spectra presented by Linschitz *et al.* for intermediates in the red and far red regions of the spectrum. A preliminary report of this work has appeared elsewhere (2).

Materials and Methods

For *in vivo* studies of phytochrome intermediates, oat seedlings were grown for 5 days in complete darkness as described elsewhere (4). Approximately 1.5 g of 2 to 4 mm coleoptile tips, freed of primary leaf tissue, were harvested under extremely dim green light. These were packed to cover the bottom of a circular cuvette approximately 2 cm in diameter.

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The cuvette was kept chilled by a circulating water bath. Direct monitoring of temperature was not possible, and it is estimated that most of the measurements were made at approximately 8°.

Instrumentation for making the various spectral measurements is described elsewhere (3, 6). Actinic light was of 3 kinds: red (Balzers "Calflex-C" heat-reflecting filter, Balzers K6 broad band interference-type filter, and Corning 2030 glass filter, intensity at sample, 1.2×10^5 ergs cm^{-2} sec^{-1}), far red (Calflex-C, Schott RG10, intensity 2.2×10^5 ergs cm^{-2} sec^{-1}), and mixed red and far red (Calflex-C, Corning 2030, intensity 4.0×10^5 ergs cm^{-2} sec^{-1}). For measurement of intermediates *in vivo*, the measuring beam was as described before (543 nm Balzer interference filter, energy at sample, 1.8×10^2 erg cm^{-2} sec^{-1}). For difference spectra, the measuring beam was obtained from a Bausch & Lomb grating monochromator.

Phytochrome was isolated and partially purified for the difference spectrum studies as described elsewhere (4). Samples were taken at 2 different stages of purity, first immediately after ammonium sulfate precipitation following elution from calcium phosphate gel (brushite), with about 5-fold purification, and second, following an additional gel filtration step with sephadex G-200, followed by ammonium sulfate precipitation and overnight dialysis to remove the residual salt (about 15-fold purification). All purification steps were carried out under dim green light and all samples showed an absorption maximum for P_R of 667 nm immediately before the experiment began. Phytochrome was assayed as described before (3), and activity is expressed as the sum of photoreversibility at 660 and 730 nm, or $\Delta(\Delta$ OD). Activity per sample varied from 1.5 to 3.0 $\Delta(\Delta$ OD). Loss during a run varied from 5 % (experiments with glycerol) to 20 % (experiments with buffer).

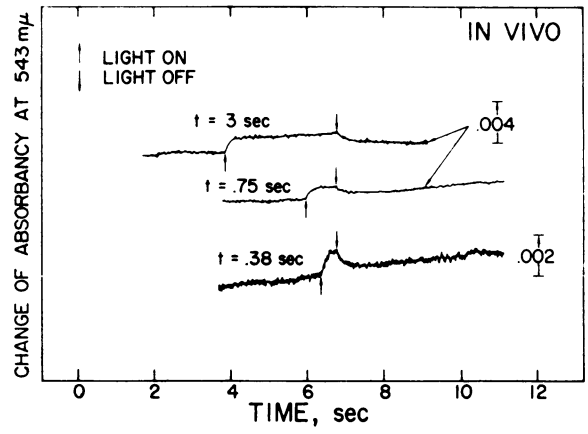

FIG. 1. Absorbancy changes at 543 nm induced by different exposure times with high intensity mixed red and far red light. Oat coleoptile sample, approximately 8°.

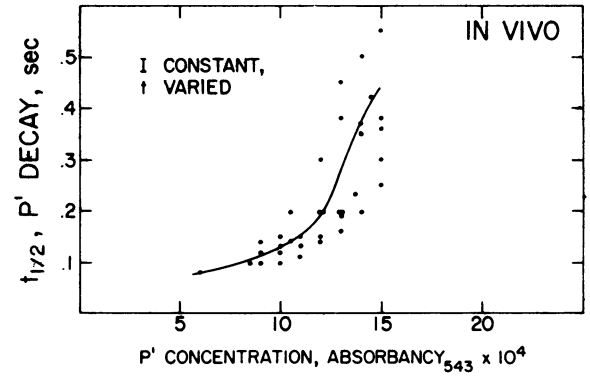

FIG. 2. Measured half times for signal decay plotted against signal height. Signal height varied by keeping intensity constant and varying exposure time. Each point represents one decay measurement.

Results

Long-lived Intermediates and Their Decay Properties in Vivo. High intensity mixed red and far red light causes absorbancy changes at 543 nm *in vivo* which are quite similar to those previously reported from isolated phytochrome solutions (1, 3). Fig. 1 shows tracings obtained when actinic light intensity was kept maximal and exposure time was varied. Note that as *in vitro* (3), the shorter the exposure time, the more rapid the decay of the signal. Half times for decay, plotted against amount of phytochrome intermediate, as determined from signal height at the end of the light exposure, are shown in Fig. 2. As with *in vitro* preparations, the half time for decay increases dramatically with intermediate concentration. The tissue samples presented a far more severe noise problem than had the liquid samples studies before (3), so detailed kinetic analysis of the decay curves was simply not possible. As with the liquid samples, however, the pattern is consistent neither with first nor second order decay of a single species.

Fig. 3 shows the results of varying signal height by keeping exposure time constant and altering actinic beam intensity. The similarity of the results with those presented before for liquid samples (3) is clear. The decay half-times do not seem to be changed by intermediate concentration, and apparent first order kinetics are observed. A plot of half times *versus* intermediate concentration is shown in Fig. 4. Despite the bad scatter caused by the noise problem with the tissue sample, no half-times shorter than 0.24 sec were observed, while shorter half times were regularly obtained with full intensity actinic light and shorter exposure time (Fig. 2).

Though signal noise precluded any precise kinetic analysis of the records, the pattern of the response with varying intensity or exposure time or both is sufficiently similar *in vivo* to that *in vitro* (3) that

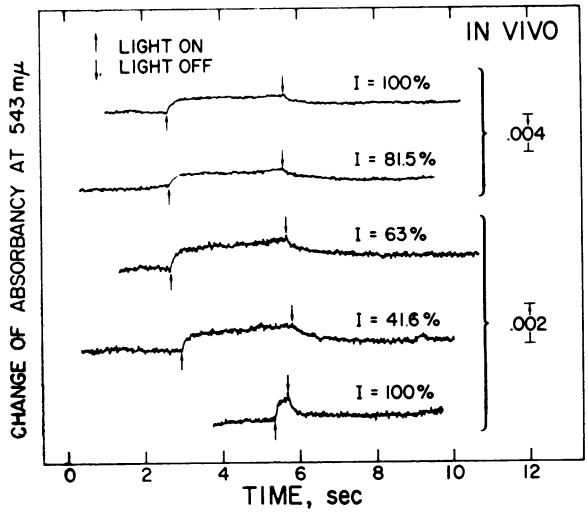

FIG. 3. Absorbancy changes at 543 nm induced by similar exposure times at different intensities of mixed red and far red light. Oat coleoptile sample, temperature approximately 8°. Bottom 2 tracings show different decay rates found when same signal height is produced in 2 different ways.

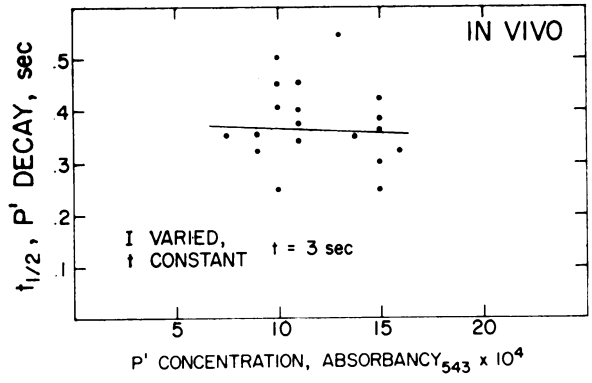

FIG. 4. Measured half times for signal decay plotted against signal height. Signal height varied by keeping exposure time constant and varying intensity. Each point represents 1 decay measurement.

one seems justified in concluding that independently decaying fast and slow intermediates are present *in vivo* as well. Thus the 2 components seen in solutions do indeed seem to have homologues in intact cells, and not to be artifacts of the purification procedure. They apparently also change with respect to their relative concentration as a function of exposure time, but not of intensity, just as was found with liquid samples (1, 3).

Proof that the observed signals obtained came from intermediates only, and not from some unknown spectral changes in protochlorophyll or chlorophyll, was more difficult to obtain than it had been with liquid samples (1, 3). However, one kind of experiment was done that sheds some light on the problem. The phytochrome in the sample was first entirely transformed to P_{FR} with actinic red light. Then the sample was given far red light, and the increase in baseline height at 543 nm, caused by the growing-in of P_R, followed. Two such records are shown on the left of Fig. 5. Transformation was complete by the end of about 3 sec, and when the far red light was turned off after about 8 sec, no spectral changes were observed in the dark. However, when the sample was now given red light, and the dropping of the baseline, representing disappearance of P_R, followed, there was an obvious dark decay of the signal following the end of irradiation (Fig. 5, right). The results were qualitatively similar to those found with liquid samples, suggesting that one is seeing accumulation of intermediates on the P_R to P_{FR} pathway only under conditions which lead to substantial pigment cycling (red, but not far red illumination). The identity of these dark-decay signals with phytochrome intermediates is further considered in the discussion.

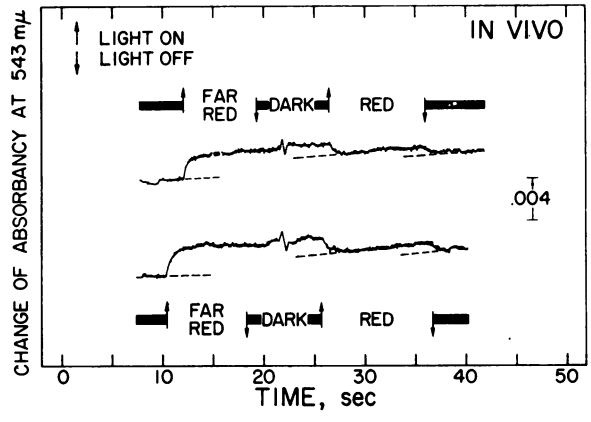

FIG. 5. Absorbancy changes induced by saturating far red and then red light at 543 nm. Sample of oat coleoptile tips received saturating red light treatment just prior to start of records shown. Note decrease in absorbancy following red, but not far red exposure. Break in tracings indicates about 60 sec for changing actinic light filters.

Difference Spectrum for Intermediates in Vitro. The design of the spectrophotometer precluded studying the properties of the intermediates in the red and far red regions of the spectrum, since the measuring beam had to be transmitted through a filter which would block the mixed red and far red actinic light from the photomultiplier. However, the instrument was ideal for investigating difference spectra between the intermediates and P_{FR} in the blue and ultraviolet regions of the spectrum. Preliminary observations suggested that it would be useful to find some way to increase signal height, to increase the accuracy of kinetic measurements and to increase signal sizes at different measuring beam wavelengths. It had been observed that samples in 0.5 M sucrose showed slower decay constants following illumina-

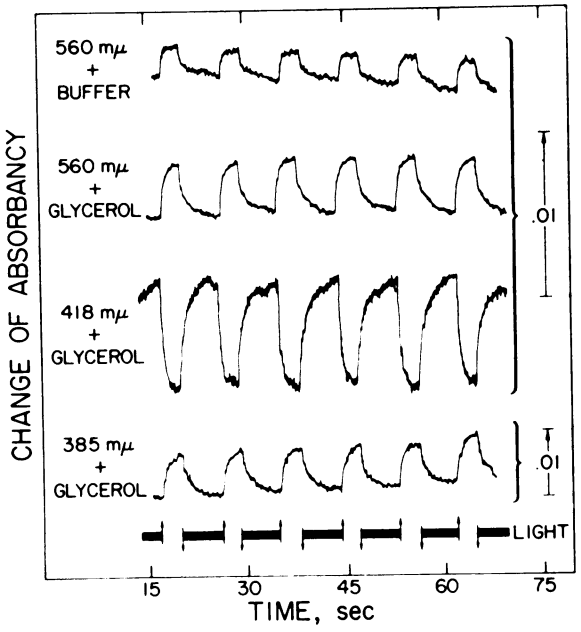

FIG. 6. Effect of glycerol on signal size, and representative negative signals at 418 nm and positive signals at 385 nm, for phytochrome intermediates *in vitro*. The time regime for illumination plus dark period was used for the difference spectra shown in Fig. 7.

tion, and correspondingly higher photostationary levels of intermediates during illumination. We therefore diluted samples either with an equal volume of buffer or with glycerol to see if the presence of glycerol would have the same result as sucrose and permit measurement of larger signals. The top 2 tracings in Fig. 6 show that glycerol did indeed give the expected enhancement in signal height (at 560 nm). Measurements were then made over a measuring beam wavelength range of 365 to 580 nm.

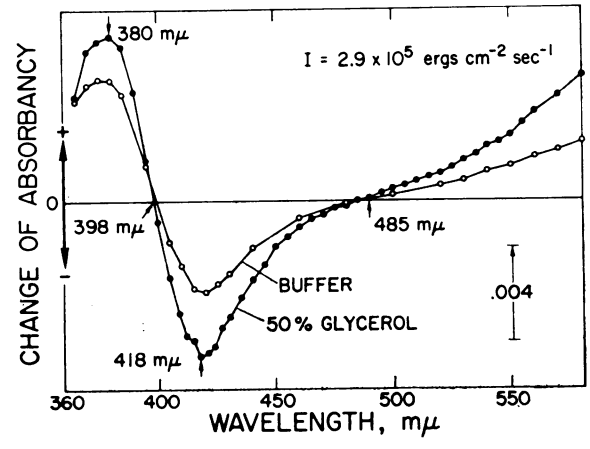

FIG. 7. Difference spectra between long-lived phytochrome intermediates and P_{FR}. Phytochrome sample probably a mixture of large and small molecular weight species (same as experiment 3, table I).

Signal height is plotted against measuring beam wavelength in Fig. 7, both for buffer and glycerol samples, and representative signals from glycerol samples are shown by the bottom 2 tracings in Fig. 6. Crossover points in the difference spectrum can be seen at 398 and 485 nm, with a maximum at 380 nm and a minimum at 418 nm. It is clear that glycerol, while altering signal height, did not significantly alter either the positions of the peaks or the positions of the crossover points.

The difference spectrum was measured 5 times, twice with buffer, and 3 times with glycerol. Table I shows maxima, minima, and crossover points for all of these spectra. In each case, absorption spectra were obtained both for P_R and P_{FR} before and after the difference spectrum measurements to determine whether the measurement process itself had altered the spectral character of the phytochrome. The maximum for P_R was in all cases 667 nm before difference spectrum measurements. When glycerol was used, this maximum was also 667 nm at the end of the experiment, but the buffer samples showed a shift of 3 to 5 nm to shorter wavelengths. Experiments 1 and 2 were done on phytochrome which had been passed through a sephadex G-200 column, and only small molecular weight fractions were pooled for the sample. Experiment 3 was done with phytochrome obtained by ammonium sulfate precipitation and resolution immediately following elution from the brushite, and as judged by the behavior of similar preparations on sephadex G-200 columns, consisted of a mixture of large and small molecular weight phytochrome. Table I shows no obvious

Table I. *Summary of Maxima, Minima, and Crossover Points for 5 Difference Spectra Between Long-lived Phytochrome Intermediates and* P_{FR} In Vitro

Sample temperature was 7 to 8°.

	Experiment				
	1	2		3	
	glycerol	buffer	glycerol	buffer	glycerol
Maximum, nm	375	370	370	375	380
Crossover, nm	399	398	398	398	398
Minimum, nm	420	418	415	420	418
Crossover, nm	*ca.*490	*ca.*480	*ca.*480	485	485

differences in spectral properties which one could associate with molecular size except possibly the position of the ultraviolet maximum, the peak for the experiment 3 material being at a wavelength 5 to 10 nm longer than that for experiments 1 and 2.

Discussion

There are several lines of evidence suggesting that the spectral changes observed during and after illumination of the coleoptile tip sample are indeed

caused by formation of long-lived phytochrome intermediates on the P_R to P_{FR} pathway. First, their accumulation under continuous red illumination, but not continuous far red illumination is precisely what was observed with liquid samples (1, 3). Second, the decay constants are comparable to those reported by Linschitz *et al.* (8) for the 2 longest-lived forms observed *in vitro* in the P_R to P_{FR} transformation. Finally, the increase in decay time with exposure time but not with exposure intensity suggests 2 intermediates decaying in parallel to P_{FR} as was the case *in vitro* where detailed kinetic analysis of signal decay was possible (1, 3). Finally, the absorption spectrum of this tissue sample, published elsewhere (4), and obtained immediately after the studies reported above, shows no evidence for significant protochlorophyll or chlorophyll being present.

The above evidence thus strongly supports the contention that more than 1 intermediate on the P_R to P_{FR} pathway, decaying independently to P_{FR}, occur in living plant tissue. This evidence does not shed light, however, on the question as to whether they arise from a single initial species of P_R *via* a split pathway to P_{FR}, or whether they arise in parallel from initially distinct species of P_R and decay to distinct species of P_{FR}. The results of Purves and Briggs (10), suggesting kinetically distinguishable species of phytochrome on the basis of complete transformation curves both for P_R and P_{FR} appear to support the second alternative, but one must use care in relating the 2 kinds of studies. One may be seeing aspects of transformation of different molecular species of phytochrome, or one may merely be observing a fortuitous correlation between unrelated phenomena.

There is 1 quantitative difference between the behavior of the system *in vivo* and *in vitro* which must be considered. When one exposes samples to continuous red light, one sees evidence for accumulation of intermediates, and therefore for pigment cycling both *in vivo* (Fig. 5) and *in vitro* (Fig. 4 in 3). However, the size of the signal in terms of the total change inducible by red and far red light is far smaller (less than 0.1 of the total change possible) *in vitro* (3) than *in vivo* (almost 0.5 of the total change possible). There are several possible explanations for this difference. First, the spectral properties either of the intermediates or the parent phytochrome as P_R or P_{FR} may be different *in vivo* than *in vitro*. Second, under identical conditions of temperature and actinic light intensity, the decay constants *in vivo* may be slower than *in vitro* (the difficulty in monitoring temperature in the tissue sample precludes determining the extent of such an effect). Third, the rate constants for formation of the intermediates may be more rapid *in vivo* than *in vitro*. Finally, any combination of the above 3 possibilities could account for the difference observed. While it is not at present possible to determine which, if in fact any of the above explanations apply, the difference does suggest that the native phytochrome is in a different physical state in the cell than it is in solution, a state which alters either its spectral properties, its rate constants in transformation, or both.

The only other work on phytochrome intermediates *in vivo* comes from Spruit's laboratory (11–14). Of some interest is an intermediate absorbing maximally at approximately 698 nm, which can be driven back to P_R. It was observed only at low temperature (−196°) in pea plumules. Cross *et al.* (5) and Pratt and Butler (9) have observed the same intermediate in purified oat phytochrome solutions, and Spruit also observed it in a crude preparation of maize phytochrome (14). This intermediate and a second low absorbancy form which arises with sample warming, however, are probably early products in phototransformation, as suggested by all 3 studies, and not the long-lived forms studied in the present paper.

It should be noted that the difference spectrum presented above represents the sum of 2 intermediates and their combined difference from P_{FR}. Though in principal kinetic analyses of the decay curves at each wavelength, followed by resolution into separate first order components, as was done previously (3), might give further detail on the individual spectra, such an analysis was not attempted since despite the larger signals obtained with glycerol, noise and baseline drift precluded the sort of accurate measurements needed. The nature of the glycerol effect is unknown, but it could be related either to the dehydrating properties of the substance, to its viscosity, or both. In any case, glycerol does not alter the difference spectrum except to increase the steady-state levels of intermediates accumulated during illumination, and as such provides a useful adjunct for their study. It is of some interest that the signals seen with glycerol are more similar to those obtained *in vivo*, in terms of signal size per unit of phytochrome, than are those with buffer. This observation may provide a lead in determining the environment of the phytochrome in the cell, suggesting perhaps an environment which could be somewhat hydrophobic or viscous or both. It is obvious that further study is needed, however, before any such conclusion is justified.

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