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PRIMARY PHOTOPROCESSES OF UNDEGRADED PHYTOCHROME EXCITED WITH RED AND BLUE LIGHT AT 77 K

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Summary

1. Red light irradiation of phytochrome (P_r) at 77 K produces an intermediate absorbing at 696 nm. The photostationary state concentration of this intermediate is rapidly established with that of P_r as the result of spectral overlap between the Q_y band of P_r and the Q_x band of the intermediate.

2. The 696 nm intermediate reverts back to P_r preferentially without yielding a substantial amount of P_{fr} upon thawing the 77 K sample to higher temperatures.

3. Blue light irradiation of P_r with or without exogenous FMN at 77 K results in the formation of two intermediates absorbing at 684 nm and 696 nm. The 684 nm intermediate is photochemically converted to the 696 nm intermediate at 77 K. Possibilities for the preferential formation of the 684 nm intermediate with blue light are discussed.

4. At 277 K, blue light irradiation of phytochrome (P_r) containing exogenous FMN increases the rate of phototransformation from P_r to P_{fr} three times over P_r having no FMN. On the other hand, exogenous FMN has no effect on the rate of transformation of P_r to P_{fr} by red light.

5. Energy transfer occurs from FMN to P_r at 77 K, initiating the photoprocesses of the P_r. The energy transfer apparently occurs within flavin-phytochrome complexes.

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Introduction

In a previous study [1], we found that the primary photoprocess in large molecular weight (120 000) phytochrome proceeds within the time scale of a few picoseconds [2], permitting the high efficiency of photomorphogenesis in plants. However, the nature of the primary photoprocess is yet to be established. One likely mechanism based on an intrapyrrolic proton transfer in phytochrome has been proposed, in analogy to the high rate of radiationless transition in free base porphyrins [1].

Although several flash photolysis and low-temperature studies of the phototransformation of small molecular weight (60 000) phytochrome, product of proteolytic degradation, have been reported (Refs. 3—6; see Refs. 7 and 8 for review), a detailed spectroscopic investigation of the primary photoprocess at 77 K has not been carried out with large molecular weight phytochrome. The importance of the large molecular weight apoprotein has been pointed out previously [1,2,9].

In the so-called high-irradiance responses of photomorphogenesis (see Ref. 10 for review), blue light is also actinically effective; e.g., in the high-irradiance response for the inhibition of hypocotyl elongation in lettuce [11]. An unknown ultraviolet-blue light photoreceptor ('cryptochrome') has been postulated for the blue light response in the high-irradiance response [12,13]. Siegelman and Hendricks [14] originally suggested a flavoprotein as one of the photoreceptors for the high-irradiance response.

In this paper, we describe the effects of blue and red light on the primary photoprocess of large molecular weight phytochrome isolated from oat seedlings as part of our attempt to search for an in vitro model for blue light action in the high-irradiance response.

Materials and Methods

Large molecular weight phytochrome was isolated from etiolated oat seedlings, as described previously [1]. Several phytochrome preparations of varying degrees of purity (A_{280}/A_{660} ranged from 1.5 to 3.2) were used without any noticeable effect on the low-temperature spectral data described herein. Unless specified otherwise, all phytochrome solutions were in 0.1 M phosphate buffer, pH 7.8. Small molecular weight P_r was prepared by tryptic digestion of large P_r [1]. All chemicals including FMN were obtained from Sigma Chemical Company. Water was deionized and redistilled before use.

A substantial fraction of the phytochrome present in different oat seedling harvests remains in an insoluble pelletable form after the initial step of isolation and purification (i.e., Brushite chromatography and $(NH_4)_2SO_4$ precipitation) of phytochrome. Because this pellet fraction contains 'endogenous' flavin in a presumably bound form, we have also examined its spectroscopic and photochemical properties. A typical absorption spectrum of the pellet is shown in Fig. 1.

Absorption spectra of optically transparent solutions at room temperature were recorded on a Cary 118C spectrophotometer. Absorption spectra of phytochrome in low-temperature snows and the phytochrome pellets were

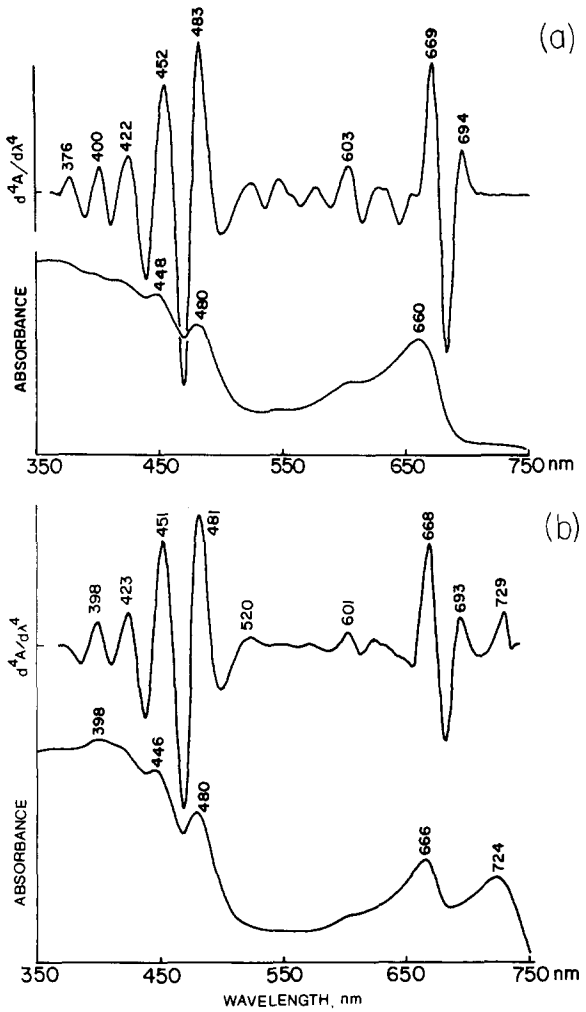

Fig. 1. (a) The absorption spectrum and its fourth derivative spectrum of large molecular weight phytochrome pellet obtained after the Brushite chromatography, suspended in phosphate buffer, pH 7.8 at 77 K. Recorded on the single beam spectrometer. (b) The 77 K absorption spectrum of large molecular weight phytochrome pellet (a) irradiated at room temperature with red light. The fourth derivative spectrum is also shown. Recorded on the single beam spectrometer.

recorded on a single beam spectrophotometer, on line with a Hewlett-Packard 2108 MX minicomputer [15], The snow solutions or pellets of phytochrome samples suspended in 0.5 ml phosphate buffer were placed in a specially built optical cuvette with pyrex or plexiglass bottom and steel cylinder siding [15], which was then immersed in liquid nitrogen (77 K) for spectral measurements. The computer-interfaced spectrophotometer was also used to obtain fourth derivative spectra (e.g., Fig. 2) as well as difference spectra (e.g., the difference between irradiated and unirradiated phytochrome samples) in order to enhance the spectral resolution of absorption spectra of phytochrome in snowy matrices at 77 K. Details of the spectrophotometer have been described elsewhere [15].

Phototransformation of P_r or P_{fr} was carried out with a 500 W tungsten pro-

jection light filtered through appropriate interference filters (669, 720 and 731 nm) and an infrared-absorbing water filter. Fluence rates used were 0.12 and 65 W/m^2 for red irradiation at ambient temperature and 77 K, respectively, and 0.68 and 33 W/m^2 for far-red irradiation at ambient temperature and 77 K, respectively. Low-temperature photolysis of phytochrome was carried out in situ using the above-metioned cuvette while immersed in liquid nitrogen. Blue light irradiation of P_r at 77 K in the presence and absence of FMN was also performed using the same light source with a 451 nm interference filter and at a fluence rate of 14 W/m^2. A Nicholas illuminator (Bausch and Lomb) with tungsten lamp (GE1460X) was also used for blue light irradiation (fluence rate 0.8 W/m^2 at 442 nm) at ambient temperature.

Results

Fig. 2 shows the absorption spectra of P_r and P_{fr} forms of phytochrome at 77 K, along with their fourth derivative spectra. The P_r form shows an absorption maximum at 668 nm, while the P_{fr} form maximally absorbs at 732 nm. The fourth derivative spectrum of the former resolves the main red-absorbing peak at 673 nm, with shoulders at 608 and 387 nm.

The absorption spectra of P_r irradiated at 669 nm for 0.16 min and 8.8 min are shown in Fig. 3, along with those of far-red-irradiated samples and their fourth derivative spectra. It can be seen that a shoulder appears at 696 nm in the absorption spectrum of P_r upon illumination with red light at 77 K, while the absorbance of the starting material, P_r, at 660–670 nm decreases somewhat. The 696 nm shoulder does not revert back to the P_r form upon irradiation with far-red light (731 nm) for 2 min or longer.

The difference spectra, i.e., absorption spectrum of irradiated P_r minus that of unirradiated P_r, are more revealing than the absorption spectra in resolving spectral location of the low-temperature intermediate produced from red irradiation, as shown in Fig. 4. The intermediate represented at 696 nm is clearly seen even after a short (0.16 min) irradiation with red light (Fig. 4), and it reaches a steady-state level within 4 min of irradiation with 669 nm light at 77

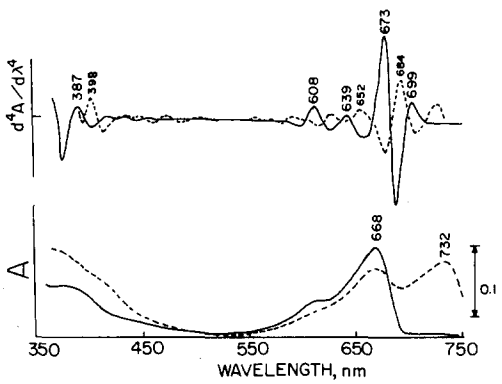

Fig. 2. The 77 K absorption spectra of large molecular weight P_r (———) and P_{fr} (- - - - - -) in phosphate buffer, along with the corresponding derivative spectra. Recorded on the single beam spectrometer. The P_{fr} spectrum was obtained in 77 K by red light (669 nm) irradiation of P_r at room temperature.

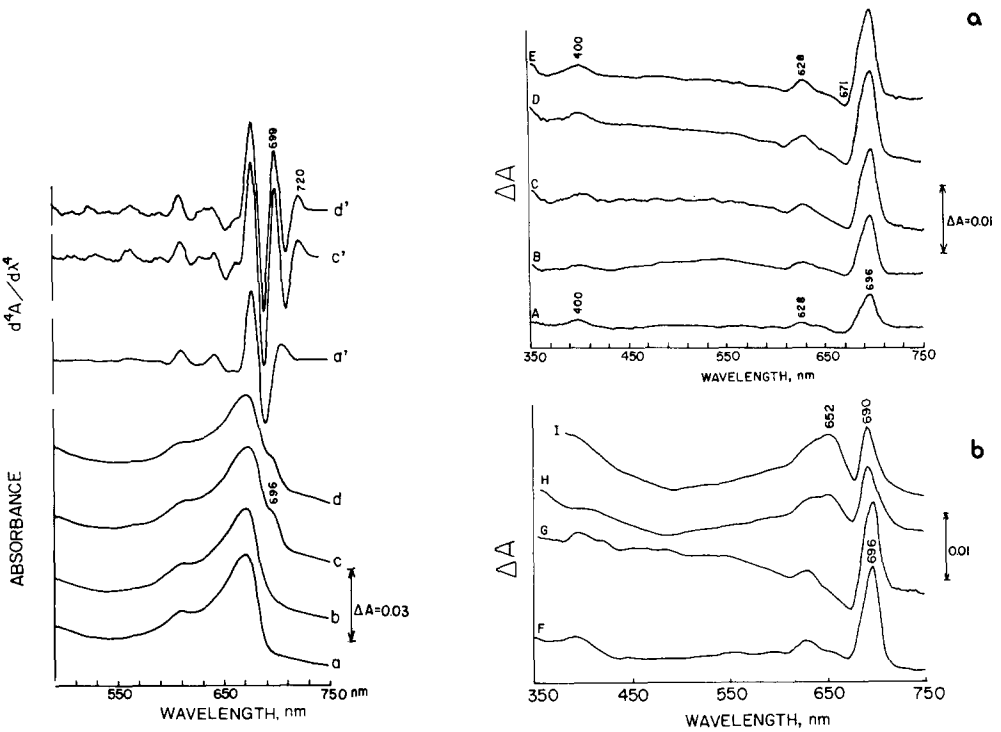

Fig. 3. The 77 K absorption spectra of large molecular weight P_r in phosphate buffer, pH 7.8, irradiated with 669 nm light for 0 min (a); 0.16 min (b); 8.8 min (c), and (c) irradiated at 731 nm for 2 min at 77 K (d). The corresponding derivative spectra are shown in primed alphabets. Recorded on the single beam spectrometer.

Fig. 4. The 77 K difference spectra (irradiated minus unirradiated) of large molecular weight phytochrome (P_r) in phosphate buffer, pH 7.8, irradiated with the 669 nm light at 77 K. (a) A, 0.16 min irradiation; B, 0.75 min; C, 1.75 min; D, 3.75 min and E, 5.75 min. (b) F, 8.75 min; G, 8.75 min red followed by 2 min far-red (731 nm); H, 50 min, and I, 60 min.

K. It can be seen from Fig. 4 that the 696 nm (or 699 nm from the derivative spectra in Fig. 3) intermediate has a higher extinction coefficient than the P_r form and its absorption overlaps that of P_r, as the change in absorbance for the latter is substantially less than the increase in absorbance in 696 nm. The 696 nm intermediate also exhibits weak absorbance maxima at 400 and 628 nm, which appear to be the result of a red shift of the absorbance maxima at 387 and 608 nm, respectively, of the P_r form of phytochrome. Far-red irradiation of the sample represented by the difference spectrum F results in an overall increase in absorbance in the region of 390—630 nm, but this may well be attributed to changes in the optical properties of the snow matrix such as scattering changes during irradiation experiments (prolonged irradiation of P_r at 77 K apparently yields product(s) with $\lambda_{max} \approx 652$ nm and 690 nm, see Fig. 4).

The 696 nm intermediate reverted back to P_r preferentially without yielding a spectrally noticeable amount of P_{fr}, as the frozen solution containing the intermediate was gradually warmed up to 288 K (Fig. 5). Thus, thawing seems

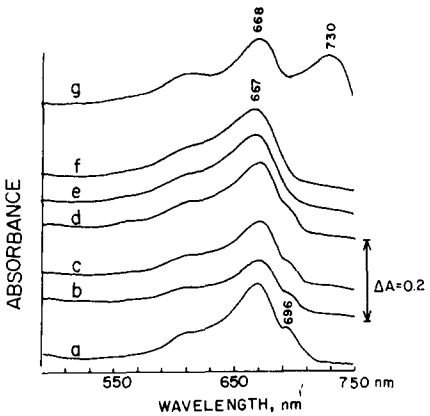

Fig. 5. The absorption spectra of large molecular weight P_r in phosphate buffer, pH 7.8, as a function of temperature after red light (669 nm) irradiation for 5 min at 77 K. Recorded on the single beam spectrometer. (a) P_r irradiated for 5 min at 77 K; (b) sample in (a) after thawing to 120 K; (c) sample in (a) after thawing to 144 K; (d) sample in (a) after thawing to 220 K; (e) sample in (a) after thawing to 271 K; (f) sample in (a) after thawing to 288 K, and (g) sample in (f) irradiated with red light for 3 min at 288 K, then frozen to 77 K.

to regenerate P_r, which transforms to a steady-state mixture of P_{fr} and P_r upon red irradiation (with 20—50% of the fluence used for the 77 K irradiation) at 288 K.

Fig. 6A shows the results of red light irradiation of small molecular weight P_r at 77 K, again demonstrating the formation of the 696 nm intermediate, which appears as a shoulder in the absorption spectra. The difference spectra shown in Fig. 6B clearly resolve the shoulder as a distinct peak at 696 nm, similar to the results shown in Fig. 4. Thawing of the irradiated samples shown in Fig. 6B did not result in significantly detectable P_{fr}.

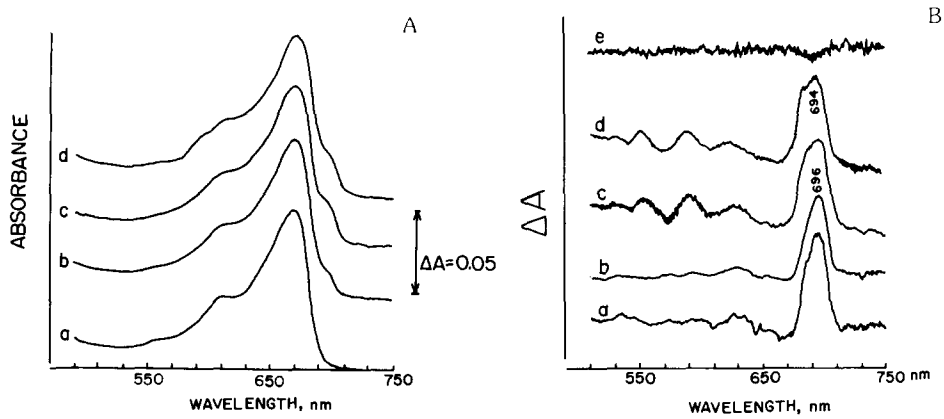

Fig. 6. (A) The absorption spectra of small molecular weight phytochrome (P_r) in phosphate buffer, pH 7.8, at 77 K, irradiated with red light. Recorded on the single beam spectrometer. (a) Small molecular weight P_r; (b) 1 min red light irradition; (c) 5 min red light irradiation, and (d) 20 min red light irradiation. (B) The 77 K difference spectra (irradiated minus unirradiated) of small molecular weight phytochrome (P_r) in phosphate buffer, pH 7.8, irradiated with the 669 nm light at 77 K. (a) After 1 min irradiation; (b) 5 min irradiation; (c) 20 min irradiation; (d) 20 min red irradiation followed by 20 min far-red (720 nm) irradiation, and (e) difference spectrum (d) — (c).

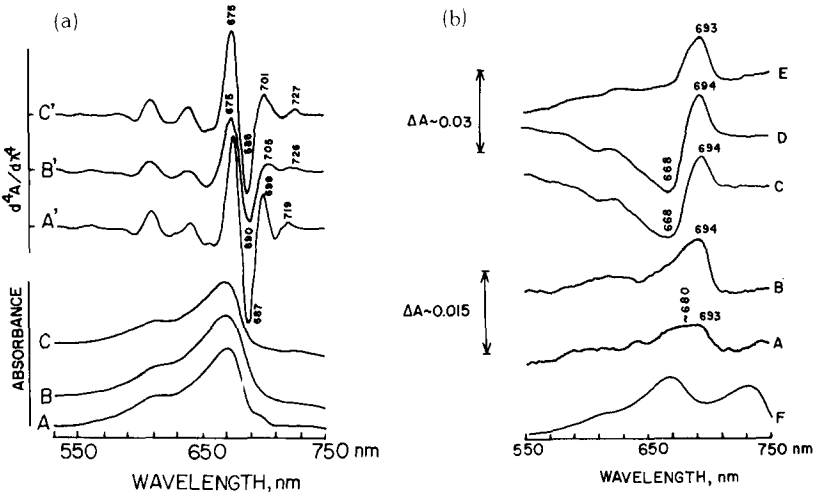

Fig. 7. (a) The 77 K absorption and fourth derivative spectra of large molecular weight phytochrome (P_r) in phosphate buffer, pH 7.8, irradiated with blue light (451 nm) at 77 K. Recorded on the single beam spectrometer. (A) 10 min irradiation; (B) sample in (A) thawed to approx. 200 K; (C) sample in (B) refrozen to 77 K. (A')—(C') corresponding fourth derivative spectra. (b) The 77 K difference spectra (irradiated minus unirradiated) of large molecular weight phytochrome (P_r) in phosphate buffer, pH 7.8, irradiated with blue light (451 nm) at 77 K. (A) 1 min irradiation; (B) 3 min irradiation; (C) 5.5 min irradiation; (D) 10 min irradiation and (E) sample in (D) thawed to approx. 200 K. Spectrum (F) is the 77 K absorption spectrum of sample in (D) thawed and irradiated with the 669 nm light for 10 min.

Irradiation of P_r with blue light (451 nm) also produces an intermediate absorbing at 690—700 nm shoulder (Fig. 7a). The difference spectra more clearly resolve the intermediate with an absorption maximum at 694 nm, as shown in Fig. 7b. It can be seen from Fig. 7b that the 694 nm intermediate is produced at the expense of P_r (absorption maximum 668 nm) at 77 K. Thawing of the blue light-irradiated sample regenerated the absorbance due to P_r, without any significant formation of P_{fr} (Fig. 7b). These results parallel those shown in Fig. 6B, suggesting that the same intermediates are produced by red and blue light irradiation of P_r at 77 K. Fig. 7b demonstrates transformation of the blue light-treated P_r to P_{fr} with red light (669 nm). This indicates that blue light irradiation at 77 K does not lead to the formation of P_{fr}.

Mixtures of phytochrome (P_r) and FMN were irradiated with 451 nm, which is preferentially absorbed by the latter (Fig. 8a). The absorption spectra as a function of blue light irradiation are shown in Fig. 8. The difference spectra recorded as a function of blue light irradiation more clearly resolve intermediates produced at 77 K (Fig. 9A). It can be seen from Fig. 9A that initially an intermediate at 684 nm increases in concentration, followed by either concomitant or consecutive formation of the 696 nm intermediate. Thus, irradiation of P_r with blue light absorbed predominantly by FMN results in a new intermediate absorbing at 684 nm, which is not observable in the blue light-irradiated solution of P_r without exogenously added FMN. Fig. 9B compares the difference spectra of blue light-irradiated solutions of P_r in the presence and absence of FMN.

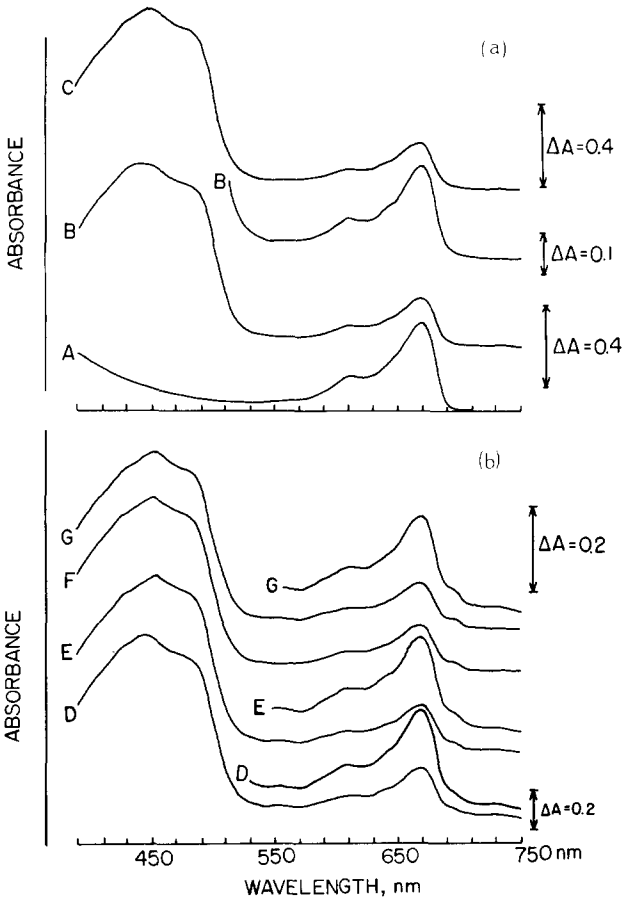

Fig. 8. The 77 K absorption spectra of large molecular weight phytochrome (4 μM P_r) in phosphate buffer, pH 7.8, irradiated with blue light (451 nm) in the presence of 76 μM FMN; [FMN]/[P_r] = 19. Recorded on the single beam spectrometer. (a) A, 5.6 μM P_r; no FMN added. No blue light; B, 4 μM P_r plus 76 μM FMN; no blue light, and C, 5 min blue light. (b) D, 10 min blue light; E, 15 min blue light; F, 20 min blue light, and G, 21 min blue light.

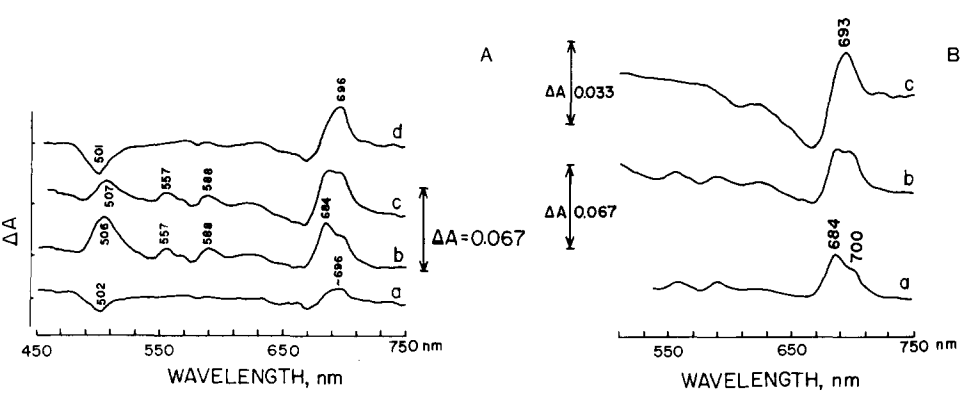

Fig. 9. (A) The 77 K difference spectra (irradiated minus unirradiated) of large molecular weight phytochrome (P_r) in phosphate buffer, pH 7.8, irradiated with blue light (451 nm) at 77 K in the presence of FMN. (a) 5 min irradiation; (b) 10 min irradiation; (c) 15 min irradiation, and (d) 20 min irradiation. (B) Comparison between the 77 K difference spectra of blue light-irradiated phytochrome (4 μM) at 77 K in the presence and absence of 76 μM FMN in phosphate buffer, pH 7.8. (a) 10 min irradiation, 76 μM FMN; (b) 15 min irradiation, 76 μM FMN, and (c) 10 min irradiation, no FMN added.

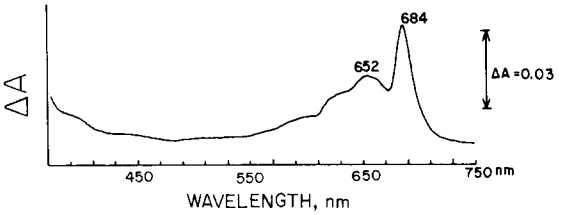

Fig. 10. The 77 K difference spectrum (irradiated minus unirradiated) of large molecular weight phytochrome in phosphate buffer, pH 7.8, irradiated with blue light for 10 min at 77 K, then thawed to approx. 250 K.

Although blue light irradiation of P_r in the absence of exogenous flavin produces no discernible spectral peak at 684 nm (Figs. 7 and 9B), it is possible to resolve a difference spectrum with the 684 nm peak after thawing the blue light-irradiated phytochrome in the absence of FMN (Fig. 10).

The effects of exogenous FMN on the phototransformation of P_r to P_{fr} were examined with blue (442 nm) and red (660 nm) lights at 277 K. At exogenous FMN to phytochrome molar ratio of 50—100, blue light irradiation of phytochrome increases the rate of transformation of P_r to P_{fr} by 3-fold (Fig. 11). At higher molar ratios, the phototransformation by blue light is inhibited due to fluorescence quenching of FMN and other factors to be discussed later. The exogenous FMN has no effect on the phototransformation of P_r to P_{fr} by red light irradiation at concentrations of FMN 50—100 times that of phytochrome. However, the rate of phototransformation from P_{fr} to P_r by far-red light decreases by 15% at the same concentration as above. The phytochrome pellet after Brushite chromatography and $(NH_4)_2SO_4$ precipitation contains endogenous flavins as indicated by absorption and fluorescence spectra (see Fig. 1 for

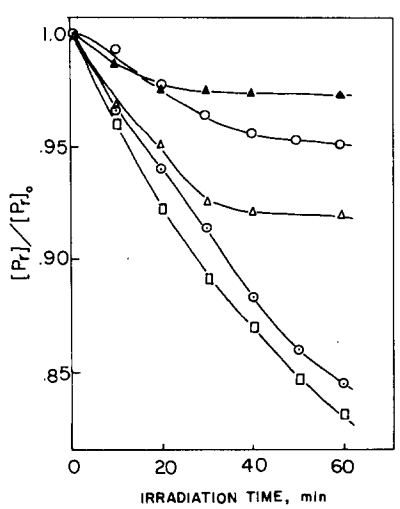

Fig. 11. The effect of exogenous FMN on the phototransformation of large molecular weight phytochrome (4 μM) in phosphate buffer, pH 7.8, irradiated with blue light (451 nm) at 277 K. ○, no FMN added; □, 50-fold molar excess FMN; ⊙, 100-fold molar excess FMN; △, 200-fold molar excess, and ▲, 300-fold molar excess FMN. $[P_r]$, P_r concentration remaining after each irradiation; $[P_r]_0$, total P_r concentration.

absorption spectrum). The absorption spectra of the pellet at 77 K irradiated with red and far-red light at room temperature demonstrate the photoreversibility of the complexed phytochrome. Blue light irradiation at 451 nm also resulted in phototransformation of P_r to P_{fr} at room temperature.

Discussion

Logically, it is to be expected that primary processes of the excited photoreceptors in photobiological systems proceed with a high efficiency and at an ultra-fast rate in order to achieve the maximum utilization of light perception for energy transduction processes such as vision and photosynthesis. Because of the fact that photomorphogenic responses of plants are extremely sensitive to red and far-red light and their photoreceptor, large molecular weight phytochrome, fluoresces only very weakly with a subnanosecond lifetime, the primary photoprocess of phytochrome (P_r) is expected to be on a subnanosecond or picosecond time scale [1,2]. Processes that take place faster than diffusion-controlled events can often be observed in rigid matrix at low temperature (e.g., phototransformation of rhodopsin to bathorhodopsin at 77 K [16]). The results presented in this paper clearly indicate that phytochrome (P_r), too, undergoes a photoprocess producing spectroscopically discernible intermediate(s) in the rigid matrix at 77 K, as discussed below.

Red light irradiation of P_r at 77 K

Red light irradiation of both large and small molecular weight P_rs at 77 K produces an intermediate absorbing maximally at 696 nm (Figs. 3, 4 and 6). Following the free electron notations, Q_y and Q_x, for the 668 and 610-nm bands (673 nm and 608 nm in the fourth derivative spectrum), respectively, of P_r shown in Fig. 2 [1], we assign the main absorption band of the above intermediate to the Q_y transition (696 nm). The Q_x transition of the intermediate is probably hidden under the P_r absorption Q_y band, as the appearance of absorption at 696 nm is not accompanied by a corresponding decrease in absorbance at 660—670 nm (Fig. 4). It should be emphasized that the 696 nm intermediate is produced and detected at 77 K without thawing the irradiated samples. This intermediate also exhibits a weak absorption peak at 400 nm (Fig. 4), corresponding to the 'Soret' band of phytochrome [1].

Previously, the light and dark reactions of phytochrome and formation of different intermediates at ambient and low temperatures have been reported [3,4,6,17,18]. However, these experiments were either done with proteolytically degraded, small molecular weight P_r and P_{fr}, or with pigments in vivo. Pratt and Butler [6] and Cross et al. [19] have confirmed the presence of several intermediates of phytochrome transformation at low temperatures. The first and the only photoproduct at 77 K was observed by the appearance of the 692 nm peak intermediate which was found to be stable in the dark below 173 K, but could be reverted back to P_r photochemically. On warming to approx. 193 K, the 692 nm intermediate shifted its wavelength maximum to 695 nm, probably through a dark reaction or thermal and solvent relaxation effects on the chromophore conformation. The latter was found to be photoconvertible back to P_r. Further warming of the sample to above 223 K, resulted in the for-

mation of another intermediate with absorption peak at 710 nm ('meta-Ra'), which eventually yielded P_{fr} with a further rise in temperature to 271 K. Subsequently, Kendrick and Spruit [18] obtained similar results, except that in vivo a 77 K intermediate exhibited an absorption peak at 698 nm, which was partially photochemically reversible at that temperature. Intermediate(s) absorbing at 692—698 nm produced by red light irradiation of small molecular weight P_r at 77 K and red flash photolysis of P_r [3—7] has been named lumi-R [4]. Lumi-R produced at 77 K apparently undergoes a series of dark relaxations to form P_{fr} at temperatures above 203 K [4], and is photoconvertible back to P_r at 203 K [6,17,19], as mentioned above. On the other hand, in vivo lumi-R undergoes dark reversion to P_r at 203 K [19]. Kroes [20] has also reported that red light irradiation of P_r at 77 K produces an intermediate (λ_{max} 693 nm) which is convertible to P_{fr} upon warming to 258 K.

There are similarities between our 696 nm intermediate and lumi-R. However, our 696 nm species did not lead to P_{fr} to any noticeable extent upon warming to 288 K. Regardless of whether our 696 nm intermediate produced from the undegraded, large molecular weight phytochrome (P_r) is prelumi-R or lumi-R, it is clear that this intermediate is produced in the primary photoprocess of P_r, as it is formed at low temperature in competition with fluorescence and other radiationless decays from the singlet excited Q_y state [1,2,21]. The possibility for an alternative prelumi-R intermediate will be discussed in the next section.

Linschitz et al. [3] observed that the initial photoproduct produced by red light flash photolysis of small molecular weight P_r had an absorbance at 696 nm and was transformed to P_{fr} at 273 K. Recently, Braslavsky et al. [22] detected an intermediate absorbing at 685 nm from a 15 ns pulse excitation of small molecular weight P_r at 273 K. They reported that this intermediate was the first detectable transient produced by excitation with pulses of 605—655 nm, and that it did not revert to P_r at 273 K. At temperatures higher than 273 K, the 685 nm transient was converted to meta-R and eventually to P_{fr} [22]. In contrast to the pulse excitation [22], the steady-state irradiation of our small molecular weight P_r does not yield the same transient at 77 K, but produces the 696 nm intermediate (Fig. 6). Thus, we assign the 696 nm species as the primary intermediate for small molecular weight P_r under our experimental conditions, i.e., aqueous snow matrix at 77 K. It would be interesting to use the pulse excitation method for the photolysis of large molecular weight P_r at 77 K for comparison.

We cannot tell if the rate of formation of the 696 nm intermediate is slower for small molecular weight P_r than that for large molecular weight P_r under slow steady-state conditions of irradiation. Subnanosecond or picosecond excitation at low temperature is necessary to determine the kinetics of the primary photochemical reactions of both phytochrome preparations.

Blue light irradiation of P_r at 77 K

Blue light irradiation of P_r produces an intermediate absorbing maximally at 694 nm. We consider this intermediate identical with the 696 nm intermediate, vide supra, produced by red light irradiation under similar conditions. The difference of 2 nm in the absorption maxima of these intermediates could be due

to the effect of FMN binding or may simply be within the experimental error. As shown in Fig. 7, the 694 nm intermediate reverts back to P_r upon thawing the 10 min-irradiated sample. The P_{fr} is produced after red light irradiation of the blue light-irradiated sample upon thawing, as suggested in Fig. 7b.

Blue light irradiation of P_r in the presence of FMN which absorbed the predominant portion of the blue light seems to yield intermediates different from those of the blue light irradiation without FMN, as can be seen from Figs. 8 and 9A. In particular, the difference spectra (Fig. 9A) clearly resolve an additional spectral peak at 684 nm (Q_y) with its Q_x band overlapping with the Q_y band of P_r. With increasing irradiation time, the 696 nm peak grows, as the 684 nm peak reaches a maximum and then decreases in intensity after 20 min irradiation with light of 451 nm which is absorbed mainly by FMN. These results seem to suggest that the 684 nm intermediate precedes the 696 nm intermediate in the blue light-irradiated sample of phytochrome. If this is the case, it is reasonable to assign the 684 nm intermediate as a prelumi-R precursor to the 696 nm species which can then be assigned as a subsequent intermediate or lumi-R, as mentioned earlier. In this regard, it is interesting that nanosecond pulse excitation of small P_r yields an intermediate absorbing at 685 nm as the first detectable transient [22]. However, we were not able to detect any 684–685 nm absorbing transients at 77 K upon red light irradiation.

It appears that the 684 nm intermediate is not uniquely formed by blue light irradiation in the presence of exogenous FMN, as the difference spectrum of blue light-irradiated sample without FMN reveals a spectral peak at 684 nm (Fig. 10). Thus, the question to be answered is whether or not the blue light irradiation results in primary processes different from those of the red light irradiation of phytochrome at 77 K. We cannot provide a definitive answer to this question at this time, as there are at least two alternative interpretations of the observed data. One is that a prelumi-R or 684 nm intermediate precedes the 696 nm species (lumi-R?) at a rate too fast to be trapped even at 77 K in the red light-induced phototransformation of P_r, while blue light irradiation preferentially produces the 684 nm intermediate which is not rapidly phototransformed to be 696 nm species by the 451 nm actinic light. In this connection, it is significant that the Q_x band of the 684 intermediate apparently overlaps with the Q_y band of P_r which is preferentially excited by 669 nm light used (cf. Figs. 7 and 9A). Another possibility is that the blue light irradiation produces the 684 nm intermediate by preferentially exciting a fraction or different conformation of P_r, and is further enhanced by the energy transfer from FMN to the P_r fraction or different conformation in the presence of exogenous FMN.

Phototransformation of P_r with blue light

In the previous section, we discussed primary photoprocesses initiated by blue light irradiation of phytochrome at 77 K. Blue light irradiation in the presence of FMN was also effective (see Fig. 9B for comparison) in producing the 696 nm (=694 nm) intermediate, as well as another intermediate absorbing at 684 nm. The rates of the photoreactions with and without FMN are approximately the same at 77 K (compare Figs. 7 and 9A; see also Fig. 9B), indicating that the blue light absorbed preferentially by FMN was efficiently transferred to the phytochrome molecule which undergoes reactions leading to the forma-

tion of 684-nm and 696-nm intermediates. Moreover, at 277 K, blue light irradiation of phytochrome containing exogenous FMN at concentrations of 50—100 times that of phytochrome increases the rate of phototransformation of P_r to P_{fr} by three times compared to irradiation of phytochrome by itself (Fig. 11).

It should be pointed out that exogenously added FMN had no effect on the rate of phototransformation from P_r to P_{fr} with red light. As shown in Fig. 11, the rate of phototransformation with blue light increases, however, as 50-fold excess FMN was added. However, at higher concentrations of FMN (300-fold excess FMN over P_r), the phototransformation with blue as well as red light was somewhat inhibited. These results can be explained in terms of a competitive inhibition of the phototransformation of both P_r and P_{fr} by FMN which binds at the chromophore-binding site of phytochrome at higher FMN concentrations (unpublished results).

There is sufficient overlap between the fluorescence emission band of flavin [23] and the absorption spectrum of P_r in the region of 500—600 nm (Figs. 1 and 3). This meets requirements for the long-range energy transfer from flavin to P_r via the Förster dipole-dipole coupling [24], as given by the following expression where R_0 is the critical distance for energy transfer:

$$R_0^6 = \frac{8.8 \cdot 10^{-25} \kappa^2 \phi_{FMN}}{n^4} \int_0^\infty I_F(\tilde{\nu})\, \epsilon(\tilde{\nu})_{P_r} \frac{d\tilde{\nu}}{\tilde{\nu}^4}$$

In this expression, κ^2 is the orientation factor for the donor (FMN) and acceptor (P_r) transition dipoles (assumed to be 2/3), ϕ_{FMN} is the fluorescence quantum yield of FMN, I_F is its fluorescence intensity, and ϵ is the molar extinction coefficient of P_r at wavenumber $\tilde{\nu}$. R_0 for the FMN-P_r system was calculated to be 25.8 Å, while R_0 for the FMN-P_{fr} system was found to be 22.2 Å. The critical distance (distance at which energy transfer is 50%) of 25.8 Å is equivalent to an average distance between FMN and P_r at approx. 10 mM. At the concentrations of 120 μM FMN and 7 μM P_r used (Fig. 9B), an average separation distance is well over 150 Å. In other words, little energy transfer from FMN to P_r is expected at these concentrations of FMN and P_r to any significant extent if FMN and P_r were present in solution independent of each other. The fact that the photolysis of P_r in the presence of FMN takes place efficiently with blue light (Figs, 9B and 11) suggests that FMN must be bound to P_r for an effective energy transfer from the blue light-excited flavin to P_r within the complex pair.

In this connection, the presence of flavin in the phytochrome pellet obtainable from the Brushite chromatography is of particular interest (Fig. 1) *. In addition to the fourth derivative spectrum of the pellet shown in Fig. 1, fluorescence excitation and emission spectra of the pellet confirmed the presence of 'endogenous' flavin (λ_{ex} 450 nm; λ_{em} 522 nm; spectra not shown). The P_r-flavin pellet undergoes phototransformation to P_{fr}-flavin readily upon irradiation with red light at room temperature, as shown in Fig. 1.

* Interactions between phytochrome and flavin have also been observed by Smith (Smith, W.O., personal communication).

Conclusions

The present results suggest that a photoreversible intermediate with λ_{max} 694—696 nm is produced at 77 K in a primary molecular process of the red and blue light-excited phytochrome of large molecular weight. We tentatively assign this intermediate as prelumi-R, which is formed within a few picoseconds. In addition, the blue light excitation of P_r yields an intermediate with λ_{max} 684 nm, which precedes the former. Apparently, blue light excitation of P_r presents a unique photochemistry in terms of primary photokinetics and/or mechanism of the phytochrome transformation. Furthermore, flavins excited by blue light can also activate P_r by excitation energy transfer. Thus, the molecular nature of blue light effect in the high-irradiance response may underscore interactions between endogenous flavin and phytochrome.

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