Difference in the Degree of Exposure of Chromophores in the Pr and Pfr Forms of Phytochrome

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Phytochrome의 Pr 및 Pfr형에서 Chromophores 開裂差에 대한 解釋

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Summary

The physiologically inactive and active forms of the photomorphogenic receptor (phytochrome), Pr and Pfr, respectively, exhibit drastically different reactivities toward permanganate oxidation. The chromophore of pfr is oxidized faster by an order of magnitude ($k=1.01\times10^{6}M^{-1}S^{-1}$) than that of Pr ($k=1.19\times10^{5}M^{-1}S^{-1}$) at 298 k, suggesting that the former is almost fully exposed from the binding crevice of phytochrome.

Introduction

Phytochrome acts as the primary photoreceptor for red light-sensitive morphogenic responses of plants, which can be reversed by far red light. Diverse photomorphogenic responses such as germination and flowering are triggered by phytochrome, which functions on the bases of the following general scheme (see review 1–3):

$$Pr \underset{\text{far red light (730nm) or in dark}}{\overset{\text{red light (660nm)}}{\rightleftharpoons}} Pfr \cdots\cdots\rightarrow \text{biological action}$$

Pr, phytochrome in the red absorbing form; Pfr, phytochrome in the far red absorbing form; $[P]_0$ and $[P]_t$, phytochrome concentrations at time=0 and t (in s), respectively; Bv, biliverdin.

In order to elucidate the molecular basis of physiological activity of Pfr in contrast to the inactive Pr form of phytochrome, we proposed a model in which the Pr→Pfr phototransformation generates a hydrophobic surface on the latter as a result of the chromophore reorientation or relocation from the binding crevice of the chromoprotein, on the basis of spectroscopic characterization of the phytochrome molecules (4–6). As a consequence of the proposed model, a preferential exposure of the Pfr chromophore is expected. In the present paper, we describe differential reactivities of the Pr and Pfr chromophores with respect to permanganate oxidation as a measure of the degree of chromophcre exposure. This method has been satisfactorily used for similar chromophore oxidation in the *Chroomonas* phycocyanin at micromolar concentrations of potassium permanganate, which do

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not damage the apoprotein (7) .

Materials and Methods

Undegraded large mol wt phytochrome (pr, 120,000 daltons) was isolated and purified from etiolated oat shoots (*Avena sativa* L., C.V Garry oat) by employing an Affi-gel Blue (Bio-Rad) affinity chromatography (8, 9) . Frozen etiolated oat tissues were extracted with buffer and chromatographed through a brushite column as described previously (8), except for a small modification of column dimension (11㎝ diameter instead of 13㎝) and elution flow rate (1.5 liters /h instead of 3 liters/h) ,Using 4 kg of tissue, normally 60−80㎎ of crude phytochrome preparations were recovered from200−300 ml of phytochrome-containing fractions of the brushite column. Twenty to twenty-five ㎎ of phytochrome preparations were then applied to an Affi-gel Blue column (2×60㎝), which was equilibrated in 0.1 M potassium phosphate buffer, pH 7.8, containing 0.1% mercaptoethanol. After washing the column with 0.1 M phosphate buffer, pH 7.8,containing 0.5 M kcal and 0.1% mercaptoethanol, phytochrome bound to the blue dye was eluted with the same starting buffer containing 10 mM FMN and then fractionated with an equal volume of saturated ammonium sulfate solution (pH 7.8). Redissolved phytochrome pellets 0.1 M sodium phosphate buffer, pH 7.8, 50 mM KCl, and mM EDTA, were subjected to a Bio-Gel 0.5m column (2.5×90㎝) and chromatographed with the same buffer at a flow rate of 30 ml/h. In final purification, 8−10 ㎎ of phytochrome with absorbance ratio (A280/A660) of 1.4 were recovered. All other chemicals including biliverdin (Bv) were purchased from Sigma Chemical Co., St. Louis, MO.

Steady state absorbance measurements were performed on a Cary 118 C spectrophotometer at 298 K under safe green light. Samples for each steady state measurement were incubated for 1 min after mixing with appropriate amounts of $KMnO_4$ stock solution. pfr samples were continuously irradiated with red light to prevent the dark reversion to Pr during incubation.

Rapid reaction kinetics were carried out on a Durrum stopped-flow spectrophotometer model D−110. Equivolumes of sample and $KMnO_4$ solution were rapidly mixed in a 2㎝ cuvette (maximum mixing deadtime=2 ms ; cuvette capacity=63 ㎕) and the decay of absorbances at 660 nm for Pr, 723 nm for Pfr, and 666 nm for biliverdin were recorded on a storage oscilloscope model Tektronix 564B.

Irradiation of phytochrome for phototransformation was carried out with,a Bausch & Lomb microscope illuminator combined with a 660 nm interference filter (Oriel C572−6600) for the red light source (7.5 W/m²), and with an infra red cutoff filter (Ealing 26−4457) for the far red light source (1.6 kW/m²) .

Unless specified otherwise, all phytochrome and $KMnO_4$ solutions were in 0.1 M sodium phosphate buffer, pH 7.8, containing 50 mM KCl and 1 mM EDTA. Biliverdin was dissolved first in acetone (10 mM stock solution) and diluted in 0.1 M phosphate buffer, pH 7.8, before use. Deionized, redistilled water was employed for all buffers and solutions used in phytochrome purification and sample preparations.

Results

Figure 1 shows the bleaching of Pr and Pfr chromophores at 660 and 720 nm bands as a function of $KMnO_4$ concentrations. Well-defined isosbestic points can be seen at 355, 422, and 580 ㎚ for Pfr (Fig. 1B) and 395 and 560 ㎚ for Pr (Fig. 1A), suggesting that the oxidation

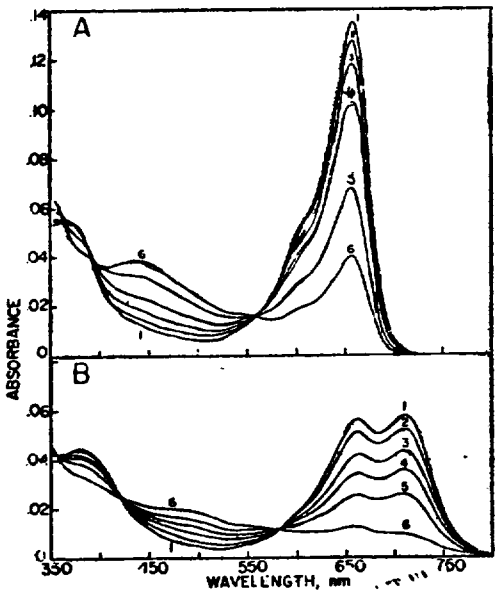

Fig 1. Absorption spectra of phytochrome in 0.1 M phosphate buffer, pH7.8, at 298K, as a function of $KMnO_4$ concentrations (spectra recorded 1 min after mixing):

(A) Pr (1.91 μM): 1, [$KNnO_4$] =0 μM; 2, 20 μM; 3, 40μM; 4, 70 μM; 5, 140 μM; 6, 210 μM. (B) Pfr (1.40 μM): 1, [$KMnO_4$] =0 μM; 2, 5 μM; 3, 10 μM; 4, 15 μM; 5, 20 μM; 6, 30 μM.

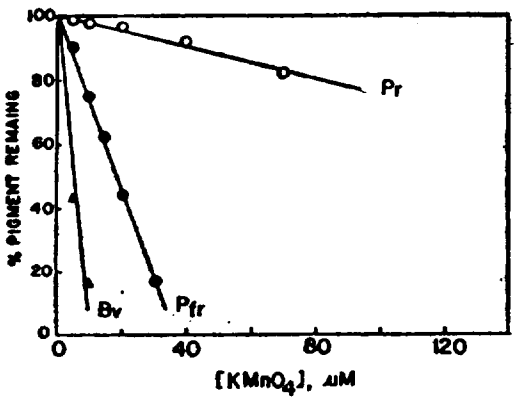

Fig 3. Dependence of the bleaching of phytochrome (1.91 μM Pr and 1.40 μM Pfr) and biliverdin (2.24 μM) on $KMnO_4$ concentrations. per cent pigment remaining was calculated after 1 min of oxidation.

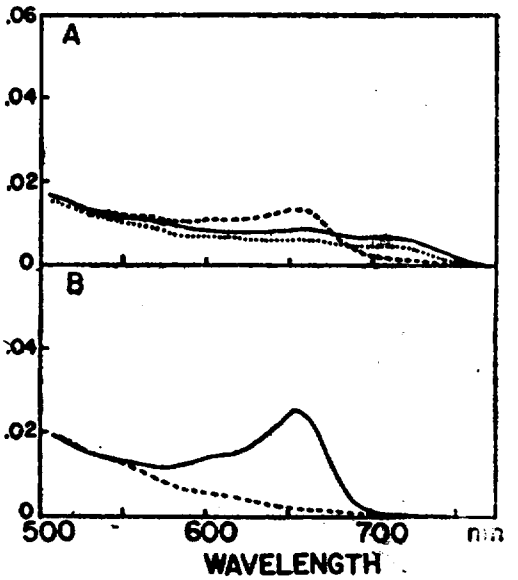

Fig 2. (A) Effects of $KMnO_4$ (30 μM) on the phototransformation of Pfr (solid line) to Pr (broken line). Note that the absorption due to Pr appears and it is photoreversible to Pfr (dotted line). The Pfr produced still absorbs at 720–730 nm, as it is oxidized slowly by the low concentration of $KMnO_4$ (compared to Fig. 2B).

(B) Effects of $KMnO_4$ (210 μM) on the phototransformation of Pr (solid line) to Pfr (broken line). Note that no appearance of absorption due to Pfr can be seen, as the Pfr photoproduced from Pr is immediately oxidized by $KMnO_4$.

of each chromophore results in one bleached product during the first 1 min of the reaction. Similar spectral changes were obtained with biliverdin (data not shown), exhibiting approximate isosbestic points at 420 and 570 nm, although the isosbestic points were not as well defined as in Fig. 1.

As can be seen in Fig. 1, the bleaching of Pfr is more exhaustive than that of Pr. This is further demonstrated in Figure 2. Figure 2A shows that a small amount of residual Pfr in the presence of $KMnO_4$ can be photo-reverted

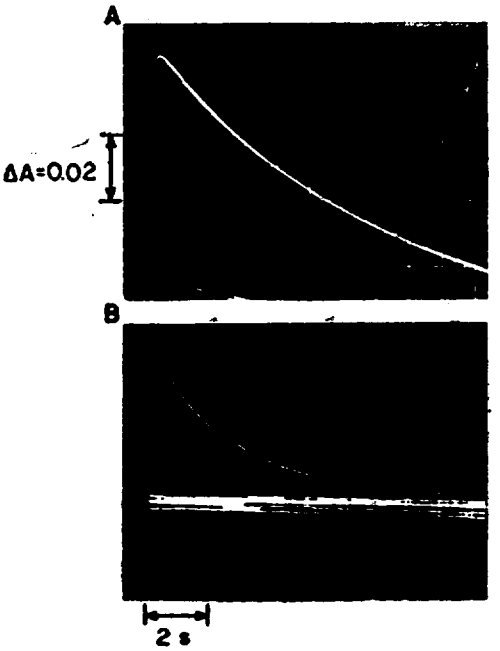

Fig 4. Oscilloscope tracings of absorbance changes upon rapid mixing of Pr (A) and Pfr (B) with $KMnO_4$. See Materials and Methods for details.

to Pr, resulting in a quantitative conversion of Pfr to Pr. On the other hand, the photoconversion of Pr to Pfr results in no detectable increase in absorbance at 720–730 nm (Fig. 2B) . This is most likely due to the fact that, as soon as Pfr is produced, it is quickly oxidized by excess $KMnO_4$, again demonstrating that the Pfr chromophore is more rapidly oxidizable than the Pr chromophore. Figure 3 shows the dependence of bleacning of phytochrome and biliverdin on $KMnO_4$ concentration. Again, Pfr is rapidly oxidized at a rate approaching that of biliverdin

Figure 4 shows oseilloscope tracings of the absorbance changes at 660 nm for Pr (Fig. 4A) and 723 nm for Pfr (Fig. 4B) recorded upon rapid mixing of phytochrome with $KMnO_4$. It can be seen that Pfr is bleached substantially faster than is Pr. First order plots of the rapid mixing kinetics shown in Fig. 4 did not yield straight lines. The kinetics of permanganate oxidation of phytochrome can be best fit to a second order kinetics on the basis of 1 : 1 stoichiometric reaction between the pigment and the permanganate, as shown in Figure 5. Again, the Pfr form is preferentiallyoxidized.

Discussion

The data presented above consistently show that the Pfr chromophore is preferentially oxi dized over the Pr chromophore (Figs. 1–5) . Since tetrapyrrole pigments of varying structures such as biliverdin, bilirubin, and phycocyanin (7) are oxidized by permanganate at more or less the same rate, a significant difference in the rate of oxidation of the phytochrome chromophore in Pr and Pfr forms can be taken as a measure of the exposure of respective chromophores.

Rate constants evaluated from Fig. 5 are presented in Table 1. It can be seen that the

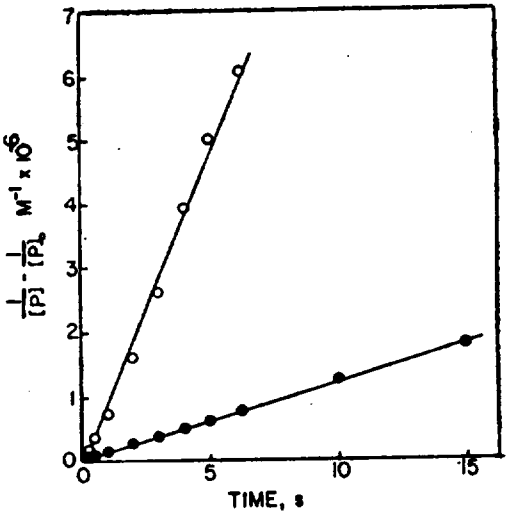

Fig 5. The second order plots for the permanganate oxidation of phytochrome in 0.1 M phosphate buffer, pH 7.8, at 298K. Solid circle, Pr ; open circle, Pfr.

second order, bimolecular rate constant for Pfr is approximately an order of magnitude greater than that for Pr. The rate constant for biliverdin is only slightly greater than that for Pfr. This suggests that the Pfr chromophore is substantially exposed; in fact, we can conclude that it behaves nearly as a freely exposed chromophore, taking into account a protein steric factor in the collisional encounter of the Pr chromophore and permanganate ion, which reduces the frequency factor in the rate constant of oxidation.

The present results are consistent with the hypothesis that the major molecular consequence of the Pr-Pfr phototransformation is a generation of hydrophobic sites on the latter, which interact with a Pfr receptor (4). The hydrophobic site on the Pfr surface or crevice can result from the vacancy created by the flexible chromophore exposure. It has been shown that the chromophore binding site on phytochrome is hydrophobic, as probed by the tryptophan fluorescence (4) and hydrophobic fluorescence probe ANS (8). Furthermore, the induced optical activity of Pfr in its CD spectrum is negligible compared to that of Pr, suggesting that the chromophore in the former is not very tightly held at its binding site (4). It is also significant that metal ion affects the absorption spectrum and stability of Pfr more extensively than Pr (10), possibly reflecting a readily accessible Pfr chromophore for coordination with metal ions, which results in the bleaching of phytochrome.

Table 1. Rate constants for the permanganate oxidation of phytochrome at 298 K

Pigment	K, $M^{-1}S^{-1}\times10^{-5}$	r*
Pr	1.19	0.999
Pfr	10.10	0.996
Biliverdin	15.80	0.984

*Correlation coefficients from the linear regression analyses.

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<국문초록>

Phytochrome의 Pr 및 Pfr 형에서 chromosom 開裂差에 대한 解釋

식물계에서 광에너지를 이용하여 일어나는 생명현상들로서 photosynthesis, photomorphogenesis 그리고 phototrophism 등을 들 수 있는 데 이들중 photomorphogenic 현상들 (예 ; 종자발아, 개화, 식물생장등)은 phytochrome 이라는 chromoproteine에 의해 지배를 받고 있다. 색소 단백질 phytochrome (M, W 120,000) 는 최초 광수용체로서 tetrapyrrole 환구조 (Bilitriene) 의 chromophore가 공유결합으로서 apoproteine에 붙어 있으며, 두가지 형태의 Phytochrome 즉 Pr (적색 광흡수 파이토크롬, λmax=660nm) 와 Pfr (원적색 광흡수 파이토크롬, λmax=730nm) 으로 광변환은 완전히 가역적이다.

$$\underset{\text{赤色}}{\text{Pr}} \xrightarrow{\text{hr (660nm)}} \underset{\text{遠赤色}}{\text{Pfr}}$$
$$\underset{\text{赤色}}{\text{Pr}} \xleftarrow{\text{hr (730nm)}} \underset{\text{遠赤色}}{\text{Pfr}}$$

Photomorphogenic respones를 직접제어하는 화학반응은 파이토크롬의 광변환 반응이며 Pfr형이 물리적인 활성화한 형태인 것이다.

이와같은 사실들은 파이토크롬이 phomorphogensis의 광수용체라는 사실과 일치한다 (11-14). 광변환 반응의 분자 기작으로 보고된 것으로 phototautomerization, photoisomerization, photodimerization 그리고 Pfr형의 anion 생성등이다 (15-19). 이들 보고된 기작중 Q. chae (Texas Tech Uinv) (15) 의 분광학적연구 결과는 phototautomerization 기작과 일치함을 보고했다. 본 연구는 소맥의 파이토크롬을 분리하여 primary photoprocess 분자 기작과 Pr→Pfr phototransformation에서 chromophore conformational change가 일어나는 가를 알아보기 위해서 파이토크롬에 대한 permanganate oxidation 반응결과 차로서 Pr형과 Pfr형의 변화를 측정하므로서 파이토크롬의 물리적인 활성과 비활성화를 실험적으로 증명하였다. Pfr형 chromophore는 magnitude ($k=1.19\times10^{6}M^{-1}S^{-1}$) 량에따라 (298k) Pr형 ($k=1.19\times10^{1}$ $M^{-1}S^{-1}$) 보다 빨리 산화되었다. 이 현상은 Pfr형이 Pr형보다 거이 노출된 상태를 의미하며, 또한 Pr→Pfr로 phototransformation 되는 것으로 결론지을 수 있다.