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Binding of 59 Kilodalton Phytochrome from *Avena sativa* to Liposomes and Cibacron Blue Dye

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귀리로 부터 얻은 59 KD phytochrome의 liposome과 Cibacron Blue Dye와의 결합성질

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초 록

암소에서 기른 귀리로부터 얻은 59 kD phytochrome의 liposome과 Cibacron Blue dye에 대한 결합성질을 규명하였다. 124 kD 및 118 kD phytochrome과는 달리 Pfr형의 59 kD phytochrome은 liposome 및 Cibacron Blue dye와 정전기적 힘으로 결합한다. 이러한 결과는 124 kD 및 118 kD phytochrome이 Pr형에서 Pfr형으로 광변환시 노출되는 소수표면이 59kD Pfr에는 존재하지 않음을 의미한다. 비교적으로 강한 소수부분이 59 kD polypeptide에 존재함이 알려져 있으므로 C-말단부위로부터 잘려져나간 55 kD tryptic domain이 118kD 및 124kD Pfr의 소수표면 노출에 중요한 역할을 하는 것으로 믿어진다.

Introduction

Phytochrome is a blue-green pigment protein which mediates various photomorphogenic reactions in higher plants. The photoreceptor protein exists in two interconvertible forms; red light absorbing (Pr) and far red light absorbing (Pfr) forms. Red light induces phototransformation from the physiologically inactive Pr to the active Pfr forms of phytochrome.

The molecular mechanism of phytochrome phototransformation is not well understood, although it is well known that a hydrophobic surface is exposed and the tetraphyrrolic chromophore is reoriented upon Pr to Pfr phototransformation (Hahn and Chae, 1986; Hahn and Song, 1981, 1982, 1987; Hahn et al., 1980, 1984a, 1984b; Kim and Song, 1981; Sarkar and Song, 1982). The physiological activity of phytochrome-mediated photomorphogenesis could be attributable to this additional hydrophobic surface in the Pfr which could serve as the binding site for membranes and/or receptor molecules (Song et al., 1979; Kendrick and Frankland, 1983).

The native oat phytochrome of apparent molecular mass of 124 kilodaltons(kD) can readily be degraded to the 118kD and further to the 59 kD phytochromes by endogenous proteases(Hahn and Chae 1986; Hahn et al., 1984b; Vierstra and Quail, 1982; Vierstra et al., 1987). Limited proteolysis during isolation and purification of phytochrome cleaves out 6 kD (or 10kD) peptide segment from the N-terminus and 55kD nonchromophore peptide domain from the C-terminus

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(Hershey et al., 1985). Cleavage out of the 6kD peptide segment causes substantial changes in the chemical and physical properties of phytochrome, including increased hydrophobicity, increased degree of exposure of the chromophore, and increased molar extinction with concomitant red shift of Qy band in the 118kD Pfr (Hahn and Chae, 1986; Hahn et al., 1980, 1984b; Vierstra and Quail, 1982, 1983). The 59kD phytochrome where the 10kD peptide from the N-terminus and the 55kD peptide from the C-terminus are removed, shows almost identical absorption and circular dichroic spectra with the 118kD phytochrome (Pratt, 1979; Song, 1983). This is of surprise because other chemical and physical properties of the 59kD and 118kD phytochromes are significantly different(Pratt, 1979). In this report we describe the binding properties of phytochrome to liposomes and Cibacron Blue dye, in order to delineate topological role of 55 kD nonchromophore peptide domain on the hydrophobic nature of phytochrome.

Materials and Methods

Degraded 59kD phytochrome was isolated from the tryptic digests of the purified 118kD phytochrome after Affi-Gel Blue chromatography. The 118kD phytochrome with A_{660}/A_{280} ratios of 0.8 was purified from etiolated oat seedlings (*Avena sativa*, cv Garry) using Affi-Gel Blue affinity chromatography procedure as described previously (Song et al., 1981; Hahn and Song, 1987). Ten mg of the purified 118kD phytochrome in 30ml of 0.1M phosphate buffer, pH 7.8, were mixed with 10μg of trypsin and incubated for 12h at 4°C. After incubation, 20μg of soybean trypsin inhibitor was added to the mixture to stop the reaction. After 10min of incubation, the solution was then applied to an Affi-Gel Blue column (2.5×10cm) equilibrated with 0.1M phosphate buffer, pH 7,8, containing 50mM KCl and 0.1mM EDTA. The column was washed with one column volume of the equilibration buffer and then eluted with equilibration buffer containing 0.5M KCl. The phytochrome containing fractions were pooled and concentrated with an Amicon PM 10 ultrafiltration membrane. Purified 59kD phytochrome as determined by SDS-PAGE with A_{660}/A_{280} ratios between 1.1 and 1.2 was obtained with this method. All experiments on phytochrome purification and sample preparation were performed in a cold chamber (278K) under safe green light.

Purified 59kD phytochrome was iodinated by the Bio-Rad procedure for the liposome binding experiments. Enzymatic iodination was initiated by adding 0.1ml of 0.1% α-D-glucose to a suspension containing 0.3mg of 59kD phytochrome, 0.3mCi of Na ^{125}I and 0.15μl of Enzymobead (Bio-Rad) in 0.1M phosphate buffer, pH 7.8, containing 50mM KCl and 0.1mM EDTA. Enzymobead consists of immobilized preparations (5~10 microns of polyacrylamide bead) of lactoperoxidase and glucose oxidase. Upon addition of glucose to a suspension, the immobilized glucose oxidase continuously generates a small, steady amount of hydrogen peroxide. The lactoperoxidase then catalyzes the peroxide oxida-

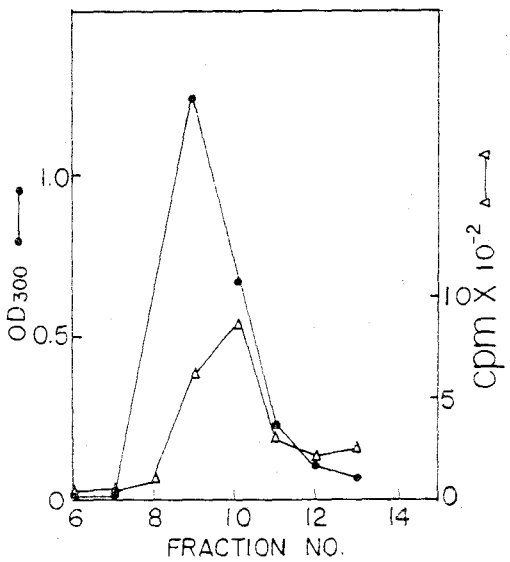

Fig. 1. A typical separation of radioiodinated phytochrome(59kD Pr) by Sephadex G-25-100 column chromatography
Two major fractions (1.5ml/tube) were pooled and used for liposome binding experiments

tion of labelled iodide to iodine. The labelled iodine reacts with phytochrome to produce radioiodinated phytochrome. The reaction mixture was incubated for 15~20 min at room temperature and chromatographed through a Sephadex G-25 column(1×18cm) equilibrated with 20mM phosphate buffer, pH 7.2, containing 10mM KCl. Received fractions were measured for absorbance at 660nm and radioactivity. Radioactivity was measured with a Beckman L200 liquid scintillation counter. Scintillation fluid contains 0.3% 2,5-diphenyloxazole, 0.02% 1,4-bis 2-(4-methyl-5-phenyl-oxazolyl)-benzene, and 25% Triton X-114. Fig. 1 shows separation of radioidinated phytochrome by Sephadex G-25-100 column chromatography.

Multilamellar liposome was prepared in 20 mM phosphate buffer, pH7.2, containing various concentrations of KCl (0.02~1.0M) at room temperature, as described elsewhere(Kim and Song, 1981). A chloroform solution (3~5ml) containing 10mg egg lecithin and 1mg cholesterol in a round bottom flask was evaporated to dryness under reduced pressure. Thin films of the lipid mixtures were dispersed in 3ml of the buffer with a vortex. The milky supension was then sonicated with a Bransonic 12 cleaner until aggregated particles were separated under a nitrogen gas atmosphere. During sonication, aliquots were taken from the suspension and examined under a microscope. Prepared multilamellar liposomes were stored at 277K and used for the binding experiments of 59kD phytochrome within several hours.

Binding of 59 kD phytochrome to liposomes was measured as follows. A half ml of multilamellar liposomes in 20 mM phosphate buffer, pH 7.2, with different concentrations of KCl was mixed with radioiodinated Pr or Pfr(7.7μg phytochrome, 26,700cpm/μg phytochrome) and incubated for 3h at 295K. The incubation mixture was applied to a Sepharose CL-4B column (1.5×18cm) equilibrated with 20mM phosphate buffer, pH 7.2, containing the same KCl concentrations as the incubation mixtures. The column was eluted with the equilibration buffer at a flow rate of 2ml/min at 295K. The collected fractions (1.5ml/tube) were measured for optical density at 300nm for liposome assay. A 0.2ml sample taken from each fraction was measured for radioactivity. Usually 3 or 4 major fractions were used for the calculation of binding stoichiometry. A typical elution profile of liposome-phytochrome-^{125}I by a Sepharose CL-4B column chromatography is shown in Fig. 2.

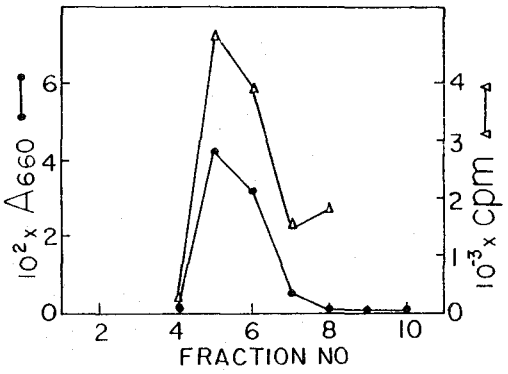

Fig. 2. A typical elution profile of liposome-59kD phytochrome-^{125}I by a Sepharose CL-4B column chromatography

Binding experiments were carried out with 1.8mg of multilamellar liposomes and 7.7μg of 59kD Pfr in 0.65ml of 20mM sodium phosphate buffer, pH7.2, containing 250mM KCl. The collected fractions(1.5 ml/tube) were measured for optical density at 300nm for liposome and radioactivity for the bound phytoch rome assay

The binding properties of 59kD phytochrome to Cibacron Blue dye were measured on Affi-Gel Blue (Bio-Rad) column equilibrated with phosphate buffer, pH 7.8. containing 50mM KCl and 0.1mM EDTA. The purified 59kD phytochrome solution in the equilibration buffer was applied to the column(50ml column volume) and eluted with equilibration buffer containing different concentrations of KCl.

Absorption spectral measurements were performed on a Cary 118C spectrophotometer. Irradiation of phytochrome for phototransformation was carried out with a Bausch & Lomb microscope illuminator combined with a 660nm interference filter(Oriels C572-6600) for the red light source (7.5 W/m^2), and with an far-red cutoff

filter(Ealing 26-4457) for the far-red light source(1.6kW/m²). All chemicals for phytochrome purification and sample preparations were purchased from Sigma Chemical Co.

Results and Discussion

Absorption spectrum of the purified 59kD phytochrome is shown in Fig. 3. This spectrum is almost identical with that of the 118kD phytochrome. The purified 59kD phytochrome is radioiodinated and used for the binding experiments to liposomes. Fig. 4 shows the binding of the iodinated 59kD phytochrome to multilamellar liposomes as a function of ionic strength at 295K. The binding of the 59kD Pr to liposomes is increased steadily whereas that of the 59kD Pfr is first decreased and then enhanced by increasing ionic strength of the medium. At lower ionic strength, Pfr binds more to liposomes than does Pr. At higher ionic strength, the Pr preferentially binds over Pfr. The result also indicates that the binding of Pr to liposome is hydrophobic in nature whereas that of Pfr is electrostatic at lower ionic strength. These results are in contrast with 118kD phytochrome in which the Pfr preferentially binds to liposome through hydrophobic interactions (Kim and Song, 1981). Thus it is assumed that the hydrophobic surface which is exposed in the Pfr of 118kD phytochrome is absent in 59kD Pfr.

Fig. 5 shows an elution profile of the purified 59kD phytochrome on Affi-Gel (Cibacron) Blue dye column. It can be seen that more than half of the applied 59kD Pr but no Pfr was eluted by the column equilibration buffer (Fig. 5A). Both forms of the 59kD phytochrome are efficiently eluted by the same buffer with higher ionic strength (0.55M KCl) (Fig. 5A). The results indicate that the binding force between the ligand (Cibacron Blue 3GA) and the 59kD phytochrome might be electrostatic with higher affinity for the Pfr form. This was further confirmed in Fig. 5B. More than 90% of the applied Pr but negligible amount of Pfr were eluted by

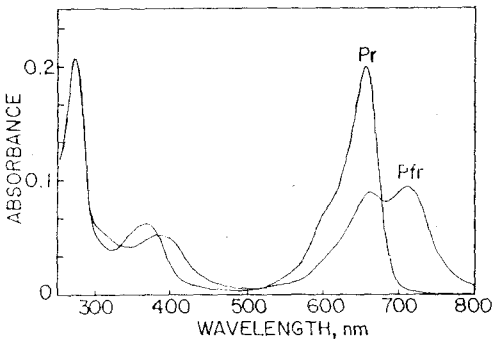

Fig. 3. The absorption spectrum of the purified 59kD phytochrome in 0.1M sodium phosphate buffer, pH 7.8, containing 0.1mM EDTA and 50mM KCl

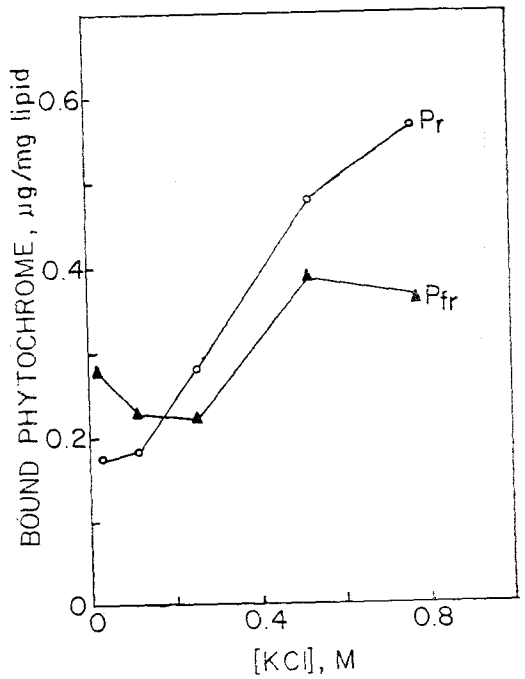

Fig. 4. Ionic strength dependence of the binding of radioidinated 59kD phytochrome to multilamellar liposomes at 295K

The concentrations indicated are the final amount of KCl in the incubation mixture, pH7.2

the same buffers with medium ionic strength (0.25M KCl). The Pfr was totally eluted by 0.55M KCl. These results are consistent with those of liposome binding experiments (*vide supra*), suggesting that most of the hydrophobic surface in the hydrophobic surface in the 118kD Pfr are lost in the 59kD Pfr.

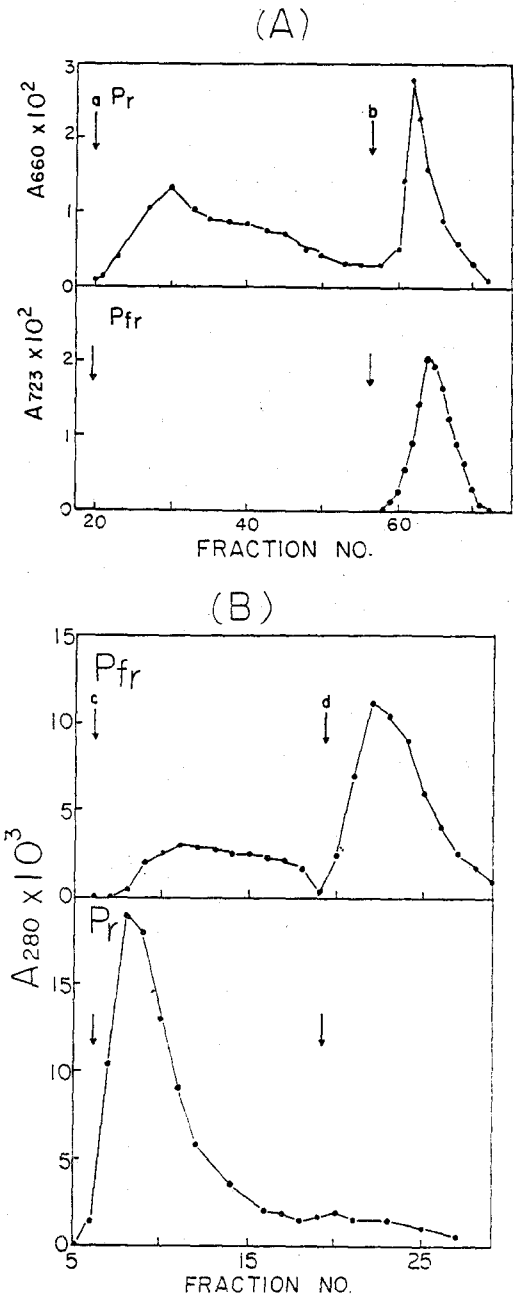

Fig. 5. Elution profiles of the purified 59kD Pr and Pfr on Affi-Gel(Cibacron) Blue dye column
(A) Applied phytochrome (2mg Pr:1mg Pfr) was first eluted with equilibration buffer(arrow a; 0.1M sodium phosphate buffer, pH 7.8, containing 50mM KCl and 0.1mM EDTA) and then eluted with the same buffer containing 0.55M KCl (arrow b). (B) Applied phytoch rome (0.5mg) was first eluted with the same equilib ration buffer containing 0.25M KCl (arrow c) and then eluted with the same buffer containing 0.55M KCl (arrow d). Column volume was 10ml

It has been well established that a hydrophobic surface is exposed upon Pr to Pfr phototransformation in the 118kD and 124kD phytochromes (Hahn and Chae, 1986; Hahn and Song, 1981, 1982, 1987; Hahn et al., 1980, 1984a, 1984b). Present data, however, strongly support the idea that the 59kD Pfr does not have the exposed hydrophobic domain. Daniels and Smith (1981) reported that the Pfrspecific interaction with Cibacron Blue 3GA is lost when the 118kD phytochrome is degraded to the 59kD phytochrome. However, present data (Fig. 5) indicate that the 59kD Pfr still preferentially binds to the Blue dye via ionic interactions. The 59kD Pfr also binds to liposomes through electrostatic interactions (Fig. 4). The binding of the 59kD Pr to liposomes is hydrophobic in nature (Fig. 4). Thus it is not surprising that the 59kD Pr subject to Affi-Gel Blue column can be eluted at lower ionic strength (Fig. 5A). Other results such as loss of the preferential binding to 8-anil inonaphthalene-1-sulfonate to the 59kD Pfr (Hahn, 1989), lack of the increased affinity of the 59 kD Pfr for the hydrophobic ligand palmitate (Tokutomi et al., 1981), and no difference in phosphorescence lifetime between the 59kD Pr and Pfr forms (Hahn and Song, 1987) are also in agreement with present data.

Hershey et al., (1985) suggests that the relatively extensive hydrophobic region in apophytochrome revealed by hydropathy analysis of the deduced amino acid sequence resides between residue 80 and 315 in the chromophore bearing domain. Thus it is assumed that the hydrophobic surface is not located in the 55kD nonchromophore domain. However, it is clear that the 55kD polypeptide plays a major role to the increase of hydrophobicity upon Pr to Pfr phototransformation in the 118 and 124kD phytochromes. The 55kD domain might involve in light-induced conformational changes which introduce an exposure of hydrophobic surface on the chromophore bearing domain in the 118 and 124kD Pfr. This idea is further supported by the fact that the N-terminal 6kD polypeptide segment is

predominantly hydrophilic(Hershey et al., 1985; Hahn and Chae, 1986), but critical the dramatic increase of hydrophobicity in the 118kD Pfr. However, exact location of the hydrophobic domain and the role of the 55kD tryptic polypeptide are yet to be determined.

Abstract

Binding properties of the degraded 59kD phytochrome from etiolated *Avena sativa* seedlings to liposomes and Cibacron Blue dye were examined. In contrast with the native 124kD and partially degraded 118kD phytochromes, the far-red light absorbing(Pfr) forms of the 59kD phytochrome binds to liposomes and Cibacron Blue dye via electrostatic interactions. Results indicate that the 59kD Pfr does not hold a hydrophobic surface which is exposed upon Pr to Pfr phototransformation of the 124 and 118kD phytochromes. Since a relatively extensive hydrophobic region is located in the chromophore bearing domain(59kD) of phytochrome(Hershey et al., Nuc. Acids Res., 13, 8543, 1986), the 55kD tryptic domain from the C-terminus plays an important role on the exposure of the hydrophobic area in the 118 and 124 Pfr to occur.

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