

# Immunochemical and Spectroscopic Evidence for Protein Conformational Changes in Phytochrome Transformations

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## ABSTRACT

Phytochrome was examined by immunochemical and spectroscopic techniques to detect differences between the protein moieties of red- and far red-absorbing phytochrome ( $P_r$  and  $P_{fr}$ ). No differences in the reaction of  $P_r$  and  $P_{fr}$  with phytochrome antibody were discernible on Ouchterlony double diffusion plates. However, the microcomplex fixation assay showed a greater degree of antibody reaction with  $P_{fr}$  than with  $P_r$ , indicating some difference in the surface characteristics of the two forms. Circular dichroism spectroscopy between 300 and 200 nanometers revealed differences between  $P_r$  and  $P_{fr}$  which may reflect differences in the protein conformation. The circular dichroism spectrum of  $P_r$  showed a negative band at 285 nanometers which was not present in the spectrum of  $P_{fr}$ , and the large negative circular dichroism band at 222 nanometers with  $P_{fr}$ , associated with the  $\alpha$ -helical content, was shifted 2 nanometers to shorter wave length with  $P_r$ , although there was no change of magnitude of this band. The absorbancy of  $P_r$  and  $P_{fr}$  is very nearly the same in the 280 nanometer spectral region, but sensitive difference spectra between  $P_r$  and  $P_{fr}$  did reveal spectra which were similar to solvent perturbation spectra obtained by others with different proteins. In total, the experiments indicate that there are conformational differences between the protein moieties of  $P_r$  and  $P_{fr}$  but that these differences are rather slight from a standpoint of gross structure.

## MATERIALS AND METHODS

All chemicals used were reagent grade. Molecular sieve gels were sized and washed extensively before use. Brushite was made up in a modification of the procedure of Levin (11).  $\text{CaCl}_2$ , 1 M, and 1 M  $\text{K}_2\text{HPO}_4$  were mixed with vigorous mechanical stirring. The precipitate was allowed to digest and was stored in the mother liquor. Before being used it was washed with water followed by 0.30 M  $\text{K}_2\text{HPO}_4$  and immediately equilibrated with 0.015 M potassium phosphate buffer, pH 7.8.

**Phytochrome Purification.** The phytochrome samples were prepared from 4-day-old etiolated oat seedlings (*Avena sativa* var Garry). All operations were done under a dim green safe-light, and care was taken to maintain the phytochrome in the  $P_r$  state at temperatures less than 5 C. Frozen leaf and coleoptile tissue was ground in a Waring Blendor with 0.050 M tris,<sup>1</sup> pH 8.5, containing 1.4% (v/v) 2-mercaptoethanol and 0.007% (v/v) SAG antifoam agent. Coarse debris was removed in a basket centrifuge. Calcium chloride was added to a final concentration of 0.015 M, and the pH was raised to 7.8 with tris. After centrifugation, EDTA was added to a final concentration of 0.005 M, and the concentration of phosphate was brought to 0.005 M with  $\text{K}_3\text{PO}_4$  still at pH 7.8. This solution was passed through Brushite (0.5 liter of gel per kg of fresh tissue) equilibrated with 0.015 M phosphate buffer, pH 7.8. Phytochrome was eluted with 0.060 M phosphate, pH 7.8, and was concentrated and purified by precipitation with 50% saturated ammonium sulfate (315.5 g of ammonium sulfate were added per liter of solution). The precipitate was solubilized in 0.060 M phosphate, pH 7.8, and was passed through a Sephadex G-100 column (5.5  $\times$  150 cm) equilibrated with 0.10 M phosphate, pH 7.8, containing 0.50 M KCl. The eluted phytochrome was precipitated by adding an equal volume of saturated ammonium sulfate and resolubilized in 0.070 M phosphate, pH 7.8. Dialysis against 0.070 M phosphate, pH 6.0, precipitated some undesired proteins which were removed by centrifugation. The phytochrome was applied to a carboxymethyl-Sephadex column (2 ml of gel per mg of phytochrome) equilibrated with 0.070 M phosphate, pH 6.0. The column was washed with the pH 6.0 buffer, and the phytochrome was eluted with 0.070 M phosphate, pH 7.8. The sample was concentrated again by precipitation with an equal volume of saturated ammonium sulfate, and the precipitate was resolubilized in 0.070 M phosphate, pH 7.8. It was then applied to a P-150 column (2.2  $\times$  150 cm) equilibrated with 0.25 M phosphate, pH 7.8. Peak fractions of phytochrome were pooled, concentrated by ammonium sulfate precipitation,

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The growth and development patterns of many plants can be controlled by brief irradiation periods of red and far red light (7). This control is mediated through a chromoprotein, phytochrome, which changes reversibly between a red-absorbing form,  $P_r$ , and a far red-absorbing form,  $P_{fr}$ , in response to red and far red light. The chromoprotein with the reversible photochromic behavior has been isolated and purified from dark-grown seedling plants (5, 11, 14, 16, 17). On the basis of different sensitivities of  $P_r$  and  $P_{fr}$  to protein denaturants, it was suggested that the chromophore change was accompanied by a change of protein conformation (4). Such a conformational change could alter the properties or activities of the chromoprotein and thus be the basis by which light activates or inactivates phytochrome-controlled processes. Attempts to show conformational changes by differences in the hydrodynamic properties of the two forms of phytochrome were negative (2). In the present paper we have sought additional evidence from immunochemical techniques, circular dichroism spectroscopy, and absorption spectroscopy to indicate structural differences between the two forms of phytochrome.

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<sup>1</sup> Abbreviations: tris: tris(hydroxymethyl)aminomethane, adjusted to the proper pH with HCl; SAG: SAG-471 silicone antifoam fluid (Union Carbide Corp.); EDTA: ethylenediaminetetraacetic acid (as the disodium salt), brought to pH 7.9 by adding KOH; CD: circular dichroism. All phosphate buffers were made from the potassium salts in the proper proportion to give the desired pH at the proper phosphate concentration.

and resolubilized in 0.01 to 0.10 M phosphate, pH 7.8. This procedure, starting with 10 kg of dark grown oat seedling tissue generally yielded about 10 mg of phytochrome in which the ratio of absorbance at 280 nm to that at 667 nm was between 0.92 and 1.20.

In this paper the ratio of absorbance of  $P_r$  at 280 and 667 nm,  $A_{280}/A_{667}$ , is used as an index of purity of the phytochrome. Mumford and Jenner (11) reported the ratio to be 1.07 for pure phytochrome. Phytochrome concentration was determined assuming  $A_{667}^{1\%} = 1.0$ .

**Preparation of Antisera.** The antisera used for these studies were produced in rabbits by injecting 1 mg of phytochrome (purity index = 1.0) in a 1:1 emulsion with Freund's adjuvant. The animals were injected under fluorescent light and were kept on their normal daily light regime. After 6 weeks, each rabbit was given an intravenous booster of 0.5 mg of phytochrome without adjuvant, and the sera were taken 10 days later.

**Ouchterlony Double Diffusion Plates.** The antibody-antigen reactions were observed by placing 0.1 ml of antiserum (full strength) and 0.1 ml of antigen (0.5 mg/ml) in adjacent wells in agar gel containing 0.15 M NaCl. Gradients of concentration are formed as the two samples diffuse toward each other and a line is observed where the concentrations of antibody and antigen are optimal for precipitation. The method (13) requires relatively large amounts of antiserum and antigen, but it is useful for assessing cross-reactions and multiple antibody-antigen systems.

**Microcomplement Fixation Assay.** Complement is a mixture of serum proteins which causes the lysis of red blood cells that have reacted with the antibody to those red blood cells. Complement is also fixed by reacting with other antigen-antibody complexes and can be used to measure the extent of the antibody reaction. In the microcomplement fixation assay the antigen-antibody reaction is allowed to proceed in the presence of a standard amount of guinea pig complement. Sheep red blood cells and the antibody to these cells produced by rabbits are then added to the reaction mixture. The amount of hemoglobin released because of the action of unfixed complement is inversely proportional to the amount of complement originally fixed and, therefore, to the extent of the antigen-antibody reaction. The amount of complement fixed depends on the size of the antibody-antigen complex. The largest aggregates are formed when stoichiometric amounts of antibody and antigen are present. If there is excess antibody or excess antigen, the complexes terminate in smaller aggregates and less complement is fixed. A curve of the amount of complement fixed at various concentrations of antigen at a given dilution of antibody with a standard amount of complement has a characteristic bell shape with a maximum at the antibody-antigen "equivalence point." The technique requires a very small quantity of antigen and is quite sensitive to the degree of antibody-antigen reaction. The method is explained in detail by Murphy and Mills (12) in their adaptation of the method of Wasserman and Levine (18).

In the present work  $P_r$  was diluted to the proper concentrations and aliquots were distributed to the assay tubes under dim green safe-light. The tubes contained a standard amount of a phosphate buffer with gelatin as a protector of the enzymatic activities of the complement, and all dilutions of phytochrome were made with this buffer. The remaining phytochrome samples were then irradiated with 659 nm red light for 1 min and the  $P_{fr}$  was distributed to other assay tubes. Standard amounts of complement and diluted antiserum were then added to the tubes. Twenty-four hours later sheep red blood cells and their antibody were added, and lysis was allowed to proceed at 37 C for about 20 min. The assay tubes were then centrifuged, and the amount of liberated hemoglobin was measured in a Zeiss PMQ II spectrophotometer at 415 nm.

**Circular Dichroism Spectra.** The Jasco CD-ORD 5 instrument equipped with a phototube with an S-20 spectral response was used to record the spectra. The temperature was maintained at 14 C with jacketed cuvettes. A 1-cm path length cuvette was used for measurements in the visible region and a 0.1-cm cuvette was used for the ultraviolet region. Care had to be taken to maintain the phytochrome in the proper form while scanning the visible region because of the actinic effect of the measuring beam during the long periods required for a scan. There was no detectable conversion due to the measuring beam at wave lengths below 400 nm.

Ellipticities were calculated on a mean residue weight basis, according to the equation

$$[\theta]_{\lambda} = \frac{3300 \Delta A \cdot m \cdot r \cdot w}{bc} \frac{(\text{cm}^2 \cdot \text{deg})}{(\text{decimole})}$$

where  $[\theta]_{\lambda}$  is the decimolar ellipticity,  $\Delta A$  the observed difference

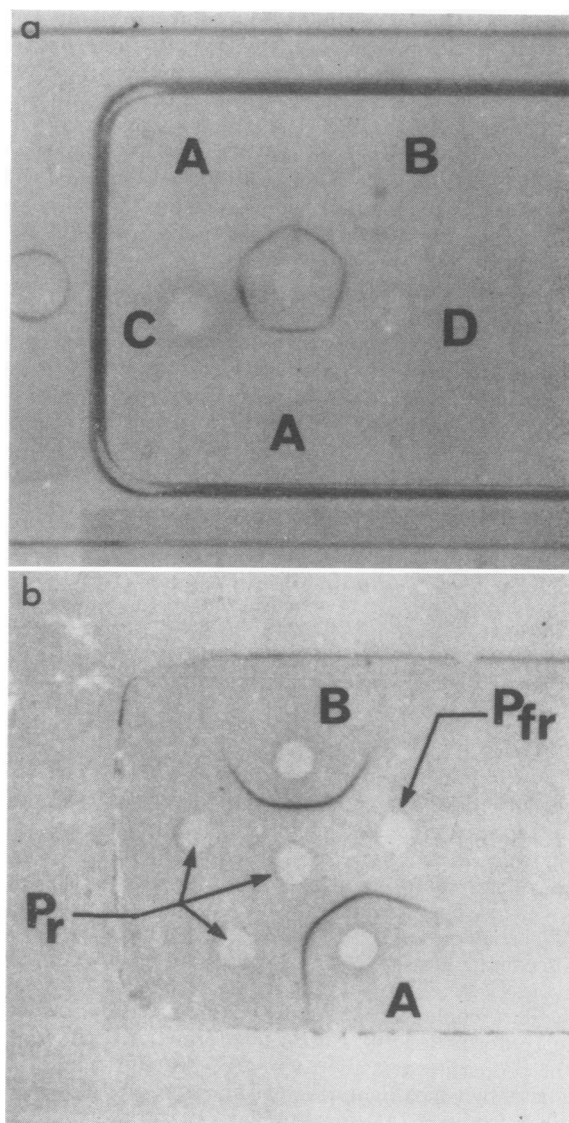


FIG. 1. Antibody reactions on Ouchterlony plates. a: Phytochrome in the  $P_r$  form at various stages of purity (outside wells) diffused toward antiserum number 8468 (center well). The purity indexes of the phytochrome were: A: 0.92; B: 3.0; C: 12.0; and D: 40.0. b:  $P_r$  and  $P_{fr}$  (purity index 0.98) diffused toward two different antisera (A: number 8468, and B: number 8469).

in absorbance of left and right circularly polarized light,  $b$  the pathlength (cm),  $c$  the concentration of protein (mg/ml), and  $m_{rw}$  the mean residue weight, taken to be 114 g/mole. The calculation of  $\alpha$ -helical content assumes a  $[\theta]_{222} = +400 \text{ deg} \cdot \text{cm}^2/\text{decimole}$  for the random coil and  $-38,000 \text{ deg} \cdot \text{cm}^2/\text{decimole}$  for the 100%  $\alpha$ -helical polypeptide (8).

## RESULTS AND DISCUSSION

The precipitin lines observed on double diffusion agar plates with phytochrome ( $P_r$ ) at purity indexes ranging from 40 to 0.92 showed single confluent lines without spurs (Fig. 1a) indicating that the antibody was directed solely against the phytochrome in these preparations. The use of phytochrome of low purity suggests that phytochrome from different sources could be compared immunologically without extensive purification.

In an attempt to demonstrate differences between the two forms of phytochrome,  $P_r$  and  $P_{fr}$  were allowed to diffuse toward antisera taken from two different rabbits (Fig. 1b). The single confluent lines without spurs indicate that no differences between  $P_r$  and  $P_{fr}$  were detectable by this technique and that there was no class of antibodies directed solely against  $P_r$  or  $P_{fr}$ . The cross-reactions showed that the antibody will precipitate both  $P_r$  and  $P_{fr}$ . Since no differences were apparent in the Ouchterlony plates, the antigen-antibody reactions were examined by the more quantitative and more sensitive microcomplement fixation assay procedure.

In typical microcomplement fixation assays (Fig. 2) with freshly purified phytochrome as antigen,  $P_{fr}$  reacted more strongly with the antibody than did  $P_r$ . The control with  $P_r$  regenerated from  $P_{fr}$  showed that the level of complement fixation was restored to the original  $P_r$  level. The higher reactivity of  $P_{fr}$  suggested that  $P_{fr}$  might have more antigenic determinants than  $P_r$ . To test this hypothesis, assays were carried out with  $P_{fr}$  (or  $P_r$ ) samples in the presence of  $P_r$  (or  $P_{fr}$ ) at a concentration 10-fold higher than the concentration at the equivalence point. If there were a class of antibodies directed against a unique set of determinants on  $P_{fr}$ , a normal or slightly depressed titration curve for  $P_{fr}$  would be obtained even in the presence of excess  $P_r$ , the height and equivalence point depending on how many extra determinants  $P_{fr}$  contained. The results in Figure 2 show that excess  $P_r$  added to  $P_{fr}$  (and excess  $P_{fr}$  added to  $P_r$ ) completely depressed the complement fixation curves, indicating

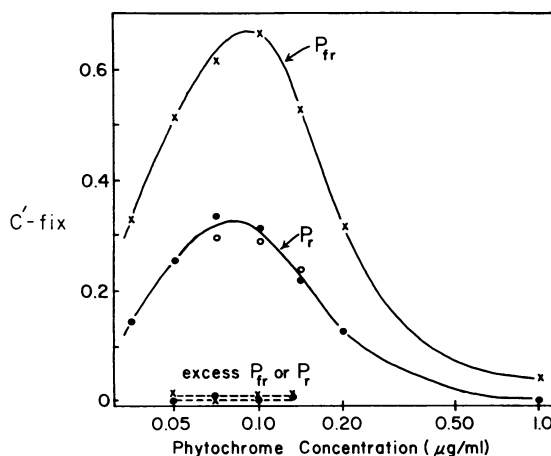


FIG. 2. Complement fixed versus concentration of  $P_r$  and  $P_{fr}$ . Antiserum number 8469 was used at a dilution of 1/2500. (●—●):  $P_r$ ; (×—×):  $P_{fr}$ ; (○—○):  $P_r$  regenerated from  $P_{fr}$ . In the competition experiments (lower curves) the competing  $P_r$  (or  $P_{fr}$ ) was added at 1  $\mu\text{g}/\text{ml}$  20 min prior to adding the  $P_{fr}$  (or  $P_r$ ) samples (●—●):  $P_r$  in the presence of excess  $P_{fr}$ ; (×—×):  $P_{fr}$  in the presence of excess  $P_r$ .

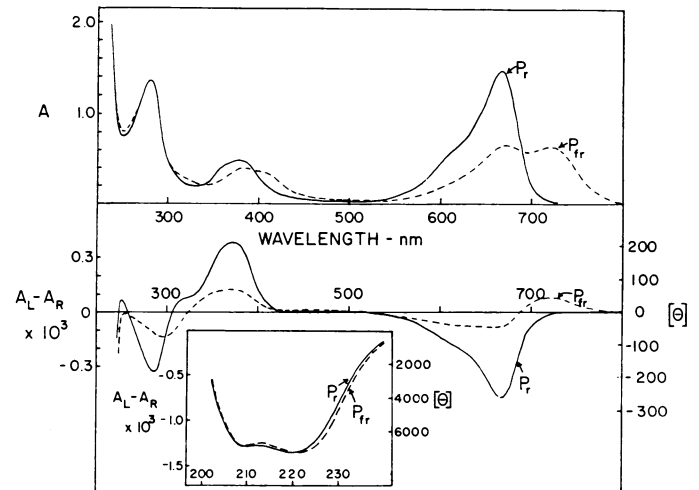


FIG. 3. Circular dichroism spectra and absorption spectra of phytochrome. The phytochrome (1.4 mg/ml, purity index 1.0) was in 0.010 M phosphate buffer, pH 7.8. A 1-cm jacketed cell at 14 C was used for the CD spectra in the visible and near-ultraviolet regions. A 0.1-cm jacketed cell with the phytochrome diluted one-half was used for the measurement of the CD spectra below 300 nm. The values of the measured ( $A_L - A_R$ ) spectra are given in the left-hand scale; on the right these values are converted into decimolar ellipticities (see "Materials and Methods"). The absorption spectra were measured in 1-cm cuvettes in the Cary-14 recording spectrophotometer at about 23 C.

that there were no antibodies directed solely against determinants unique to either  $P_{fr}$  or  $P_r$ . We conclude that  $P_{fr}$  and  $P_r$  possess common determinants. The greater reactivity of  $P_{fr}$  in the complement fixation assay can be attributed to a greater binding constant for the  $P_{fr}$ -antibody complex, perhaps resulting from a spatial change of the determinants. Apparently, the surface characteristics were altered by the transformation of  $P_r$  to  $P_{fr}$  so that a stronger antibody reaction resulted.

The absorption spectra and CD spectra of  $P_r$  and  $P_{fr}$  from 800 to 200 nm are shown in Figure 3. The CD spectra from 800 to 300 nm are due to optical activity associated with the chromophore absorption bands of  $P_r$  and  $P_{fr}$ . The CD spectrum of red-irradiated sample shows some contribution from  $P_r$  because red light establishes a photo-stationary state of about 80%  $P_{fr}$  and 20%  $P_r$ . The CD spectra, in fact, provide reasonable confirmation of that value for the photo-stationary state which was calculated previously from kinetic data on the photoconversion of  $P_r$  and  $P_{fr}$  (3). The optical activity may be due to optically active atoms in the chromophore or an asymmetric interaction between the chromophore and the protein or to a combination of both effects. Siegelman *et al.* (15) proposed a model for the photoisomerization of phytochrome in which the chromophore of  $P_r$  has two optically active carbon atoms while the chromophore of  $P_{fr}$  has none. The finding that the 730 nm absorption band of  $P_{fr}$  shows some positive ellipticity does not rule out this model. The larger negative CD bands of  $P_r$  could be due to optically active centers in the  $P_r$  chromophore while the smaller positive CD band of  $P_{fr}$  could be due to asymmetry of the environment around  $P_{fr}$  chromophore. Kroes (9) has shown the same CD spectra in the region of 800 to 300 nm.

Below 300 nm we associate the optical activity with protein absorption bands although we have not ruled out the possibility of chromophore optical activity in this region. The negative CD band at 280 nm in the spectrum of  $P_r$ , but not  $P_{fr}$ , suggests a change of asymmetry near an aromatic amino acid. At shorter wave lengths the large negative ellipticity with minima near 222 and 209 nm is indicative of  $\alpha$ -helix. Transformation of  $P_{fr}$  to  $P_r$  is accompanied by a shift of the 222 nm minimum by about 2

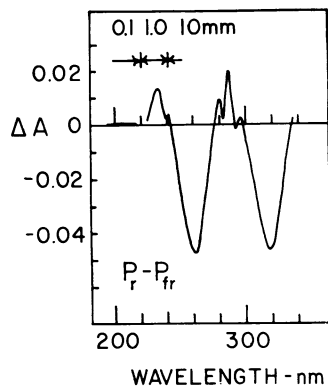


Fig. 4. Ultraviolet difference spectrum of  $P_r$  minus  $P_{fr}$ . The phytochrome (1.5 mg/ml, purity index 0.92) was in 0.010 M phosphate buffer, pH 7.8 at about 23 C. The spectrum was measured with a Cary-14 recording spectrophotometer with 0.1-, 1.0-, and 10.0-mm cells for the spectral regions indicated.

nm to shorter wave length. The shift is small but readily confirmed since the photoreversibility between the two forms permits repeated comparisons to be made between  $P_r$  and  $P_{fr}$  at fixed wave lengths. Such measurements at wave lengths in the steeply declining region of CD between 235 and 225 nm consistently showed less negative ellipticity with  $P_r$  than with  $P_{fr}$ . The precise nature of the change indicated by the shift of the 222 nm CD band is not known, but it is reasonable to suggest that the shift reflects a change in the structure of the polypeptide backbone which is relatively small from a standpoint of gross structure. Assuming 100% purity for the phytochrome, an  $\epsilon_{665}^{0.1\%}$  of 1.0 for  $P_r$ , and the specific ellipticities for the  $\alpha$ -helix and random coil given for polylysine (8), the  $\alpha$ -helix content of  $P_r$  and  $P_{fr}$  were calculated to be about 27%.

The absorption difference spectrum between  $P_r$  and  $P_{fr}$  (Fig. 4) shows considerable detail in the absorption band region of the aromatic amino acids. Maxima at 280, 287, and 297 nm with minima at 283 and 293 nm suggest different environmental influences on some of the aromatic amino acids in  $P_r$  and  $P_{fr}$ . We cannot distinguish spectral changes due to the protein from those of the chromophore. The minimum at 320 nm is undoubtedly due to the chromophore since protein has relatively little absorption at wave lengths greater than 300 nm. The absorption in the 280 nm absorption band, however, is primarily due to the protein, and similar difference spectra with up to three maxima have been observed in solvent perturbation difference spectra of proteins and model mixtures of amino acids (1, 6).

We conclude that the results of the immunochemical assay indicate structural differences between  $P_r$  and  $P_{fr}$ . These differences are mirrored in the circular dichroism and absorption spectra of the two forms.

The above work was carried out on phytochrome with a purity index of 0.92. A later modification of the purification procedure (the G-100 Sephadex column was replaced with a diethylaminoethyl cellulose column) gave phytochrome with a purity index of 0.78. (W. R. Briggs of Harvard University previously obtained phytochrome with this purity index using a very similar purification procedure, personal communication.) The qualitative conclusions reached in this paper with the earlier phytochrome would not be altered by the presence of small amounts of contaminating protein. Using more pure phytochrome would merely have made the differences between  $P_r$  and  $P_{fr}$  greater.

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