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THE PHOTOTRANSFORMATION OF PHYTOCHROME PROBED BY 360 MHz PROTON NMR SPECTRA

Pill-Soon Song[1,*], Hemanta K. Sarkar,[1] Hani Tabba[2] and Kevin M. Smith[2]

[1]Department of Chemistry, Texas Tech University, Lubbock, Texas 79409 U.S.A.
[2]Department of Chemistry, University of California, Davis, CA 95616 U.S.A.

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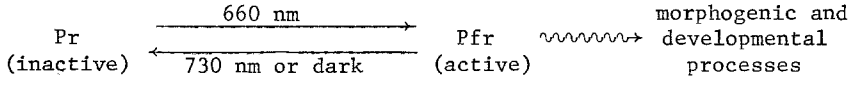
The 360 MHz proton NMR spectra of the Pr and Pfr forms of phytochrome have been recorded to probe the nature of the phytochrome phototransformation (Pr → Pfr). The NMR spectra of aliphatic protons in Pr and Pfr proteins are similar, suggesting that the conformation of both proteins are not drastically different. However, the NMR spectrum of aromatic and the -NH- proton resonance region of Pfr differs significantly from that of Pr, including the absence of a resonance at 6.15 ppm in the former. Differences in the NMR spectra of small and large mol wt phytochromes have also been noted.

INTRODUCTION

Phytochrome (Pr) is phototransformed into the physiologically active form (Pfr) by 660 nm light, triggering a variety of responses in plants [See recent reviews by Rüdiger and Pratt (1,2)]:



A number of studies have focussed on the question of whether or not significant conformational changes result from the phototransformation of Pr to Pfr (3-14).

Our view is that the chromophore reorients and becomes exposed as a result of the Pr → Pfr phototransformation (8, 10-12, 14). The chromophore reorientation then generates an additional protein surface (hydrophobic) which entails a local conformational change in the Pfr form.

The present study of the 360 MHz proton NMR spectra of the Pr and Pfr forms of phytochrome was undertaken to ascertain whether or not (a) a large con-

* To whom correspondence regarding this paper should be addressed.

Abbreviations: DSS, 2,2-dimethyl silapentane 5-sulfonic acid; NMR, nuclear magnetic resonance; Pr, red light absorbing form of phytochrome; Pfr, far-red light absorbing form of phytochrome; ppm, parts per million.

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formational change of protein occurs and (b) amino acid residues and/or the chromophore gain sufficient flexibility in the Pfr form as the result of exposure of the protein surface. Recently, the Pr chromophore and its chromopeptide have been characterized by NMR, but the large and small mol wt phytochromes and their apoproteins have not been studied by the same technique (15).

MATERIALS AND METHODS

Large mol wt phytochrome (~120,000 as determined by gel electrophoresis) was isolated and purified from etiolated oat seedlings (Avena sativa L., Garry oat) according to Song et al. (16) modified from the Affi-gel Blue affinity procedure (17). Small mol wt phytochrome (~60,000) was prepared by the tryptic digestion of large phytochrome as follows. The Brushite fraction of phytochrome was treated with trypsin (1 µg trypsin/5 mg protein) for 12 h at 4° C. After 12 h, approximately the same amount of trypsin inhibitor was added to the trypsin-treated phytochrome preparation and incubated for ca. 15 min, followed by centrifugation to remove any precipitate. A clear supernatant was then applied to an Affi-gel Blue (Bio Rad) affinity column and equilibrated with 0.1 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA and 14 mM 2-mercaptoethanol. After ca. 1 h, the column was washed with about two-column volumes of 0.1 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA and 14 mM 2-mercaptoethanol and 50 mM KCl. The tryptic digested phytochrome was then eluted with 10 mM lumichrome-free FMN (16) in the starting buffer, pooled, fractionated with ammonium sulfate, redissolved in 0.1 M sodium phosphate buffer, pH 7.8, and then applied to a Biogel 0.5 m column equilibrated with the same buffer. The purified fraction of specific absorbance ratio (A_{660}/A_{280}) ~1.17 was collected, refractionated with ammonium sulfate and then dissolved in D_2O-sodium phosphate buffer (0.1 M), pD 7.8, for NMR measurements. After preparation, the Pr phytochrome had a mol wt of 60,000.

NMR spectra were recorded (with respect to DSS) in a 360 MHz Nicolet NMR spectrometer at the University of California NMR facility (Davis, CA) using 5mm-diameter NMR tubes. The phytochrome concentrations used were 1 mg/ml and 0.9 mg/ml for small and large phytochromes, respectively, at 280 and 278 K, respectively. Phytochrome phototransformations, Pr → Pfr and Pfr → Pr, were carried out with a Bausch & Lomb microscope illuminator, red interference filter (Oriel C572-6600; 8 W/m^2) and far-red cutoff filter (Ealing 26-4457; 1.6 kW/m^2).

RESULTS

Figure 1 shows the 360 MHz NMR spectra of small Pr and Pfr in the upfield region after 5000 and 2500 computer accumulated scans, respectively. The two spectra are virtually superimposable, when differences in signal-to-noise ratios are taken into consideration. This result suggests that the conformation of the folded peptide core in phytochrome remains largely unaltered upon Pr → Pfr phototransformation. It should be noted that the NMR spectra seen in Fig. 1 can be assigned to various methyl protons and α-hydrogens (18, 19). The resonances observed at <1 ppm (relative to DSS) are possible due to methyl protons in the vicinity of aromatic amino acid residues and/or the chromophore.

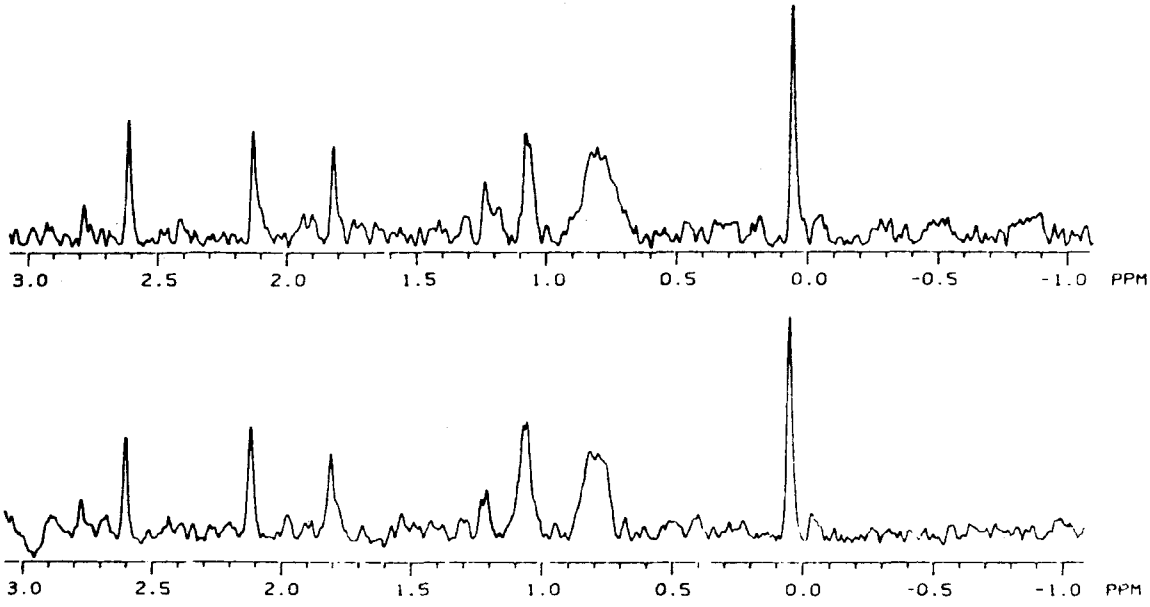
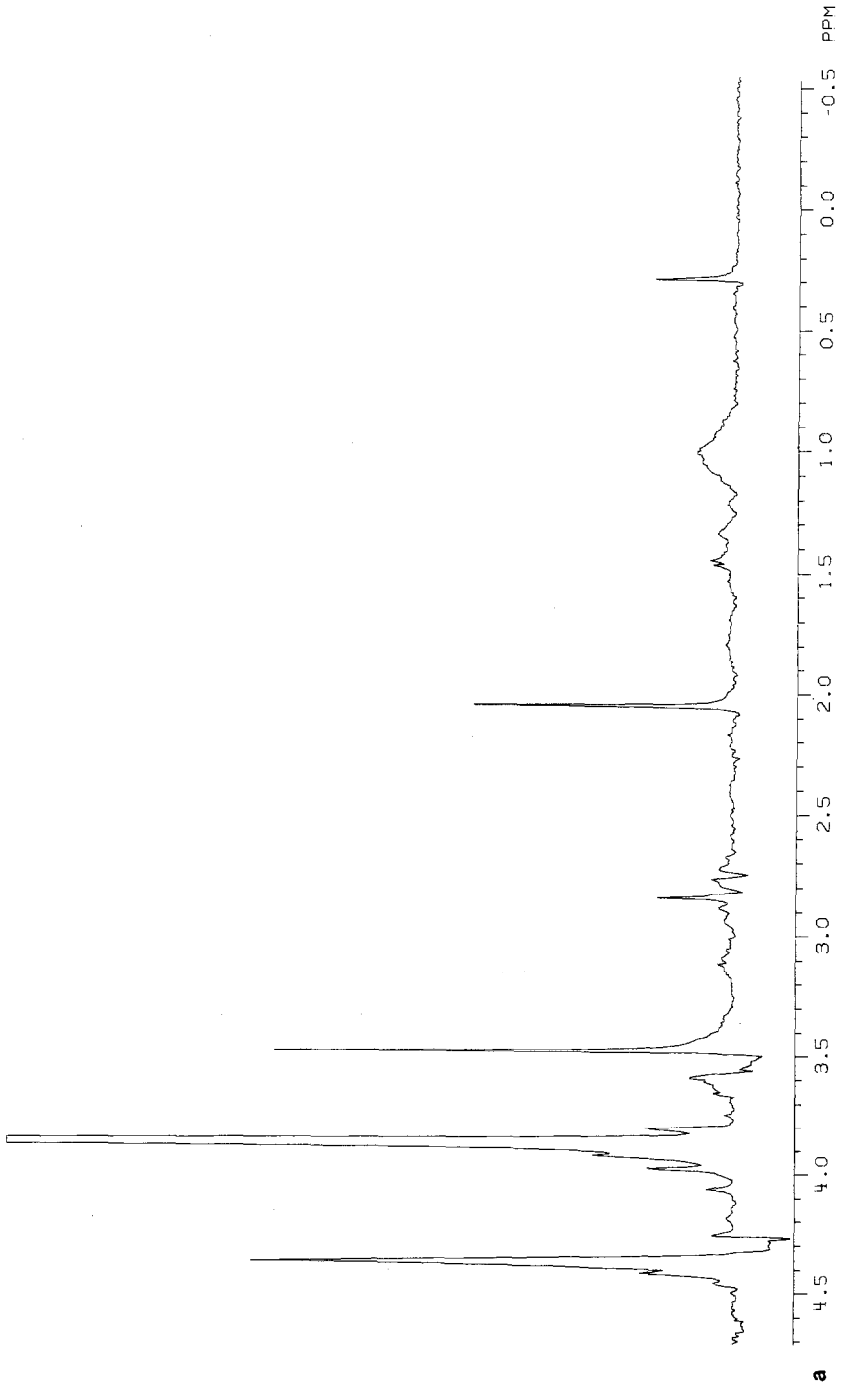


Figure 1 - The 360 MHz proton NMR spectra of phytochrome (small mol wt, 60,000) in D_2O, 0.1 M sodium phosphate buffer, pD 7.8, at 280 K. Top panel: Pr; lower panel: Pfr.

A significant NMR change upon Pr → Pfr transformation is shown by the 3.70 ppm signal in Pr, which shifts to 3.76 ppm. The photoreversion of Pfr to Pr is also accompanied by an upfield shift from 3.76 to 3.71 ppm. However, the peak at 3.81 ppm does not shift during phototransformation and photoreversion (photocycling). β- and δ-protons of serine and proline residues, respectively, are known to exhibit resonances in this region (18, 19), but we cannot make more definitive assignments for the phytochrome resonances at the present.

Spectral changes similar to those shown in Fig. 1 were also found upon the Pr → Pfr transformation of large phytochrome, but other less resolved changes were also noticeable. For example, the 0.04 ppm peak in small Pr is apparently shifted to 0.29 ppm in large Pr. Peaks at 0.80, 1.05, and 1.20 ppm for small Pr appear as a broad band centered at 1.00~1.05 ppm. Also, in large Pr and Pfr, proton resonances appear at 3.47 and 3.51 ppm, respectively, in apparent contrast to 3.70 and 3.76 ppm for small Pr and Pfr, respectively (Fig. 2).

Large Pr shows a peak at ~4.4 ppm, which disappears upon Pr → Pfr phototransformation (Fig. 2). This disappearance signifies that the proton(s) responsible for the observed resonance in Pr becomes exchangeable in Pfr; however, it


PPM
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0.5
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1.5
2.0
2.5
3.0
3.5
4.0
4.5
a

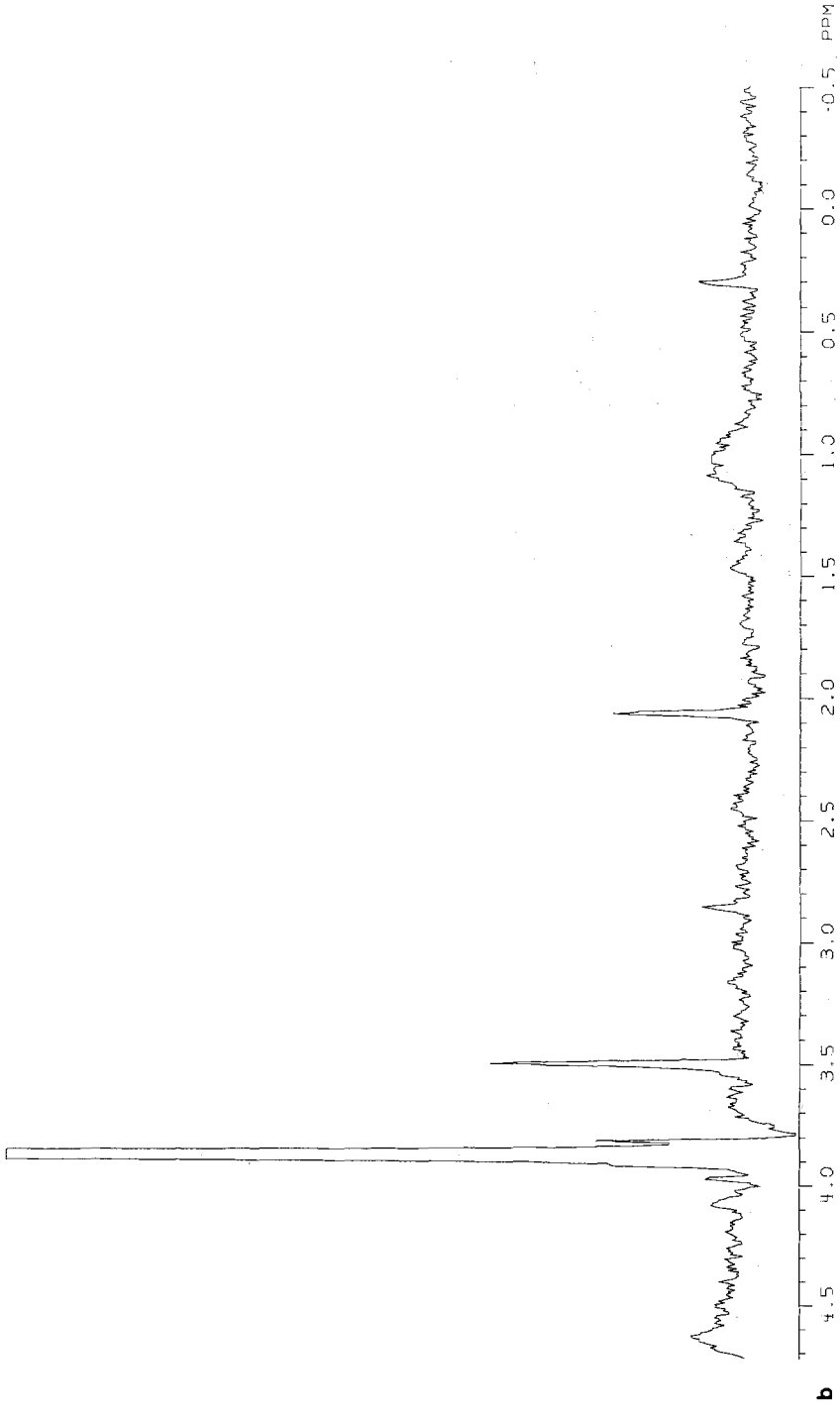

Figure 2 - The 360 MHz proton NMR spectra of phytochrome (large mol wt, 120,000) in D_2O, 0.1 M sodium phosphate buffer, pD 7.8, at 278 K. a: Pr; b: Pfr.

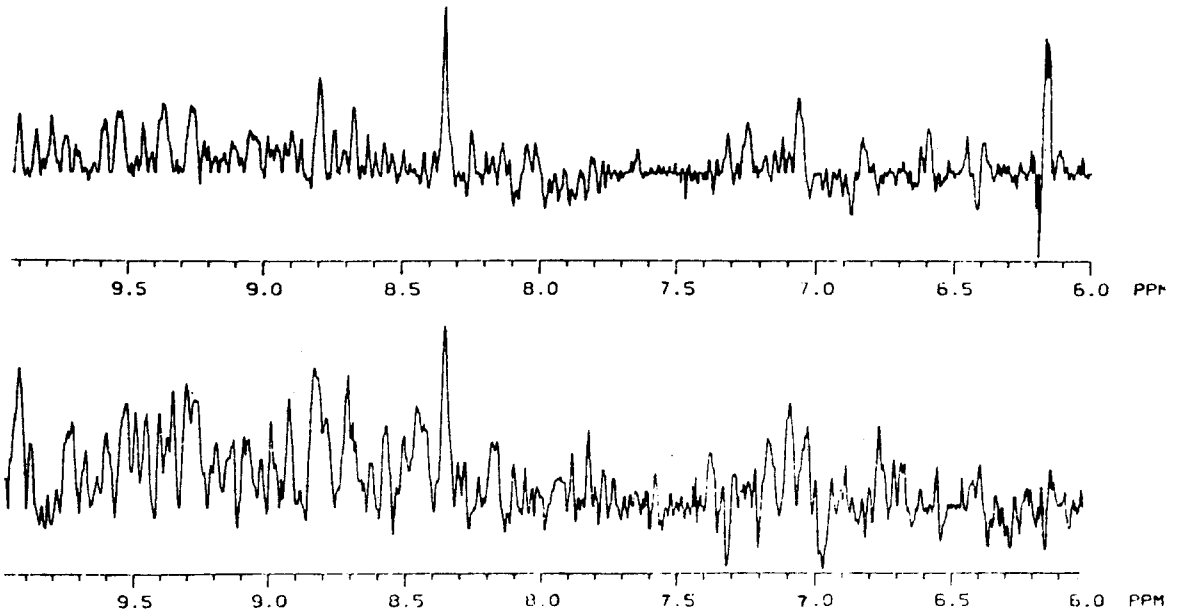

Figure 3 - The 360 MHz proton NMR spectra of phytochrome (small mol wt) in D_2O, 0.1 M sodium phosphate buffer, pD 7.8, at 280 K. Top panel: Pr; lower panel: Pfr.

is more likely that the disappearance of the 4.4 ppm signal is due to a conformational freeze upon Pr → Pfr phototransformation.

Small phytochrome did not show NMR peaks in the region of 4.00~4.41 ppm (spectra not shown). Thus, we suggest that the above NMR signal arises from the non-chromophore domain of the large mol wt phytochrome, which is tryptically cleavable.

The most interesting differences between the NMR spectra of Pr and Pfr were found in the down-field region (Fig. 3). This region represents the proton resonances of aromatic amino acid residues such as histidine, tyrosine, phenylalanine, and tryptophan, along with the chromophore. The prominent peak at 6.15 ppm appears to be a doublet, which is absent in Pfr. It is also significant that the photoreversion of Pfr does not regenerate the 6.15 ppm peak (Fig. 4). However, the proton resonance spectrum in the aliphatic region is essentially indistinguishable after photocycling (Fig. 4).

DISCUSSION

Although assignments of individual proton resonances are not feasible with any degree of certainty at this time, several significant points can be made

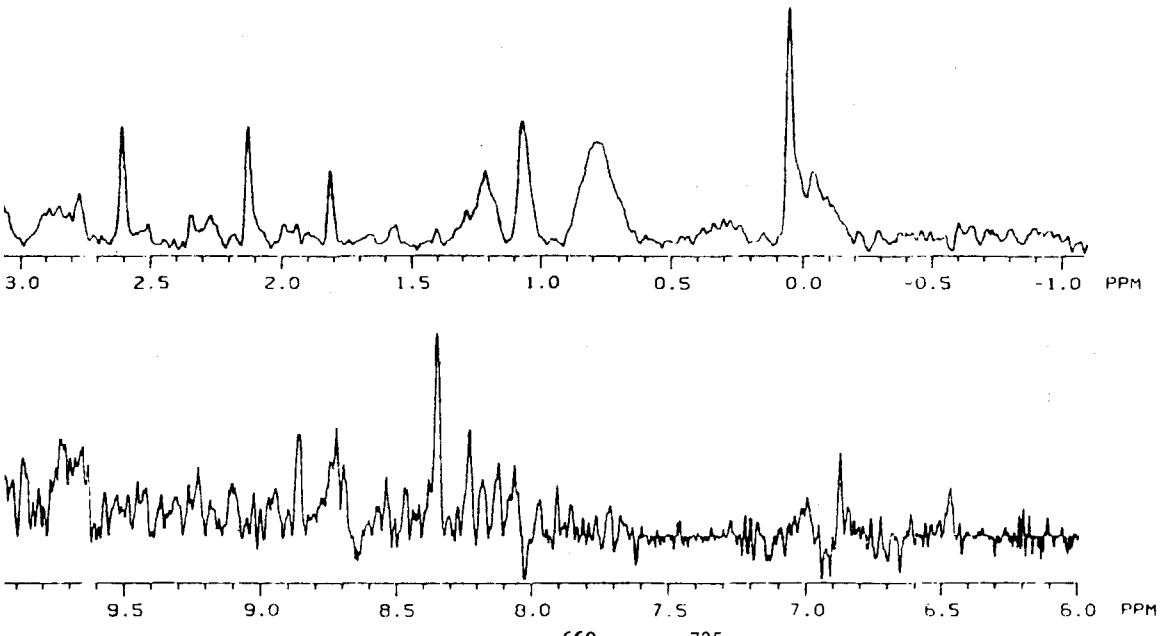

Figure 4 - The effect of photocycling ($Pr \xrightarrow{660\ nm} Pfr \xrightarrow{725\ nm} Pr$) on the 360 MHz NMR spectrum of phytochrome (Pr; small mol wt) in D_2O, 0.1 M sodium phosphate buffer, pD 7.8, at 280 K.

regarding the nature of phototransformation of phytochrome. In particular, the core of folded peptide domains remains largely unaltered upon Pr → Pfr phototransformation, since the NMR spectra of the aliphatic proton region are very similar, independent of the mol wt of the phytochrome examined, i.e. small vs. large phytochromes (Figs. 1 and 2). The photocycling of phytochrome does not produce any significant changes in this region (Fig. 4). Thus, one can suggest that the folded peptide conformations of Pr and Pfr are not noticeably different, consistent with previous CD results (5,7). However, there appears to be a local conformational change, as revealed by the shift of a proton signal from 3.70 ppm in Pr to 3.76 ppm in Pfr (small mol wt), possibly as the result of exposure of serine or proline residues in the Pfr form. Similar changes for large mol wt phytochrome are described in the Results section and presented in Fig. 2.

It has been suggested that the most prominent molecular change in the phototransformation of Pr to Pfr is the increased degree of exposure of the chromophore in the latter (7, 8, 10, 13, 14). The present NMR data (Figs. 3 and 4) are consistent with this model. The 6.15 ppm peak disappears upon Pr → Pfr transformation in D_2O, and photoreversion, Pfr → Pr, does not regenerate the same resonance.

This is direct evidence that certain amino acid residues and/or the chromophore are exposed in the Pfr form, thus accounting for the irreversible loss of the resonance peak due to hydrogen-deuterium exchange. Some of the likely protons for this resonance (doublet; Fig. 3) are histidyl-NH- and tyrosyl-OH protons (18, 19); the chromophore-NH- proton is another possibility (20). In addition, several new peaks appear in the Pfr form, involving aromatic protons that may be assigned to histidyl, tyrosyl and/or tryptophyl residues (Fig. 3). This observation further supports the above conclusion.

It is tempting to speculate that the shift of the peak at 3.70 ppm in small Pr to 3.76 ppm in small Pfr (3.47 to 3.51 ppm in large phytochromes; Fig. 2) arises from the chromophore reorientation, as these resonances may be assigned to histidyl and/or prolyl residues, both of which occur in the chromo-peptide (15).

In large phytochrome, the 4.4 ppm peak in Pr also disappears upon phototransformation to Pfr (Fig. 2). However, no parallel disappearance of such a peak was found with small Pr. Thus, there is a subtle conformational change of a peptide segment, which may be cleaved off by tryptic digestion of large Pr. The 4.4 ppm peak may be assigned to the α-hydrogens of the peptide segment.

Although detailed assignments are not feasible, due to the resolution of the NMR spectra recorded on the 360 MHz NMR, we have been able to demonstrate significant qualitative differences between the Pr and Pfr forms of small and large phytochromes. The differences found seem to support the notion that several amino acid residues and the chromophore become exposed as the result of phototransformation of phytochrome from Pr to the Pfr form. Further work is planned to delineate the NMR spectra of phytochrome using a 500 MHz NMR.

ACKNOWLEDGEMENTS

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REFERENCES

1. Rüdiger, W. (1980) Structure and Bonding 40, 101-140.
2. Pratt, L.H. (1982) Ann. Rev. Plant Physiol. in press.
3. Butler, W.L., Siegelman, H.W., and Miller, C.O. (1964) Biochemistry 3, 851-857.

4. Rice, H.V. and Briggs, W.R. (1973) Plant Physiol. 51, 939-945.
5. Tobin, E.M. and Briggs, W.R. (1973) Photochem. Photobiol. 18, 487-495.
6. Cundiff, S.C. and Pratt, L.H. (1975) Plant Physiol. 55, 207-211.
7. Song, P.S., Chae, Q., and Gardner, J.D. (1979) Biochim. Biophys. Acta 576, 479-495.
8. Hahn, T.R., Kang, S.S., and Song, P.S. (1980) Biochem. Biophys. Res. Commun. 97, 1317-3123.
9. Hunt, R.E. and Pratt, L.H. (1981) Biochemistry 20, 941-945.
10. Hahn, T.R. and Song, P.S. (1981) Biochemistry 20, 2602-2609.
11. Sarkar, H.K. and Song, P.S. (1981) Biochemistry 20, 4315-4320.
12. Sarkar, H.K. and Song, P.S. (1982) Biochemistry in press.
13. Hahn, T.R. and Song, P.S. (1982) Biochemistry in press.
14. Song, P.S. (1982) In The Biology of Photoreceptors (ed. by D. Cosens and D.V. Prue) Society of Experimental Biology, Symposium No. 36, Cambridge University Press, in press.
15. Lagarias, J.C. and Rapoport, H. (1980) J. Am. Chem. Soc. 102, 4821-4828.
16. Song, P.S., Kim, I.S., and Hahn, T.R. (1981) Anal. Biochem. 117, 32-39.
17. Daniels, S. and Smith, W.O., Jr. (1981) Plant Physiol. 67 (Suppl.), 130.
18. Knowles, P.F., Marsh, D., and Rattle, H.W.E. (1976) In Magnetic Resonance of Biomolecules John Wiley and Sons, New York.
19. Roberts, G.C.K. and Jardetzky, O. (1970) Adv. Prot. Chem. 24, 447.
20. Smith, K.M., Sharkus, L.C., and Dallas, J.L. (1980) Biochem. Biophys. Res. Commun. 97, 1370-1375.