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Chemistry of the Phytochrome Photoconversions [and Discussion]

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Chemistry of the phytochrome photoconversions

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The photoconversions of phytochrome, $P_r \rightleftharpoons P_{fr}$, occur both *in vivo* and *in vitro*. Structural differences between P_r and P_{fr} are discussed for chromophore and apoprotein. The chemical structure of the P_r chromophore has been established. The P_{fr} chromophore was recently demonstrated to be the 15*E* isomer. The red shift of absorption to 730 nm in native P_{fr} is discussed as interaction between chromophore and apoprotein. The nature of this interaction is still unknown. Small changes in the apoprotein surface are of particular interest because they could be part of the signal chain in photoperception.

1. Introduction

An important criterion for the involvement of phytochrome in photoperception is the red–far-red photoreversibility of physiological responses. This is based on the photochromic properties of phytochrome. Phytochrome exists in two forms, P_r, absorbing at 660 nm, and P_{fr}, absorbing at 730 nm. The photoconversions can be carried out in both directions, *in vivo* and *in vitro*:

$$P_r \underset{730\ nm}{\overset{660\ nm}{\rightleftharpoons}} P_{fr}.$$

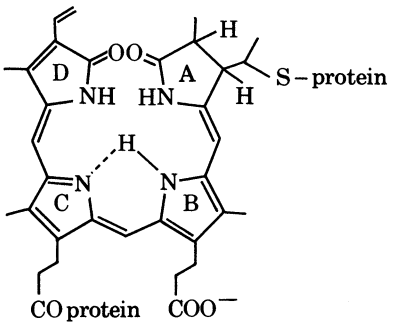
It has long been known that phytochrome is a biliprotein (for a review on early work see Mitrakos & Shropshire (1972)). If one considers the light signal used by dark-grown plants for photoperception via P_r, it is obvious that the first step must be the absorption of photons by the bilin chromophore. This excitation leads to the modification of either the chromophore or the chromophore–apoprotein interaction, which has to be considered as the primary photoreaction. A chain of further modifications, probably dark relaxations, occur subsequently. Intermediates of this chain have been characterized by spectroscopy (Kendrick & Spruit 1977). The final product of this chain is P_{fr}, from which the biological signal starts, as generally accepted (see papers by Quail, Raven and Mohr in this symposium, but see also the paper by Smith for a critical view).

The same general scheme of primary photoreaction and subsequent dark relaxations is also true for photoconversion of the physiologically active P_{fr} to the physiologically inactive P_r. The primary photoreaction must deal with the chromophore here also, whereas dark relaxations can affect apoprotein as well as chromophore. Intermediates of the backward reaction are different from those of the forward reaction (Kendrick & Spruit 1977).

It is hoped that an understanding of the difference between P_r and P_{fr} will help to reveal the nature of the biological signal. The present paper deals with chemical properties of both phytochrome forms that are changed during the photoconversions. Emphasis is given to the chemistry of the chromophores of P_r and P_{fr}.

2. Structure and conformation of the P_r chromophore

The chemical structure of the P_r chromosphore has been elucidated in a number of independent papers (for review see Rüdiger 1980). Important steps were ultraviolet–visible spectroscopy under defined conditions (Grombein *et al.* 1975), analysis of products of oxidative degradation (Klein *et al.* 1977), identification of the cleaved chromophore with a product of total synthesis (Rüdiger *et al.* 1980), and high-resolution ^1H n.m.r. spectroscopy of a chromopeptide obtained from P_r (Lagarias & Rapoport 1980). Structure **1** has been deduced from these studies. The thioether linkage between chromophore and peptide chain was also revealed by the above studies



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Bile pigments are flexible molecules, which can exist in a great number of conformations. Theoretical calculations (Chae & Song 1975; Sugimoto *et al.* 1976; Pasternak & Wagniére 1979) predicted a dramatic influence of the conformation upon spectral properties especially on the oscillator strength ratio of red to blue absorption bands. Examples of the experimental verification of these predictions are given in figure 1. The examples are bile pigments in which the conformations are fixed by chemical 'cross-linking'. The fixed conformation is closed (or

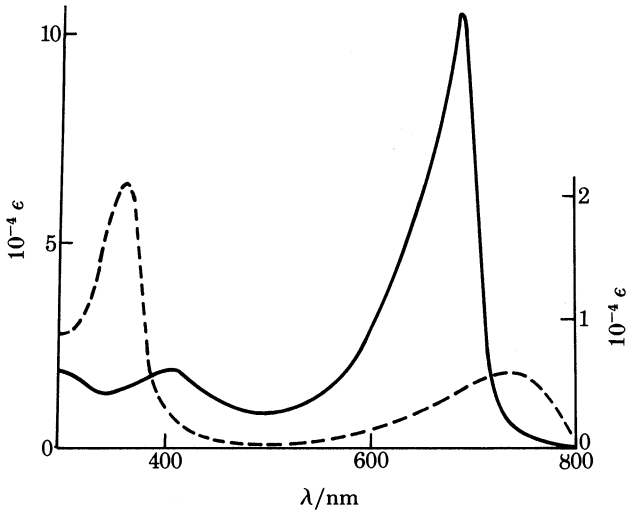
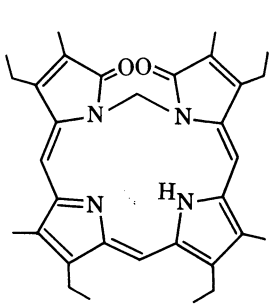


Figure 1. Electronic spectra of bile pigment cations with chemically fixed conformations. – – –, Spectrum of **2** in acidic $CHCl_3$ (right ordinate) (redrawn from Falk & Thirring 1981); ——, spectrum of **3** in acidic methanol (left ordinate) (Scheer & Kufer, unpublished results).



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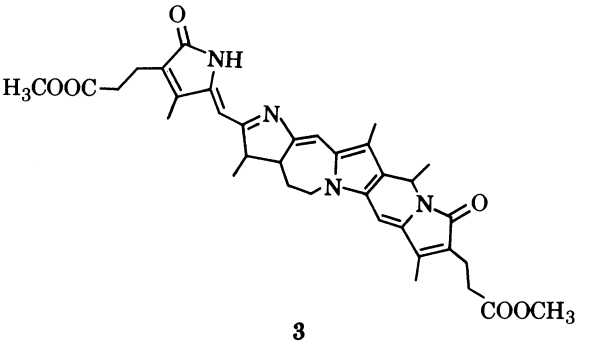

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helical) in **2** and open (or extended) in **3**. In accordance with the prediction, **2** has a small and **3** a large oscillator strength ratio of red (700–750) nm to blue (350–400 nm) bands.

A comparison of the absorption spectra of the P_r chromopeptide and native P_r (figure 2) demonstrates that the chromophore has a more closed conformation in the former and a more extended conformation in the latter. It can be assumed that the (native) protein fixes a conformation of the chromophore that is otherwise unfavourable. A semicircular (or semi-extended) conformation has been calculated for native P_r (Song *et al.* 1979). Denaturation of P_r (e.g. with 8 M urea or 6 M guanidinium chloride) leads to the same spectral properties as for the P_r chromopeptide (Grombein *et al.* 1975). This resembles the situation in phycocyanin, an accessory biliprotein pigment of photosynthesis in blue-green and red algae (Scheer & Kufer 1977). A difference concerns the stability: denatured phycocyanin can be renatured, whereas renaturation is not possible with denatured P_r.

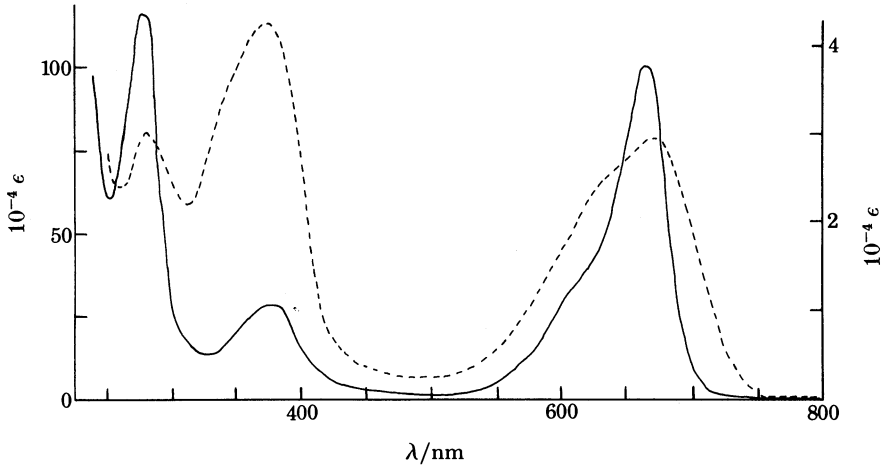

FIGURE 2. Electronic spectra of large phytochrome in the P_r form (——, left ordinate) and P_r chromopeptide (– – –, right ordinate). Phytochrome from oat ($A_{667}/A_{280} = 0.87$) with $\epsilon_{667\,nm} = 10.2 \times 10^4$ (after Roux *et al.* 1982). Independent measurements in our laboratory yielded $\epsilon_{665\,nm} = 10.9\text{–}11.8 \times 10^4$ per phytochromobilin chromophore (Brandlmeier *et al.* 1981). P_r chromopeptide in 1 % aqueous formic acid (after Thümmler & Rüdiger 1983).

It can be speculated that plants developed strong red bands in the chromophores of phycocyanin and phytochrome for specific use of red light and minimal interference of blue–u.v. light. However, the functions seem to be quite different. Phycocyanin has been optimized for energy transfer. The protein stabilizes the chromophore, i.e. it inhibits photochemical and other

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radiationless deactivation processes. Absorbed light is emitted as fluorescence with high quantum efficiency. Phytochrome has been optimized for photoconversion. The absolute quantum yield for both $P_r \rightarrow P_{fr}$ and $P_{fr} \rightarrow P_r$ transformations has been determined as 0.17 (Pratt 1975). This high quantum efficiency implies that other radiationless deactivation processes are inhibited. Phytochrome exhibits an almost complete lack of fluorescence at room temperature (Song *et al.* 1979).

3. Structure of the P_{fr} chromophore

Early investigations demonstrated that the P_{fr} chromophore is less stable than the P_r chromophore; the native protein serves to stabilize the chromophore. The relevant literature contains much speculation on possible structures of the P_{fr} chromophore (recently summarized by Lagarias & Rapoport 1980). Some of these speculations could be ruled out by rather simple experiments. Oxidative degradation revealed that the β side chain of the chromophore and its linkage to the protein are identical in P_{fr} and P_r (Klein *et al.* 1977). This ruled out changes of double bonds in the side chains (Siegelman *et al.* 1968) or the cleavage of the thioether linkage (Song *et al.* 1979) during the $P_r \rightarrow P_{fr}$ transformation. Early spectroscopic measurements (Grombein *et al.* 1975) demonstrated differences between denatured P_{fr} and denatured P_r; the meaning of these results were questioned because of possible artefacts during denaturation (Kendrick & Spruit 1977). A difference between the P_{fr} chromophore and the P_r chromophore could finally be established by preparation of chromopeptides from both phytochrome forms.

(*a*) *Preparation and isolation of chromopeptides from* P_{fr}

A small chromopeptide was at first isolated by Fry & Mumford (1971) after pepsin digestion of phytochrome. Those authors did not find any difference in the composition of chromopeptide starting with either P_r or P_{fr}. Such a difference between P_{fr} chromopeptides and P_r chromopeptides was, however, found if pepsin digestion was carried out under carefully controlled conditions (Thümmler *et al.* 1981). The conditions had to be obeyed because otherwise the P_{fr} peptide was easily transformed into the P_r peptide. Digestion and isolation had to be performed in darkness or under green safety-light and between pH 2 and 4; the incubation time at 37 °C had to be kept at a minimum. It was shown that white light and strong acids and bases catalyse the transformation into the P_r peptide. A slow, temperature-dependent 'reversion' of the P_{fr} peptide occurred also in the dark. Isolation included column chromatography on Biogel P-10 and silica gel with step gradients of acids (acetic acid, formic acid or, later, diluted hydrochloric acid) (Thümmler & Rüdiger 1983). This method allowed separation of P_{fr} peptides from colourless peptides and from P_r peptides but not the separation of single P_{fr} chromopeptides from each other. This heterogeneity was confirmed by amino acid analysis (Thümmler & Rüdiger 1983).

(*b*) *Chemical and spectroscopic properties: comparison with model chromophores*

The ultraviolet–visible spectra of the P_{fr} chromopeptide and the P_r chromopeptide obtained from it by photoconversion are shown in figure 3. The spectra resemble those of *Z*, *E* isomeric biliverdin chromophores (Thümmler *et al.* 1981). The hypsochromic shift of the red band in the *E* configurated model compounds was explained by partial uncoupling of one pyrrole ring from the conjugated system due to steric hindrance (Falk *et al.* 1978). *E* configurated model

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compounds are unstable, like the P_{fr} chromopeptide. Irradiation of the cation with white light, and contact with strong acids and bases yield the *Z* configurated isomers.

The best model for the P_{fr} chromopeptide proved to be a chromopeptide from phycocyanin (Thümmler & Rüdiger 1983). Pepsin digestion of phycocyanin yielded chromopeptides which contain the all-*Z* configurated chromophore **4**. This chromophore can be converted to its *E* configurated isomer by a procedure outlined in figure 4. The decision between the two possible

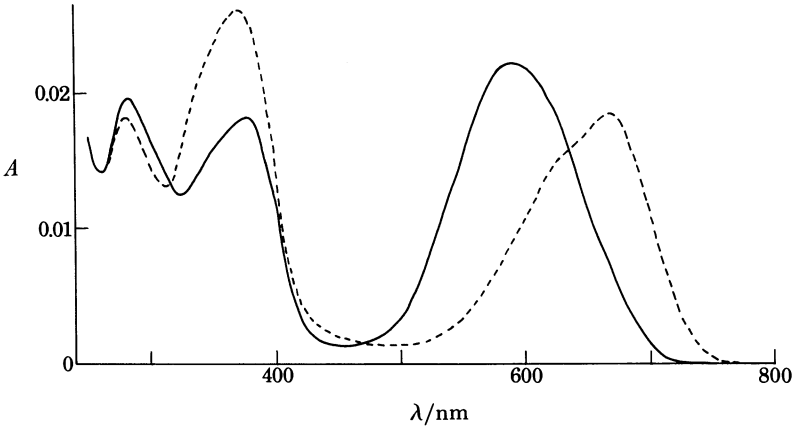


FIGURE 3. Electronic spectra of P_{fr} chromopeptide (——) and P_r chromopeptide (– – –) in 1% aqueous formic acid (after Thümmler & Rüdiger 1983).

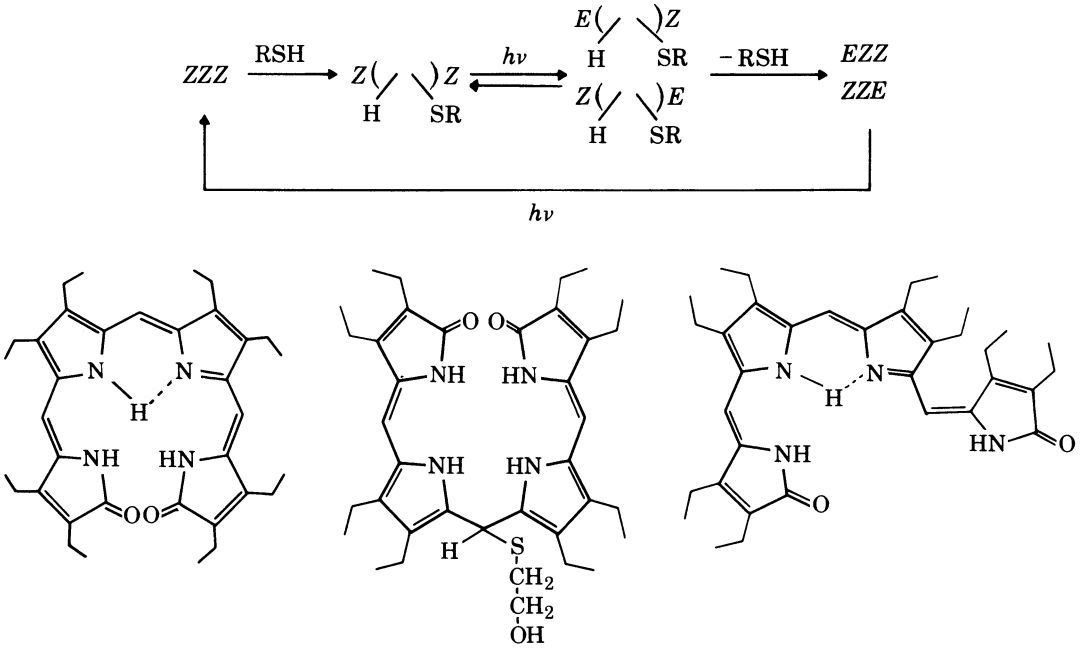
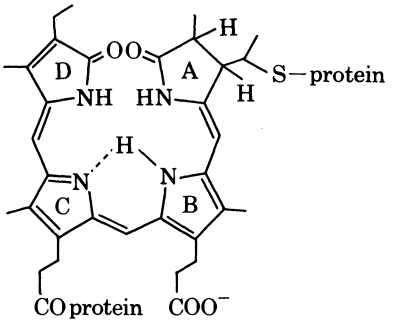


FIGURE 4. Reaction sequence for preparation of *E* configurated bile pigments by a procedure of Falk *et al.* (1980). Structures are given for octaethylbiliverdin (from left to right): all-*Z* educt, adduct, *E* configurated product.

structures, the 4*E* and the 15*E* configurated chromophore, was obtained by high-resolution ^{1}H n.m.r. spectroscopy: only the 15*E* configurated phycocyanin chromopeptide **5** is formed by the procedure of figure 4 (Thümmler *et al.* 1983). The methine signals are particularly

valuable for localization of the isomerized methine double bond (Rüdiger *et al.* 1983). The high resolution ^{1}H n.m.r. spectrum of the P_{fr} chromopeptide revealed that this also contains the 15*E* configuration (Rüdiger *et al.* 1983; Thümmler *et al.* 1983). Because **6** is the structure of the P_{fr} chromophore in the chromopeptide it can be concluded that the *Z*, *E* (or *cis*, *trans*) isomerization at C15 is an essential step of the $P_r \rightleftharpoons P_{fr}$ photoconversion.



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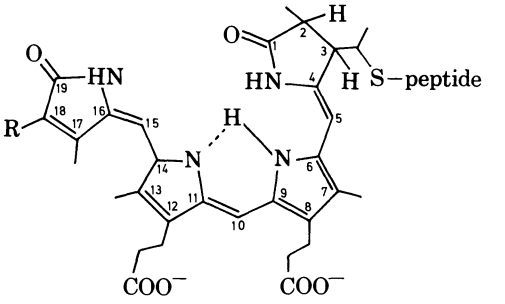

6 R=CH=CH$_2$
5 R=C$_2$H$_5$

It should be mentioned here that **6** is more reactive towards oxidation and reduction than the *Z* configurated isomer **1**. Therefore oxidation or reduction of **6** yields some **1** besides the proper oxidation or reduction products (Thümmler *et al.* 1981). The catalysis of dark reversion of native P_{fr} by reductants (Mumford & Jenner 1971) has been explained by the same mechanism (Thümmler *et al.* 1981). The chromophore is more easily oxidized in native P_{fr} than in native P_r. This has been interpreted as difference in the accessibility of the chromophore (Hahn *et al.* 1980) but the above difference in reactivity has yet to be considered in this connection.

(*c*) *The role of the apoprotein*

The typical absorption band of native P_{fr} (λ_{max} = 730 nm) is only formed at the last intermediate step of the $P_r \rightarrow P_{fr}$ photoconversion (Pratt *et al.* 1982). The direct precursor is a relatively weakly absorbing intermediate (called P_{bl} or *meta*-Rb) that has a broad absorption maximum at about 650 nm (Cross *et al.* 1968). A 'closed' conformation has been proposed for this intermediate (Burke *et al.* 1972), which implies that it contains less chromophore–protein interaction than P_r or P_{fr}, which both are assumed to have more extended chromophore conformations due to interaction with the protein. Similar weakly absorbing or 'bleached' forms can also be obtained by treatment of P_{fr} with urea or proteolytic enzymes (Butler *et al.* 1964), with 8-anilinonaphthalene-1-sulphonate (Hahn & Song 1981) or by rigorous removal of water from P_{fr} (Balangé 1974; Tobin *et al.* 1973).

We have checked whether the intermediate *meta*-Rb and bleached phytochrome forms derived from P_{fr} contain the *E* configurated chromophore **6** or whether the bleaching process is accompanied by a reversion to the *Z* configurated chromophore **1**. For this purpose, bleached solutions were acidified. The solution then contained the chromophore cation linked to denatured protein. Starting with P_{fr}, this treatment yielded a product spectroscopically identical with the P_{fr} chromopeptide. The experiment is illustrated here with *meta*-Rb (figure 5). It turned out that all bleached phytochrome preparations still contained the chromophore **6**, which could be transformed into **1** by irradiation of the acidic solution with white light. Apparently, the $Z \rightarrow E$ isomerization is not related directly with the absorption shift to

730 nm. It must therefore be one of the early intermediate steps of the $P_r \rightarrow P_{fr}$ transformation. By analogy with rhodopsin (Wald 1968), it could be a good candidate for the primary photoreaction. However, the deuterium isotope effect found for the $P_r \rightarrow P_{fr}$ phototransformation has been interpreted in terms of the primary reaction involving an intramolecular proton transfer (Sarkar & Song 1981). The exact chemistry of the primary photoreaction remains to be elucidated.

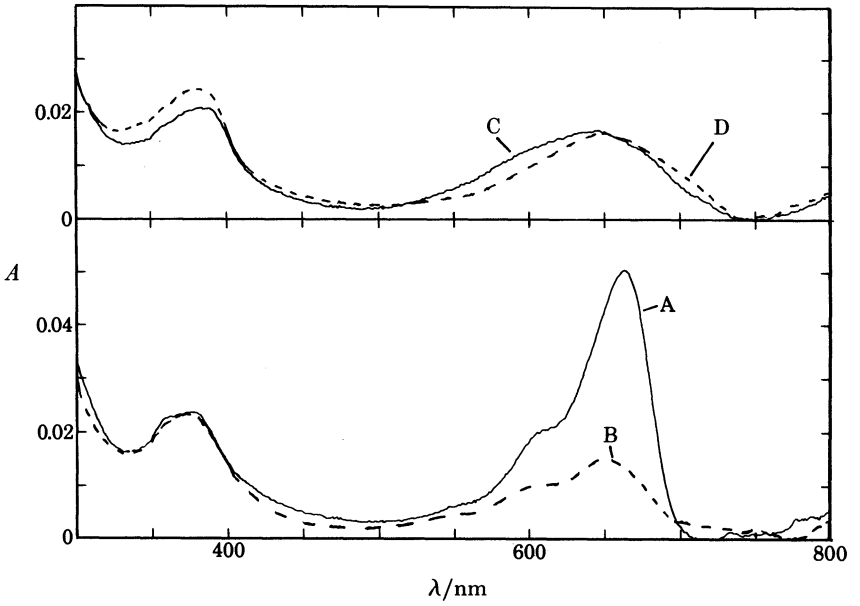

FIGURE 5. Electronic spectra of phytochrome preparations in 66 vol. % aqueous glycerol at −40 °C. Lower traces: A, phytochrome in the P_r form, B, the same after saturating irradiation at 660 nm. Upper traces: C, B after acidification with HCl (this corresponds to the P_{fr} chromopeptide); D, C after irradiation with white light (this corresponds to the P_r chromopeptide) (Thümmler & Rüdiger, unpublished).

A consequence of the $Z \rightarrow E$ isomerization is the prediction that chromophores 1 and 6 should have different positions in relation to the bulk apoprotein. Different dichroic orientation of P_r and P_{fr} in cells of *Mougeotia* and other organisms has been known since a long time (Haupt 1972). It was not known, however, whether this signifies different orientation of the whole phytochrome molecule in the membrane or of the chromophore within the apoprotein. The latter possibility was more recently supported by chemical and spectroscopical investigations on phytochrome in solution (Hahn *et al.* 1980; Hahn & Song 1981) and by investigation of immobilized phytochrome with polarized light (Sundquist & Björn 1983).

4. Differences between P_r and P_{fr}: the apoprotein

The photoconversion of phytochrome could not lead to photoperception by plants if photochemical changes were restricted to the chromophore and eventually to the immediate protein environment of the chromophore. Differences between P_r and P_{fr} other than spectral shifts were postulated early in phytochrome research, but many attempts to establish such differences failed, as critically reviewed by Pratt (1978, 1982). In summary, no drastic conformational changes of the protein were found. The biological significance of minor changes (probably at the protein surface) remains unclear because only proteolytically degraded

phytochrome was studied in early investigations. The isolation of native phytochrome (124 kDa) has only recently been described (Vierstra & Quail 1982; Kerscher & Nowitzki 1982). Some investigations with partly degraded phytochrome (114–118 kDa) will be considered here because these results are probably also relevant for native phytochrome.

Hunt & Pratt (1981) established differences in the accessibility of several amino acids that point to a conformational change (eventually small) at the surface. This may be related to the finding of a hydrophobic surface area in P_{fr} that is not found in P_r (Hahn & Song 1981; Tokutomi *et al.* 1981). Photoreversible pH changes caused by unbuffered phytochrome solutions (Tokutomi *et al.* 1982) could be due to the exposure of charged amino acid residues by the photoconversion.

All of these changes could occur in only a restricted area of the protein surface because no difference between P_r and P_{fr} could be detected with immunological methods (see Pratt 1978). The changes are currently of particular interest: they could be part of the signal chain leading from P_{fr} to the biological responses in photoperception.

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Discussion

R. Bonnett (*Department of Chemistry, Queen Mary College, London, U.K.*). With respect to the question of whether the protein or the linear tetrapyrrole undergoes a photoinduced change, it should be borne in mind that although the protein can undoubtedly perturb the chromophore, it is the linear tetrapyrrole that absorbs the photon. In other words, of all the chemical groups present, it is the linear tetrapyrrole that becomes excited first. Of course, it may pass on its excitation energy, but the geometrical isomerization of the excited linear tetrapyrrole is a very plausible route, and is strongly supported by Professor Rüdiger's present evidence.

My questions have to do with the chromopeptides. Firstly, what is the nature of the

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chromopeptide: how may amino acids are present? Secondly, do I understand correctly from the nuclear magnetic spectra at 500 MHz that the sample of P_{fr} chromopeptide subjected to the irradiation experiment is an approximately 1:1 mixture of P_r and P_{fr} chromopeptides?

W. Rüdiger. The chromopeptides from P_{fr} were prepared by the same method as previously from P_r (Fry & Mumford 1971; Lagarias & Rapoport 1980). The main product in that case was an undecapeptide containing some smaller peptides. Our amino acid analysis (Thümmler *et al.* 1983) confirmed that the same peptides were obtained from P_{fr}. Further fractionation of P_{fr} chromopeptides was unfeasible owing to rapid dark reversion to P_r chromopeptides. This is also the reason why the sample of P_{fr} chromopeptide contained considerable amounts of P_r chromopeptide at the time of the n.m.r. measurement. The exact percentage cannot easily be determined but, according to electronic and n.m.r. spectra, the sample contained somewhat more P_{fr} than P_r chromopeptide.

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