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Tetranitromethane Oxidation of Phytochrome Chromophore as a Function of Spectral Form and Molecular Weight

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ABSTRACT

Tetranitromethane bleaches *Avena* phytochrome. The phytochrome (far-red absorbing form; Pfr) chromophore of 124 kilodalton (kD) phytochrome is oxidized 8 times more rapidly than the red absorbing form (Pr). Proteolysis of the 124 kD molecule to the extensively studied mixture of 118 and 114 kD polypeptides increases the rate of oxidation of Pfr 5-fold without affecting the rate of Pr oxidation. As a result, the Pfr form of 118/114 kD preparations is oxidized at a rate 40 times greater than the Pr form. Further proteolytic degradation of the chromoprotein to 60 kD results in an additional increase in the oxidation rates of both Pr and Pfr. These differences in reactivity to tetranitromethane indicate that the chromophore of Pfr is either intrinsically more chemically reactive and/or physically more accessible than the Pr chromophore and that the reactivity/accessibility of both spectral forms is increased by proteolysis. The enhanced reactivity of the Pfr chromophore after proteolytic cleavage of the 6 to 10 kD polypeptide segment(s) from the 124 kD species is further evidence that these segment(s) affect the environment of the native photoreceptor.

Phytochrome serves as the principal photoreceptor for many of the light-mediated developmental responses in plants (12, 15, 18). The molecule consists of a linear tetrapyrrole chromophore covalently linked to a polypeptide (2, 10) and can exist in two photointerconvertible forms, Pr and Pfr. The molecular mechanism of phytochrome action remains unknown, but Pfr is generally considered to be the physiologically active form (15, 18). Efforts to elucidate this mechanism have concentrated on characterizing the isolated molecule and on detecting chemical differences between Pr and Pfr (for review, see 16, 23).

Over the past decade, most experimentation with purified phytochrome has involved the use of an approximately 120 kD chromoprotein that was widely considered to be undegraded (15). Recently, it has been demonstrated that the 118 and 114 kD polypeptides that actually comprise these '120' kD phytochrome preparations from *Avena* are artifactually derived by limited proteolysis from an apparently native 124 kD molecule (27). Since this proteolysis substantially alters many of the spectral properties of phytochrome (28–30), it is likely that other information obtained with 120 kD preparations may also require reevaluation.

Various reagents have been used to probe the Pr and Pfr forms of phytochrome for differences in the reactivity and/or accessibility of the chromophore and specific amino acid residues (1, 3, 4, 7, 17, 19). Hahn *et al.* (6) have reported that the Pfr chromophore of 118/114 kD phytochrome is more readily bleached by permanganate oxidation than the Pr chromophore and have suggested that this occurs because the Pfr chromophore is more accessible. In the present study, the susceptibility of the chromophore to chemical oxidation is further investigated for the Pr and Pfr forms of the newly purified 124 kD molecule and compared with the susceptibility of the proteolytically degraded 118/114 kD and 60 kD chromoproteins. Tetranitromethane (TNM[1]) is employed here as a more specific oxidant of the phytochrome chromophore than permanganate. TNM is also used to modify tyrosines (11, 21) and has been reported to effectively bleach 118/114 kD *Avena* phytochrome (7).

MATERIALS AND METHODS

All phytochrome preparations were obtained from etiolated oat seedlings (*Avena sativa*, cv Garry). The 118/114 kD phytochrome (as determined by SDS-PAGE), with A_{666}/A_{280} ratios > 0.8, was purified as Pr from frozen tissue using modifications of the Affi-Gel Blue affinity chromatography procedure developed by Smith and Daniels (20) as described previously (24).

The 60 kD chromoprotein was obtained from the tryptic digests of 118/114 kD phytochrome after Affi-Gel Blue chromatography. Ten mg of purified 118/114 kD phytochrome in 30 ml of 0.1 M phosphate buffer (pH 7.8) were mixed with 10 μg of trypsin and incubated for 12 h at 4°C. After incubation, 20 μg of soybean trypsin inhibitor was added to the mixture to stop the reaction. After 10 min of incubation, the solution was then applied to an Affi-Gel Blue column (2.5 × 10 cm) equilibrated with 0.1 M phosphate buffer (pH 7.8), containing 50 mM KCl and 0.1 mM EDTA. The column was washed with one column volume of the equilibration buffer and then eluted with equilibration buffer containing 0.5 M KCl. The phytochrome-containing fractions were pooled and concentrated with an Amicon PM 10 ultrafiltration membrane. Purified 60 kD phytochrome (as determined by SDS-PAGE) with A_{666}/A_{280} ratios between 1.1 and 1.2 was obtained with this method.

The 124 kD phytochrome was purified as described previously (29). Briefly, this procedure included: homogenization of the tissue with the phytochrome as Pfr in a buffer containing 50% ethylene glycol and 4 mM phenylmethylsulfonylfluoride, followed by polyethyleneimine and $(NH_4)_2SO_4$ precipitation, hydroxyapatite chromatography, Affi-Gel Blue affinity chromotography, and gel filtration. The 124 kD preparation (as determined by SDS-PAGE) had A_{666}/A_{280} ratios > 0.9.

Steady state absorbance measurements and reaction kinetics were performed with a Cary 118C spectrophotometer at 2°C under safe green light. Samples for each steady state measurement were incubated for 2 min after mixing with appropriate amounts of 2 mM TNM stock solution. The phytochrome phototransformations were performed with a Bausch and Lomb

[1] Abbreviation: TNM, tetranitromethane.

microscope illuminator combined with a red interference (Oriel C572-6600; fluence rate 8 w/m^2) or far red cut-off (Ealing 26-4457; fluence rate 1.6 kw/m^2) filters.

SDS-PAGE was conducted on 7.5% acrylamide gels according to the method of Laemmli (9). Biliverdin and bilirubin were dissolved in acetone (10 μM stock solution) and diluted in 0.1 M K-phosphate (pH 7.8) before use. A TNM stock solution (2 mM) was prepared in 0.1 M K-phosphate (pH 7.8) and used within 3 h. Phytochrome preparations were in 0.1 M K-phosphate, 9.1 mM Na_4 EDTA, and 50 mM KCl (pH 7.8). Reported extinction coefficients (25, 30) were used for the calculation of phytochrome concentrations. All chemicals including model compounds and TNM were purchased from Sigma Chemical Co.

RESULTS

Hunt and Pratt (7) first reported that TNM bleaches both the Pr and Pfr forms of 118/114 kD *Avena* phytochrome with the Pfr form preferentially affected. We confirm these observations with the undegraded 124 kD molecule and with the degraded 118/114 and 60 kD preparations (Figs. 1 and 2). Results from various kinetic and competition experiments demonstrate that this bleaching results from oxidation of the chromophore rather than modification of specific tyrosine residues on the phytochrome polypeptide as was proposed by Hunt and Pratt (7). First, two linear tetrapyrroles, biliverdin and bilirubin, with structures similar to the phytochrome chromophore have pseudo-first order rate constants for TNM oxidation (~1.2 × 10^{-2} s^{-1} for 0.9 μM chromophore and 113 μM TNM [Table I]) approximately 33 times higher than that for free tyrosine (~3.7 × 10^{-4} s^{-1} for 10 μM tyrosine and 1.4 mM TNM [data not shown in Table I]). Second, a 140-fold molar excess of free tyrosine over biliverdin or bilirubin has no effect on the oxidation rate of the tetrapyrroles by TNM (unpublished data). Third, a 100-fold molar excess of free tyrosine over purified 118/114 kD phytochrome has no effect on the oxidation rate of the phytochrome chromophore as Pr or Pfr (unpublished data).

Comparison of the oxidation rates of Pr and Pfr for the three mol wt preparations of phytochrome indicates that the Pfr chromophore is more readily oxidized by TNM than the Pr chromophore in each case and that these oxidation rates are enhanced by proteolysis (Figs. 1 and 2). Based on the pseudo-first order rate constants for TNM oxidation (Table I), the Pfr form of 124 kD phytochrome is 8 times more reactive toward TNM than the same size Pr. Chromophore oxidation of the 124 kD molecule as Pr and Pfr is 200 and 25 times slower, respectively, than the tetrapyrroles, biliverdin and bilirubin. Proteolysis to the 118/114 kD species does not significantly affect the rate of Pr oxidation but does increase the rate of Pfr oxidation 5-fold. As a result, the Pfr form of 118/114 kD preparations is 40 times more sensitive to TNM than the Pr form. Further proteolysis to the 60 kD chromoprotein increases the reactivity of both Pr and Pfr to TNM with Pfr being 15 times more sensitive than Pr. The chromophore oxidation of the 60 kD species is still significantly slower than that of the free tetrapyrroles (38 and 2.4 times as Pr and Pfr, respectively).

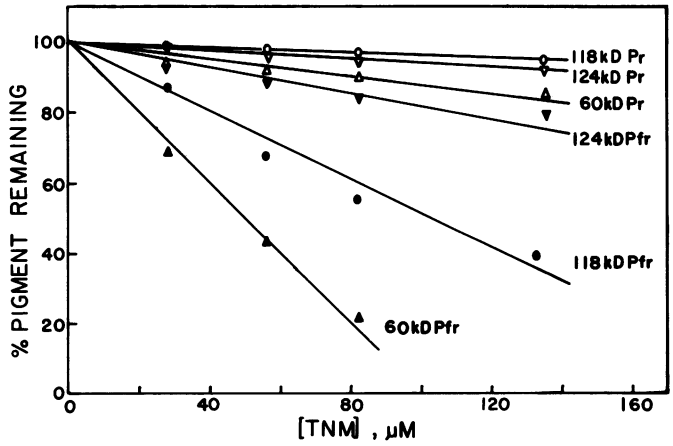

FIG. 1. The effect of TNM concentration on the bleaching of the different spectral and mol wt forms of *Avena* phytochrome. Phytochrome and model chromophore (biliverdin, bilirubin) concentrations were 0.9 μM for each preparation. Percent pigment remaining was assayed after a 2-min incubation with TNM at 2°C in the dark. Note that 118 kD phytochrome is contaminated with 114 kD protein.

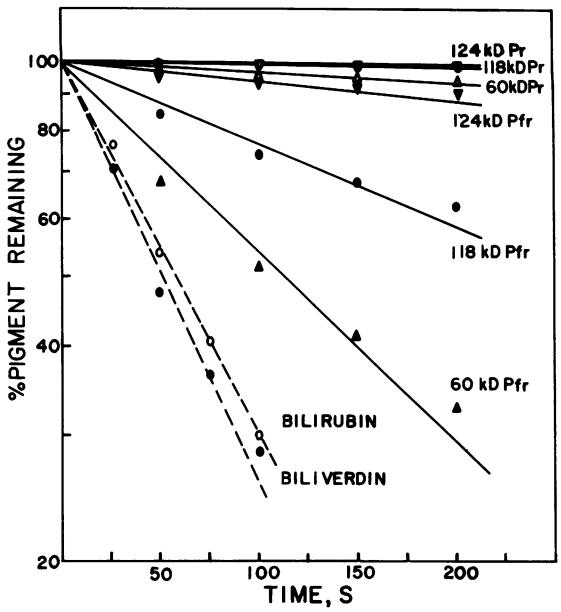

FIG. 2. First order kinetic plots for TNM bleaching of free tetrapyrroles, biliverdin, and bilirubin, and the different spectral and mol wt forms of *Avena* phytochrome. Chromophores (0.9 μM) were incubated in 133 μM TNM at 2°C. Note that 118 kD phytochrome is contaminated with 114 kD protein.

In the above experiments, TNM was added to the preparations in the dark after phototransformation. We have also observed that phototransforming phytochrome in the presence of TNM increases the rate of chromophore bleaching. For example, the oxidation of Pfr during red irradiation is 2 times more extensive than for Pfr in complete darkness (Fig. 3). More bleaching occurs during phototransformations from Pr to Pfr than from Pfr to Pr (Fig. 4).

DISCUSSION

These results are not consistent with the previous conclusion that TNM-induced bleaching results from modification of several tyrosines involved in phytochrome phototransformation and maintenance of the Pfr form (7, 16). While it is likely that phytochrome tyrosines are in fact modified by TNM, presumptive inhibition of this modification by excess free tyrosine does not affect the sensitivity of the photoreceptor to this reagent. Therefore, we conclude that such bleaching is a consequence of direct oxidation of the phytochrome chromophore. The observations of Hunt and Pratt (16) that photoconversion of Pr to Pfr after incubation with TNM enhances the sensitivity of phytochrome to bleaching is consistent with our observations that the kinetics for chromophore oxidation by TNM increases during phototransformation (Fig. 3). This increased sensitivity during photoconversion may indicate that one or more of the phototransformation intermediates are substantially more reactive or

Table I. *The Pseudo-First Order Rate Constants of the Oxidation of Avena Phytochrome and Model Chromophore by TNM (133 μM) at 2°C (Data from Fig. 2)*

Phytochrome Preparations and Model Chromophores (0.9 μM)	Pr ($s^{-1} \times 10^5$)	Relative Rate	Pfr ($s^{-1} \times 10^5$)	Relative Rate	Rate (Pfr) / Rate (Pr)
124 kD phytochrome	5.8	1.00	48.8	1.00	8.3
118/114 kD phytochrome	5.6	0.97	227.0	4.69	40.5
60 kD phytochrome	33.1	5.71	516.0	10.66	15.6
Bilirubin	1220	210.3		25.20	
Biliverdin	1260	217.2		26.03	1.0[a]

[a] Rate (biliverdin)/rate (bilirubin) for the model chromophores.

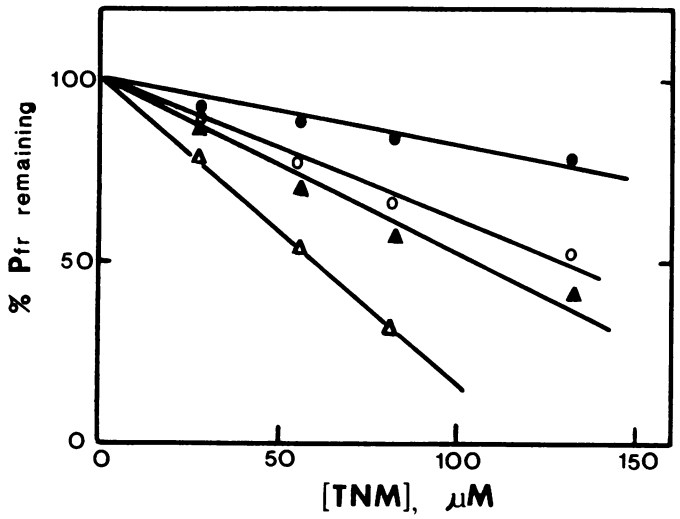

FIG. 3. The effect of red light irradiation on the bleaching of the Pfr forms of *Avena* phytochrome by TNM. After preliminary saturating irradiation with red light, phytochrome samples (124 and 118/114 kD phytochrome [0.9 μM as Pr]) were incubated for 2 min with TNM at 2°C during red light irradiation (124 kD [O——O]; 118/114 kD [Δ——Δ]) and in complete darkness (124 kD [●——●]; 118/114 kD [▲——▲]).

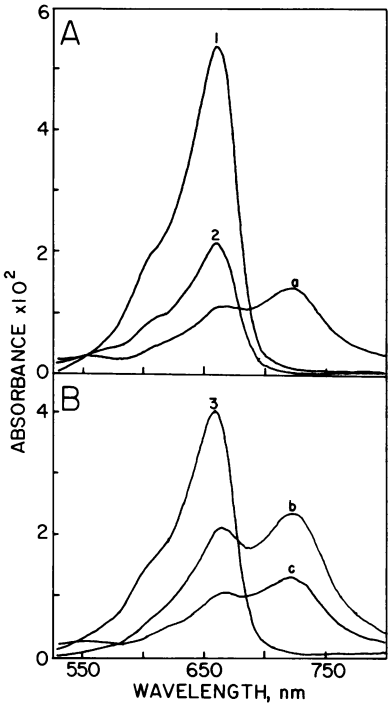

FIG. 4. The effect of phototransformation on the chromophore bleaching of 124-kD phytochrome. A, 124-kD Pr (spectrum 1) was phototransformed to Pfr (spectrum a) by 2 min of red light irradiation in the presence of 133 μM TNM. Cycled Pfr was further transformed back to Pr (spectrum 2) by 2 min of far red light *in situ*. B, 124-kD Pfr (spectrum b) was phototransformed to Pr (spectrum 3) by 2 min of far red light irradiation in the presence of 133 μM TNM. Pr was once more cycled to Pfr (spectrum c) by 2 min of red light *in situ*. Note that in both cases (A and B) Pr $\xrightarrow{TNM}$ Pfr yields less than 50% of the expected amount of Pfr, whereas Pfr $\xrightarrow{TNM}$ Pr yields 80 to 90% of the expected amount of Pr.

accessible than Pr or Pfr.

The differential sensitivity of the chromophore to TNM oxidation as a function of both spectral form and mol wt could result in either case from: (a) differences in intrinsic chemical reactivity of the chromophore without differences in accessibility; (b) differences in accessibility without differences in chemical reactivity; or (c) both. Moreover, it is possible that the molecular basis for differential sensitivity of Pr and Pfr is distinct from the basis for differential sensitivity of the various size classes of phytochrome. For example, Pr to Pfr photoconversion may alter the chemical reactivity of the chromophore whereas proteolytic degradation may alter its accessibility to the medium.

An argument favoring differential accessibility of the chromophore without chemical change is based on theoretical estimates of the reactivity of proposed Pr and Pfr chromophore structures, in addition to points described later. Such estimates, obtained using the frontier electron and perturbation methods, suggest that the chemical reactivity of the Pr and Pfr chromophores are not significantly different (8). (Thus, the model chromophores of two distinctly different structures, bilirubin and biliverdin, are oxidized at the same rate [Table I].) If this result is correct, both the higher rates of oxidation that results from Pfr formation and proteolytic degradation must be a consequence of a greater exposure of the chromophore to the medium. Furthermore, the relative rates of oxidation of the Pr and Pfr forms do not follow the same proportionate dependence on the size of protein (Table I). However, this conclusion remains tentative in the absence of direct evidence that the proposed chromophore structures are valid for the molecules studied here.

Evidence that the chromophores of Pr and Pfr may differ in their chemical reactivities comes from studies with phytochrome chromopeptides. Chromopeptides isolated from Pfr after deliberate proteolytic digestion are spectrally distinct and substantially more sensitive to oxidation and reduction than chromopeptides from Pr (26). Because the peptide portion of these chromopeptides are only approximately eleven amino acids in length (10), physical accessibility of the chromophore to TNM is unlikely to have been a significant factor in the differential response. Thus, the chromophore of Pfr appears to be chemically different from the Pr chromophore. In addition, it is possible that the intrinsic chemical reactivity of the chromophore is also altered by prote-

olysis. The notion is based on observations that proteolysis of 124 kD phytochrome to the 118/114 kD species and finally to the 60 kD species does affect the spectral and photochemical properties of phytochrome (13, 14, 28–30).

At the present time, we cannot distinguish between the alternative interpretations for the observed effects of TNM on 124 kD phytochrome. It seems probable that both differential chemical reactivity and accessibility of the Pr and Pfr chromophores are involved.[2] It may also be argued that the chromophores of the Pr ⇄ Pfr phototransformation intermediates are significantly more exposed and/or reactive than the Pr chromophore (Fig. 4). The increased sensitivity of Pfr but not Pr to TNM bleaching after proteolytic cleavage of the 6 to 10 kD polypeptide segment(s) from 124 kD phytochrome is consistent with our previous observations that these peptides are important to the molecular integrity of Pfr (5, 28–30). How these 6 to 10 kD polypeptide segment(s) affect the various properties of Pfr remains to be determined (22).

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[2] However, in order to attribute the 8-fold difference in oxidation rate (Pr *versus* Pfr) of the 124 kD phytochrome entirely to the intrinsic chemical reactivity difference, it must be assumed both the Pr and Pfr chromophores must be fully exposed. Since there is strong evidence that the Pr chromophore is buried (22), it seems reasonable to propose that the differential oxidation rates reflect a preferential accessibility of the Pfr chromophore relative to the Pr chromophore (P. S. Song, personal communication).