On the Chemical Reactivity of the Phytochrome Chromophore in the P_r and P_{fr} Form

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The reactivity of the phytochrome chromophore and related tetrapyrroles towards ozone and tetranitromethane was investigated. Both oxidizing reagents cause bleaching of the main absorption band of the pigment. The rate constants for this bleaching were determined under conditions of pseudo first order reaction kinetics. The rate constants for the reaction with ozone are similar for native phytochrome and for freely accessible tetrapyrroles (biliverdin, small chromopeptides from phytochrome) indicating that accessibility is not the limiting factor for the reaction with ozone. Under a variety of conditions, the P_{fr} chromophore reacts by about 10% faster than the P_r chromophore. This may reflect the true difference in reactivity. The rate constants for the reaction with tetranitromethane are much larger for biliverdin, bilirubin and small chromopeptides from phytochrome than for native phytochrome. The limiting factor for this reaction in native phytochrome therefore is the accessibility of the chromophore by the reagent. Previous conclusions on the difference in exposure of the tetrapyrrole chromophore in P_r and P_{fr} are confirmed.

Introduction

Phytochrome, a photoreceptor for plant photomorphogenesis, is a chromoprotein that exists in two forms, P_r and P_{fr}. The P_r form is physiologically inactive, whereas the P_{fr} form is considered the physiologically active form [1].

The molecular topology of P_r and P_{fr} has been studied with the final aim to find out those differences between P_r and P_{fr} which are responsible for the physiological activity of P_{fr}. One point of particular interest in this connection is the higher reaction rate of the P_{fr} chromophore compared to the P_r chromophore during oxidation with permanganate [2] and tetranitromethane [3] or reduction with borohydride [4]. This has been interpreted as protein protection of the P_r chromophore but exposure of the P_{fr} chromophore which is more pronounced in partially degraded [2, 4] than in native [3] phytochrome. However, the reactivity of the P_{fr} chromophore during oxidation and reduction is higher than that of the P_r chromophore even in a small chromopeptide [5] in which the chromophore should be accessible for the reagents in both forms. This difference has been explained as an intrinsic difference of chemical reactivity of the chromophores the configuration of which was demonstrated to be 15 *Z* in the P_r form and 15 *E* in the P_{fr} form [6–9]. To establish the effect of intrinsic chemical reactivity on the one hand and of chromophore exposure or protection on the other hand, we have studied the oxidation of the P_r and P_{fr} chromophore by tetranitromethane in small chromopeptides in relation to native phytochrome. Furthermore, ozone was used as oxidizing reagent because it is small enough to penetrate to relatively "inacessible" sites within the protein. It should therefore be suitable to test the intrinsic reactivity of the chromophore in both P_r and P_{fr}.

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Materials and Methods

Phytochrome

Native Phytochrome (124 kd) was isolated from etiolated oat seedlings (*Avena sativa* L., cv. Pirol, Baywa, München) according to the procedure of Vierstra and Quail [10] with the following modifications: 500 g of seedlings (germinated for 72 h at 28 °C) were extracted with Tris-HCl/50% ethylene

glycol containing 20 mM ascorbic acid instead of sodium bisulfit. Elution from hydroxylapatite was achieved with 100 mM potassium phosphate buffer. The subsequent Affi-Gel Blue procedure was omitted. Phytochrome in the pooled fractions eluted from hydroxylapatite ($A_{666}/A_{280}=0.05$ to 0.1) was precipitated with ammonium sulfate instead.

The pellet (containing 5 mg phytochrome) was washed twice with 1.5 ml 100 mM potassium phosphate buffer, pH 7.8 (4 °C). Most of contaminating proteins (and some phytochrome) were dissolved by this procedure. The remaining deep blue pellet was finally dissolved in 3 ml 20 mM potassium phosphate buffer, pH 7.8 (4 °C) giving a purity index $A_{666}/A_{280}=$ 0.33. This solution was submitted to a Bio-Gel A-1.5 M column and eluted with the same buffer. Phytochrome with A_{666}/A_{280} better than 0.55 was obtained and used for ozonolysis. Use of Tris-HCl buffers and EDTA or 2-mercaptoethanol for ozonolysis was strictly avoided.

Degraded phytochrome (120 kd) was partly purified by brushite chromatography according to [11] form 3.5 day old etiolated oat seedlings (*Avena sativa* L. var. Pirol, Baywa, München, FRG). Degraded phytochrome was used for preparation of P_r chromopeptides (about 2.5 kd) as previously described [6]. The Biogel P-10 fraction of the P_r chromopeptide was applied to a silicia gel column which had been equilibrated with distilled water. After washing with distilled water it was eluated with 50% aqueous HCOOH. Biliverdin was purchased from Sigma (Dihydrochloride, purity approx. 80%) and used without further purification.

Electronic spectra and kinetics at a fixed wavelength were taken either with a spectrophotometer model DMR 22 (Zeiss, Oberkochen, FRG), a dual wavelength spectrophotometer ZWS II (Sigma Instruments, Berlin) or with a spectrophotometer model Lambda 3 (Perkin Elmer, Norwalk, USA).

Ozonolysis

2 ml of the pigment solution (phytochrome, chromopeptide or biliverdin) were placed in a 3.5 ml quartz cuvette (pathway 1 cm) and cooled to 5 +/− 1 °C. A stream of ozone/oxygen was bubbled through a capillary at a flow rate of 5 ml min^{-1}. Ozone was produced either in a micro scale discharge tube inserted before the capillary or an Orec ozonater model 03V5-0 (Phoenix, USA). Simultaneously bleaching of phytochrome was recorded by measuring absorbance at the maximum of the long wavelength band. The kinetic run was recorded on a 8110 electronic recorder with data processing and storage capability (Bascom-Turner Instruments, Mass. USA).

Urea denaturation of the phytochrome samples was carried out in the measuring cuvettes. To avoid to much dilution of the samples, the urea was added in solid form to a final concentration of 8 M.

To avoid precipitation of phytochrome upon acidification, conc. HCl was added to the samples after urea denaturation.

Results and Discussion

The conditions for bleaching with ozone had to be chosen in such a way that pseudo-first order kinetics are obtained. This is the case at rather fast flow rates of ozone. As shown in Fig. 1 for phytochrome, first order kinetics are observed after a short lag-phase. Only at the end of the bleaching period deviation from linearity in the semilogarithmic plot occurs.

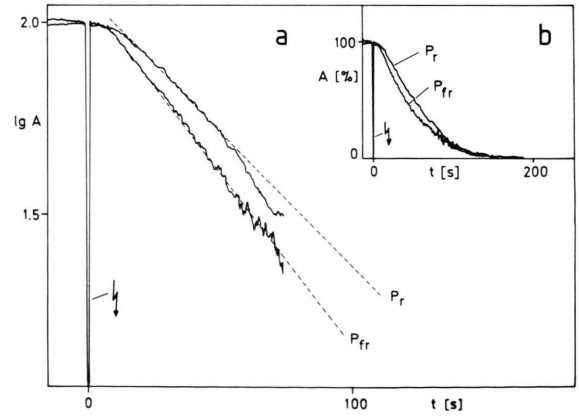

Fig. 1. Ozonolysis of Phytochrome (124 kd). Conditions: 5 °C, 20 mM potassium phosphate buffer, pH 7.8 (4 °C) phytochrome concentration 0.68 μM, oxygen-flow rate at 5 ml min^{-1}, a) linearized plot lgA with A ($t=0$) normalized to 100% *versus* time assuming pseudo first order kinetics. A has been recorded at 666 nm for P_r and at 730 nm for P_{fr} (obtained through saturating irradiation with red light). Linear graphs are obtained by least square fitting. The arrow denotes start of electric discharge for ozone production. b) (Insert figure.) Actual plot of kinetics with A ($t=0$) normalized to 100%.

Similar curves were obtained for the bleaching of other tetrapyrroles with ozone. For the evaluation of the kinetic data, only the linear part of the semilogarithmic plot was taken.

The kinetics of bleaching of various tetrapyrroles with ozone are very similar to each other: A short initial lag-phase is followed by a linear phase in the semilogarithmic plot whereas deviation from linearity is observed at the end of the bleaching period. The rate constants in the linear phase (Table I) for bleaching of protein-bound tetrapyrroles (phytochrome, phycocyanin) are very close to those of biliverdin and a small P_r-peptide in which the tetrapyrrole chromophore should be freely accessible. The small molecule ozone seems to easily penetrate to sites within the protein (chromophore site in P_r) which are inaccessible for other reagents. Any differences in the reaction kinetics between P_r and P_{fr} should therefore be due to differences in the reactivity rather than in the accessibility of the chromophore.

Table I. Bleaching of various tetrapyrroles with ozone.

Compound	Solvent	Rate constant $(s^{-1}) \times 10^2$
Phytochrome (P_r)	phosphate buffer pH 7.8	1.95–2.6
Biliverdin	Tris/HCl pH 7.5	2.55
Biliverdin	Tris/HCl pH 4.0	1.88
Biliverdin	isoamylalcohol	2.4
Biliverdin	isoamylalcohol/2.5% HCOOH	2.4
P_r Peptide	50% HCOOH	2.5
P_{fr} Peptide	methanol/5% pyridine	3.5
Phycocyanin	5 mM Tris/HCl	1.7

Table II. Bleaching of 124 kd phytochrome as P_r and P_{fr} with ozone. All values are rate constants in $(s^{-1}) \times 10^2$.

No. of experiment	Remark	P_r	P_{fr}	Rate P_{fr} / Rate P_r
1 a	native	0.42	0.46	1.09
1 b	denatured*	0.51	0.51	1.00
2 a	native	2.6	3.1	1.19
2 b	denatured*	1.8	2.0	1.11
3 a	native	1.95	2.2	1.12

* With urea/HCl

The comparison of the bleaching of P_r and P_{fr} is shown in Table II. The flow rate of ozone was varied from one experiment to the other so that the rate constants also varied. However, the conditions were identical for P_r and P_{fr} within one experiment. The data of Table II clearly demonstrate that the rate constants for P_r and P_{fr} are similar within one experiment but that those of P_r are always somewhat smaller than the corresponding values of P_{fr}. This is true for native phytochrome as well as for denatured phytochrome, *i.e.* independent of the apparent accessibility of the chromophores. We conclude that this small difference of about 10% reflects the higher reactivity of the P_{fr} chromophore. The difference is much smaller than the difference previously detected for bleaching with permanganate [2] or tetranitromethane [3]. Therefore, in that case the difference in reactivity must be mainly due to the fact that the chromophore in P_r is shielded by the protein whereas it is exposed to the medium in P_{fr}. This is especially true for partially degraded phytochrome [2].

Table III. Tetranitromethane oxidation of phytochrome and bile pigments. All values are rate constants in $(s^{-1}) \times 10^5$.

Compound	P_r	Relative rate	P_{fr}	Relative rate	Ratio P_{fr}/P_r	Remarks
124 kd phytochrome*	5.8	1.00	48.8	8.41	8.41	[3]
118 kd phytochrome*	5.6	0.97	227.0	39.14	40.35	[3]
60 kd phytochrome*	33.1	5.71	516.0	88.97	15.58	[3]
Chromopeptide** (neutral)	4800	827.59	5100	879.31	1.06	this work
Chromopeptide+ (acidic)	5700	982.76	6600	1137.93	1.16	this work
Bilirubin*	1220	210.34				[3]
Biliverdin*	1260	217.24				[3]

* In 0.1 M potassium phosphate buffer, pH 7.8, 50 mM KCl and 9.1 mM EDTA, 275 K.
** P_r-peptide in methanol and P_{fr}-peptide in methanol (5% pyridine), 283 K.
\+ P_r-peptide in 50% HCOOH and P_{fr}-peptide in acidic methanol (5% HCl), 283 K.

We had also to consider the possibility that the difference in the reaction rate between P_r and P_{fr} could be larger for other oxidants than for ozone. We therefore investigated the tetranitromethane reaction with intact phytochrome and small chromopeptides obtained from P_r and P_{fr}.

From the results shown in Table III, it can be suggested that the P_{fr} chromophore is more exposed than the P_r chromophore. However, the degree of exposure clearly depends on the size of protein, indicating in particular that the 6000-dalton molecular mass peptide chain partially shields and interacts closely with the P_{fr} chromophore. Comparison of the reactivity of the chromopeptides of P_r and P_{fr} forms seems to indicate that the latter is slightly more reactive than the former also with tetranitromethane. Both chromopeptides are more reactive than biliverdin or bilirubin which have hitherto been used as model chromophores.

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