Volume 234, number 2, 497–499 FEB 06116 July 1988

The amino-terminal structure of oat phytochrome

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Received 13 May 1988; revised version received 1 June 1988

Phytochrome from etiolated oat seedlings was digested under carefully controlled conditions with trypsin. A dodecapeptide which contains the blocked amino-terminus of phytochrome was isolated from the digestion mixture and analyzed. According to amino acid analysis and the results obtained in FAB mass spectrometry, the sequence of mature phytochrome starts with *N*-acetylserine, corresponding to serine-2 derived from the DNA sequence.

FAB mass spectrometry; Proteolysis; HPLC; Peptide sequencing; Trypsin; (*Avena sativa* L.)

1. INTRODUCTION

Phytochrome is a well-investigated photoreceptor for light-regulated development of higher plants [1,2]. Most investigations on the protein part have been performed with phytochrome from etiolated oat seedlings for which isolation procedures have been described [3–6]. During photoconversion of the inactive Pr-form to the physiologically active Pfr-form, structural changes occur not only in the tetrapyrrole chromophore [7] but also in the protein moiety according to the results of partial proteolysis [4,8,9] and of other methods [10–12]. It has been concluded from these investigations that the amino-terminal part of phytochrome is exposed to the environment in the Pr-form but inaccessible from outside in the Pfr-form. Sequencing of proteolytic fragments of phytochrome [9,13] allowed the exact localization of sites for proteolytic attack within the entire protein sequence derived from DNA sequence analysis [14]. Since the amino-terminus of phytochrome is blocked [9,15] it was not possible to perform Edman degradation directly with native phytochrome and fragments derived from the amino-terminus. It was therefore unknown whether the initiator methionine is still present in mature phytochrome. We describe here the isolation and analysis of small peptides derived from the amino-terminus of oat phytochrome.

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2. MATERIALS AND METHODS

Phytochrome (124 kDa) was isolated from 3.5-day-old etiolated oat seedlings (*Avena sativa* L.; c.v. Pirol, Baywa, Munich) as in [5] with the following modifications: final purification was achieved by precipitation with ammonium sulfate (2% saturation) instead of polyvinyl pyrrolidone. The precipitate was dissolved in 0.5 ml/mg phytochrome of 2 mM Hepes, pH 7.8, containing 1 mM EDTA. The absorbance at 667 nm of the resulting solution was about 2.0 with a ratio A_{667}/A_{280} of between 0.90 and 0.99 in the Pr-form.

The resulting solution of Pr (0.5 ml, $A_{667} = 2.0$) was incubated with 0.2% (w/w) trypsin at 4°C for 30 min. The reaction was then stopped by acidification with formic acid (final pH 2.5). The mixture was then applied to HPLC separation on a column (0.46 × 12.8 mm) packed with Hibar LiChrospher 100, RP-18 (Merck, Darmstadt) and eluted with a gradient of solvent A (0–80%, v/v, within 80 min) in solvent B at a flow rate of 1.2 ml/min (solvent A: 0.1% trifluoroacetic acid in water; solvent B: 0.1% trifluoroacetic acid in acetonitrile). The UV detector was set at 206 nm. Aliquots (50 μl) of the main fractions (650 μl) (see fig.1) were hydrolyzed with 6 N HCl containing 10% trifluoroacetic acid at 160°C for 20 min. The resulting amino acids were determined in an amino acid analyzer (System 6300, Beckman, Palo Alto, CA). The bulk (600 μl) of the serine-rich fraction 7 was subjected to analysis by FAB mass spectrometry.

The peptide (~1 nmol) was dissolved in glycerol/20% acetic

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

acid (1:1) and monitored on a type HSQ 30 mass spectrometer (Finnigan MAT, Bremen) equipped for FAB ionization.

In accordance with earlier publications [8,12], we count the amino acid residues of the primary translation product and not of the processed phytochrome protein.

3. RESULTS AND DISCUSSION

For the preparation of small amino-terminal fragments from phytochrome, we used the same conditions of trypsin digestion which yielded a 122 kDa fragment starting with V-22 [9]. According to the sequence, tryptic cleavage sites within the first 21 amino acids are expected at Arg-13, Arg-15 and Arg-21. The resulting amino-terminal peptides with calculated molecular masses around 1325, 1595 and 2252 Da, respectively, should in any case contain a high percentage of serine. The peptide mixture was separated by HPLC (see fig.1) and the resulting main peptide fractions (T_1–T_5) were analyzed. According to the amino acid analysis (table 1), the peptides arose from cleavage at Arg-13, Arg-15, Arg-21, Arg-53 and Arg-62; no cleavage was detected at Arg-5 due to the sequence Arg-Pro. Only peptide T_3 contained a large amount of serine but no methionine (table 1). The yield of serine (70% of the calculated amount) is in accordance with serine analyses performed by others [16]. Peptide T_3 was further analyzed by FAB mass spectrometry. As shown in fig.2, signals in the molecular mass range were obtained at *m/z* 1237, 1259 and 1281/1282. According to earlier observations, HPLC separation of peptides leads to extensive H-Na exchange. We therefore attribute the signals at *m/z* 1259 and 1281/2 to the mono- and disodium salt, respectively, and the signal at *m/z* 1237 to the peptide proper.

This molecular mass is only compatible with tryptic cleavage of phytochrome at Arg-13. Furthermore, it confirms the absence of methionine (see table 1) in peptide 3, since the molecular mass should in this case be found in the range *m/z* 1325

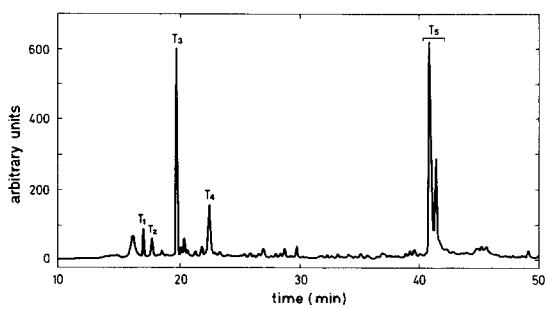

Fig.1. HPLC separation of tryptic peptides from phytochrome on a reversed-phase column with trifluoroacetic acid/acetonitrile. Detection by absorbance at 206 nm (arbitrary units given). The bulk of undigested phytochrome and the 122 kDa fragment are eluted later (not shown).

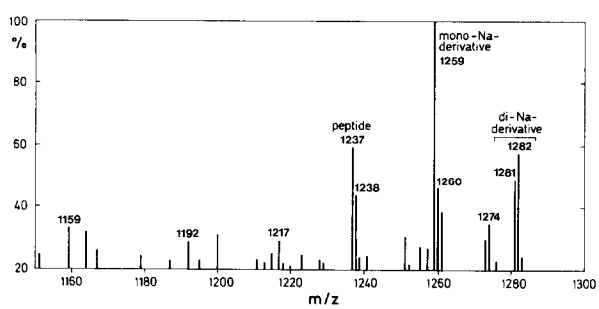

Fig.2. Analysis of peptide T_3 by FAB mass spectrometry. Only peaks with intensities ≥20% of base peak intensity are given.

Table 1

Amino acid composition of tryptic peptides (T_1–T_5) of oat phytochrome after limited proteolysis

Peptide:	T_1	T_2	T_3	T_4	T_5
Position:	16–21	14–21	2–13	54–62	22–62
Asx Asn		1.1 (1)		1.1 (1)	4.6 (1)
Asp					(4)
Ser	1.6 (2)	1.7 (2)	5.8 (8)		2.5 (3)
Thr					2.0 (2)
Glx Glu	2.4 (2)	2.4 (2)		2.2 (2)	8.4 (4)
Gln					(4)
Pro			0.9 (1)	1.7 (2)	1.8 (2)
Gly				2.1 (2)	2.5 (3)
Ala	1.0 (1)	1.0 (1)	1.0 (1)		4.0 (4)
Cys					
Val				1.0 (1)	3.0 (3)
Met					
Ile					
Leu					4.4 (4)
Tyr					2.3 (2)
Phe					1.5 (2)
Lys					1.0 (1)
His					
Arg	1.1 (1)	2.3 (2)	1.3 (2)	1.1 (1)	2.1 (2)
Total	(6)	(8)	(12)	(9)	(41)

Tryptophan was not determined. The real number of residues, derived from the sequence [14], is shown in parentheses. The positions of the peptides are numbered according to the unprocessed primary translation product [9,13]

Table 2

Calculation of the mass of the blocking group of peptide T_3

Sequence	Molecular mass calculated	Molecular mass determined	Mass of X calculated
I	1325 + *X*	1237	–
II	1195 + *X*		42

Molecular masses given in Da

Sequence I = X M S S S R P A S S S S S R
Sequence II = X S S S R P A S S S S S R

+ *X*. As shown in table 2, the peptide must still contain the amino-terminal blocking group of phytochrome for which a mass of 42 was calculated. The most likely group which fits with these data is the acetyl group. This group is found relatively often as an amino-terminal substituent in those proteins which have a blocked amino-terminus [17].

We conclude that the primary translation product is processed to final phytochrome not only by covalent linkage of a tetrapyrrole chromophore but also by cleavage of amino-terminal methionine and acetylation of the resulting free amino group of Ser-2. As pointed out in section 1, the amino-terminal part of the peptide chain is involved in the photoreversible conformational change. It may be that a charged amino-terminus would interfere with such photoreversible changes by undesired ionic interaction and that acetylation prevents this interaction.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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