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Accumulation of a translation intermediate of D1 protein by light–dark transition in isolated spinach chloroplasts

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In an in vitro translation experiment using spinach chloroplasts, a novel protein band of about 17.5 kDa appeared by light to dark transition. The protein never accumulated in detectable amounts either in continuous illumination or in continuous darkness. The 17.5 kDa protein accumulated upon light–dark transition, on the other hand, disappeared by the subsequent illumination. Accumulation of the protein in light, however, was observed when stromal level of ATP in chloroplasts was lowered after preillumination by the addition of various chemical compounds which, irrespective of the mode of action, eventually decrease the ATP level, e.g. atrazine, carbonyl-cyanide-*m*-chlorophenyl hydrazone and glycerate. The dark-accumulated protein was concluded to be a translation intermediate of D1 protein based on the facts that this component precipitates with specific antibodies and is resistant to lysylendopeptidase treatment. The suppression by chloramphenicol of both appearance upon light–dark transition and disappearance by the subsequent illumination of the protein also supported this conclusion. The phenomenon was discussed in terms of pausing in the translation of *psbA* mRNA.

D1 protein; Intact chloroplast; Light-regulated synthesis; Photosystem II; *psbA*; Translation intermediate

1. INTRODUCTION

The D1 protein is a subunit of photosystem II reaction center (PSII RC) [1], which, on the other hand, is known to be one of the most rapidly metabolizing proteins in illuminated chloroplasts [2]. The rapid rate of metabolism is now believed to represent photodegradation of the subunit as a result of functioning of PSII RC and light-regulated de novo synthesis of the protein, where a specific coupling between the degradation and the synthesis has been postulated to exist [3,4].

Chloroplasts have multiple copies of genomic DNA and contain the whole genetic information expression system, i.e. DNA polymerases, RNA polymerases, basic machineries for translation and regulators for various stages of gene expression [5]. The gene for D1 protein, *psbA*, is present on chloroplastic genome [6] and the mRNA level is stable in the dark. Thus, the site of control by light of the expression of D1 protein is not just at the transcriptional but mainly at the translational level [7]. A recent study demonstrated that the light-induced stromal level of ATP is one of the determinants for the regulation in isolated chloroplasts under certain circumstances [8].

On the other hand, earlier studies postulated discontinuous translation of D1 protein since the translation intermediates of 15–25 kDa were transiently labeled in a pulse-chase experiment [9]. Toeprint analysis in order to determine the distribution of ribosomes on polysome-associated *psbA* mRNA also indicated that ribosomes pause at discrete sites during translation of D1 protein [10]. The significance of this phenomenon in translation regulation was discussed in terms of co-factor association during synthesis of D1 protein [10].

In the present study the accumulation of a novel protein band of about 17.5 kDa was detected upon light–dark transition in isolated chloroplasts, which presumably corresponds to a translation intermediate of D1 protein. The phenomenon has been analyzed in this paper in search for regulatory mechanisms at the stage of translation in the expression of D1 protein.

2. MATERIALS AND METHODS

2.1. *Translation of D1 protein in isolated chloroplasts*

Intact chloroplasts were isolated from fresh spinach leaves as described in [11]. The reaction mixture for translation contained spinach chloroplasts equivalent to 500 μg Chl ml^{-1}, 500 μCi ml^{-1} L-[^{35}S]methionine (ca. 1000 Ci mmol^{-1}; ICN Biomedical Inc., Irvine, CA, USA), 330 mM sorbitol and 50 mM Tricine-KOH (pH 8.4). The translation reaction was conducted for definite time intervals at 25°C, either in the dark or in the light. An actinic light of the intensity of 50 W m^{-2} was provided by a slide projector equipped with a tungsten halogen lamp (1000 W) and UV- and heat-absorbing filters.

2.2. *Protein analysis*

In the protein analysis, radio-labeled chloroplasts were suspended in 20 mM Tris-HCl (pH 7.5) with vortexing, and then the thylakoidal fraction was collected by micro-centrifugation and resultant pellets were solubilized in a sample buffer containing 125 mM Tris-HCl (pH 6.8), 5% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol and 5% (w/v)

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SDS. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described by Laemmli [12].

After electrophoretic separation, radiolabeled proteins were detected either by fluorography or by autoradiography using an imaging analyzer (Type, BAS-2000; Fuji Film Co. Ltd., Tokyo, Japan). In case of fluorography, gels were fixed, soaked in an enhancer (ENLIGHTENING; Du Pont Chem. Co., Wilmington, DE, USA), dried and then exposed to X-ray films (Type, New RX; Fuji Film Co. Ltd., Tokyo, Japan) at −80°C. In case of autoradiography using Fuji imaging analyzer, the fixed gels were dried and then exposed to a recyclable imaging plate (Type, BAS-III; Fuji Film Co. Ltd.).

2.3. *Immunoprecipitation*

Polyclonal antibodies against D1 protein were provided by Dr. Mullet (Texas A&M University, TX, USA) [10]. ^{35}S-labeled thylakoid membranes were solubilized by digitonin (0.5%) and NP-40 (0.5%) for 30 min and centrifuged at 100,000 × *g* for 20 min at 0°C. The resultant supernatant was incubated with antibodies for 5 h at 0°C and then antigen–antibody complexes were collected by centrifugation in the presence of Protein A Sepharose CL4B (Sigma).

2.4. *Quantitation of ATP in chloroplasts*

ATP was extracted from chloroplasts as in [13] and then determined photometrically by using a luciferin–lusiferase assay kit (Lucifer-LU, Kikkoman, Chiba, Japan) [8].

3. RESULTS AND DISCUSSION

In the experiment shown in Fig. 1A, isolated spinach chloroplasts were incubated for 5 min in the reaction mixture for translation (see Materials and Methods) under white light (50 W·m^{-2}) in the presence of [^{35}S]methionine at 25°C (0 min in Fig. 1A). After turning off the actinic light, incubation was further continued for 0.5–20 min under the same condition (0.5–20 min in Fig. 1A). Fluorographic analysis of newly synthesized proteins in thylakoid membranes obtained by hypotonic treatment of chloroplasts revealed that light illumination caused the appearance of heavily radiolabeled bands of D1 protein and its precursor of about 30 kDa [8], together with some other minor bands, parts of which presumably correspond to proteolytic fragments and/or translation intermediates of various thylakoidal proteins, as is documented [9,14]. During the subsequent dark labeling period following translation in light, a novel protein band of about 17.5 kDa, as estimated by electrophoretic mobility on 15% acrylamide concentration in analyzing gel, gradually appeared on the fluorogram Fig. 1A). The protein never accumulated in detectable amounts under continuous illumination, but appeared only after light–dark transition. However, only a short interval of light illumination of about one minute was enough to saturate the amount of accumulated protein. The 17.5 kDa band thus accumulated by cessation of actinic illumination disappeared almost completely within 5 min of subsequent light incubation, as shown in Fig. 1B. The formation of 17.5 kDa protein band by cessation of light illumination was also observed in isolated pea chloroplasts under the same conditions (data not shown).

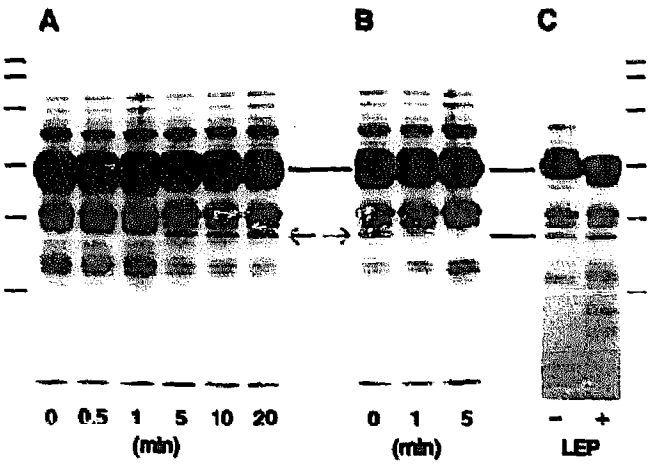

Fig. 1. Appearance of a novel protein band upon light–dark transition. Spinach chloroplasts were radiolabeled for 5 min with [^{35}S]methionine in the light (0 min) and then incubated in the dark for 0.5–20 min (A). After 20 min dark incubation, the reaction mixture was illuminated for 0–5 min (B). Thylakoid membranes from chloroplasts after 20 min dark incubation described above were solubilized and incubated, either in the presence (+) or absence (−) of lysylendopeptidase (100 μg·ml^{-1}), and then subjected to SDS-PAGE and fluorographic analysis (C). Arrows indicate the position of novel protein band appeared by light–dark transition. Bars on both sides are positions of mol. wt. marker proteins: 94, 67, 43, 30, 20.1 and 14.4 kDa, respectively, from top to bottom. 15% (w/v) acrylamide gel containing 6 M urea was used for analysis.

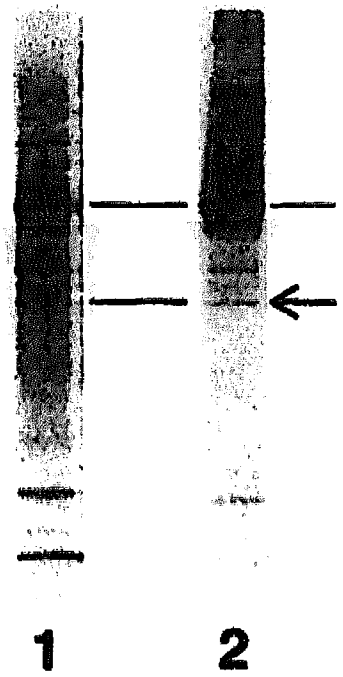

Fig. 2. Autoradiographic analysis of immunoprecipitated proteins using antibodies against D1 protein. Thylakoid membranes obtained in Fig. 1 (A; 20 min) were used as starting materials (lane 1). Detergent-solubilized proteins were immunoprecipitated as described in Materials and Methods (lane 2). A bar and an arrow indicate positions of the mature sized D1 protein and the novel protein accumulated upon light–dark transition, respectively.

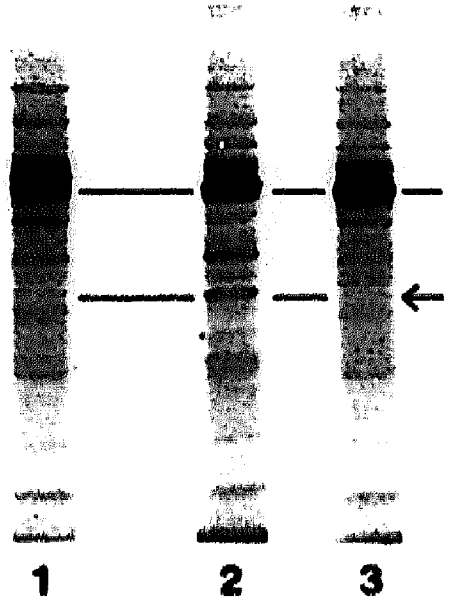

Fig. 3. Inhibition by chloramphenicol of the appearance of 17.5 kDa protein upon light–dark transition. Lanes 1 and 2 correspond to 0 and 20 min dark incubation in Fig. 1A. Chloramphenicol was added to the translation mixture at 200 $\mu g \cdot ml^{-1}$ at the cessation of 5 min illumination and the mixture was further incubated for 20 min in darkness (lane 3). A bar and an arrow indicate positions of mature-sized D1 protein and 17.5 kDa protein, respectively.

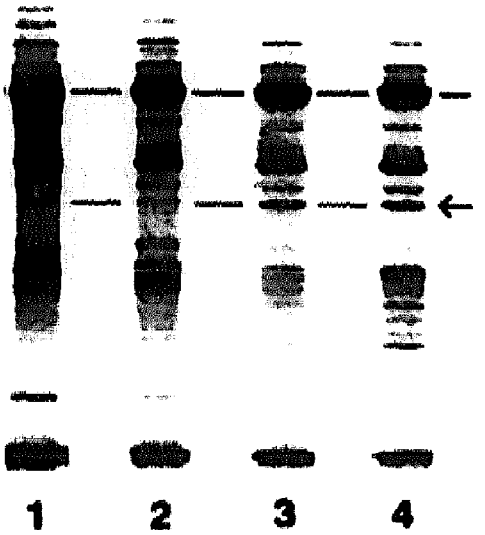

Fig. 4. Appearance of 17.5 kDa protein by the addition of atrazine in illuminated spinach chloroplasts. After 5 min of light-labeling period, atrazine was added to chloroplasts at the concentration of 10 (lane 1), 40 (lane 2), and 100 (lane 3) μM, respectively, and then incubated in the light for another 20 min. Lane 4 corresponds to sample dark-incubated for 20 min after cessation of actinic illumination. A bar and an arrow on the right side indicate positions of the mature D1 protein and the 17.5 kDa protein, respectively.

suggesting that the phenomenon is not restricted to spinach.

The new protein band appeared that by light–dark transition was detected in the fraction immunoprecipitated using D1-specific antibodies (Fig. 2) and thus it is concluded that this component is a part of the D1 protein. The above conclusion was supported by the fact that this protein band is resistant to lysylendopeptidase treatment (Fig. 1C), consistent with the deduced amino acid sequence of D1 protein of spinach [6]. A partial proteolytic fingerprinting experiment (data not shown) further supported this conclusion and suggested, based on previous observations [14], that the 17.5 kDa protein corresponds to the fragment containing N-terminal half of D1 protein.

There are two possibilities for the origin of the new protein band of 17.5 kDa which appeared upon light–dark transition. One of the possible origins might be degradation of D1 protein as described by Edelman et al. [14]. However, the view is not consistent with kinetic behavior of appearance of this protein band. In the present study, the amount of accumulated protein during the dark after light period saturated only by a brief preillumination of about 1 min under the light intensity used, as mentioned above. On the other hand, in the case of the degradation product of Edelman et al., the amount of the degraded protein was shown to depend on the length of light illumination [14].

An alternative possibility is that the protein band observed here is a paused translation intermediate of the D1 protein. The fact that both appearance upon light–dark transition (Fig. 3) and disappearance during the subsequent light period of this protein (data not shown) were prevented by the addition of chloramphenicol (200 $\mu g \cdot ml^{-1}$) which inhibits elongation in chloroplastic protein synthesis is in favor of this possibility. This fact together with kinetic evidence mentioned above strongly supports the interpretation that the novel protein band observed in this study is a paused

Table 1

Levels of ATP in isolated spinach chloroplasts incubated under different conditions

Conditions	ATP* (nmol mg^{-1} Chl)
5 min light	8.14
10 min dark after 5 min light	3.26
10 min light in the presence of**:	
100 μM atrazine	3.44
40 μM atrazine	3.96
10 μM atrazine	5.52

*ATP was determined as in Materials and Methods.
**Chloroplasts were preilluminated for 5 min under the conditions for translation.

translation intermediate of D1 protein, as suggested by Kim et al. in barley [10].

In order to understand the mechanism of elongation pausing of D1 protein upon light–dark transition, atrazine was added to the translation mixture after 5 min of illumination and translation was further continued for another 20 min in the light (Fig. 4). The result indicated that presence of atrazine elicits the appearance of the 17.5 kDa component even in light, in a concentration-dependent fashion. In a previous study it was clearly demonstrated that higher concentration of atrazine decreases the ATP level in illuminated chloroplasts to that of dark control which corresponds to the level of thylakoid-bound ATP [8] (Table I). This fact suggests that the functioning of cyclic phosphorylation is insignificant under the conditions in sustaining the ATP level in stromal compartment of chloroplasts (without externally added cofactors). This fact together with experimental evidence shown in Fig. 4 led us to hypothesize that elongation of the D1 protein proceeds even at a relatively lower concentration of ATP, except for at a specific point where a high level of ATP is required by an as yet unknown mechanism. For this reason, the lowering of ATP by the addition of atrazine after preillumination results in accumulation of a paused intermediate. The interpretation was further supported by the fact that the appearance of this protein band was also observed in light in the presence of carbonyl-cyanide-*m*-chlorophenyl hydrazone (CCCP) or of glycerate (data not shown) both of which decrease the stromal level of ATP in chloroplasts by entirely different mechanisms [8].

In a recent study, Kim et al. [10] observed several species of D1 translation intermediates in barley chloroplasts. They proposed that ribosome pausing facilitates co-translational binding of cofactors such as chlorophyll to D1 protein and aids the integration of D1 protein into the thylakoid. However, the translation intermediate observed in this study might be different from those reported by Kim et al. [10], since the experimental conditions and the result obtained are largely different from each other; Kim et al. observed the formation of several intermediates of D1 protein under continuous illumination, while in the present study, only one sharp band of 17.5 kDa was observed under dark after brief preillumination.

The size of 17.5 kDa approximately corresponds to 160 amino acids, although estimated values by SDS-polyacrylamide gel electrophoresis can never be exact especially for hydrophobic membrane proteins. Taking into account the inaccuracy of molecular weight estimation, we may expect, for example, a situation where binding of special pair chlorophyll (P-680) to the 198th histidine on D1 protein requires a higher concentration of ATP, in the repairing process. Another possibility of pausing of elongation is that the integration into thylakoids of a helix, for example putative membrane spanning helixes C or D, is inhibited under low ATP concentrations. Further analysis evidently is needed to clarify the control mechanism.

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REFERENCES

[1] Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
[2] Ellis, R.J. (1981) Ann. Rev. Plant. Physiol. 32, 111–137.
[3] Kyle, D.J., (1987) in: Photoinhibition (Kyle, D.J., Osmond, C.B. and Arntzen, C.J. eds.) pp. 197–226, Elsevier, Amsterdam.
[4] Mattoo, A.K., Marder, J.B. and Edelman, M. (1989) Cell 56, 241–246.
[5] Mullet, J.E. (1988) Annu. Rev. Plant Physiol. Plant Mol. Biol. 39, 475–502.
[6] Zurawski, G., Bohnert, H.J., Whitefeld, P.R. and Bottomley, W. (1982) Proc. Natl. Acad. Sci. USA 79, 7699–7703.
[7] Fromm, H., Devic, M., Fluhr, R. and Edelman, M. (1985) EMBO J. 4, 291–295.
[8] Kuroda, H., Inagaki, N. and Satoh, K. (1992) Plant Cell Physiol. (in press).
[9] Mullet, J.E., Klein, P.G. and Klein, R.R. (1990) Proc. Natl. Acad. Sci. USA 87, 4038–4042.
[10] Kim, J., Klein, P.G. and Mullet, J.E. (1991) J. Biol. Chem. 266, 14931–14938.
[11] Mullet, J.E. and Chua, N.-H. (1983) Methods Enzymol. 97, 502–509.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Inoue, Y., Kobayashi, Y., Shibata, K. and Heber, U. (1978) Biochim. Biophys. Acta 504, 142–152.
[14] Greenberg, B.M., Gaba, V., Mattoo, A.K. and Edelman, M. (1987) EMBO J. 6, 2865–2869.