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ATP-dependent protein synthesis in isolated pea chloroplasts

Evidence for accumulation of a translation intermediate of the D1 protein

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In the presence of externally added ATP, in the dark, isolated pea chloroplasts accumulate two proteins of molecular masses of about 22 and 24 kDa which precipitate with specific antibodies raised against the D1 protein. By chasing in the light, these proteins disappeared on the fluorogram concomitant with the appearance of the precursor- and mature-sized D1 proteins. Polysome analysis indicated that the 22-kDa component is associated with membrane-bound ribosomes and is thus ascribed to a translation intermediate of the D1 protein. On the other hand, the 24-kDa component could not be found in the polysome fraction under the experimental condition used, suggesting the possibility that this component is a degradation product of the D1 protein. The conclusion from this analysis is that the synthesis and/or stable accumulation of the D1 protein requires factor(s) caused by illumination, in addition to ATP, in isolated pea chloroplasts.

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

1. INTRODUCTION

The D1 protein is a subunit of photosystem II (PS II) reaction center, providing, together with the D2 protein, the site for primary photochemistry [1,2]. This protein is also known to be one of the most rapidly metabolizing components in illuminated chloroplasts [3,4]. The light-dependent synthesis of this protein is now understood to represent the reparative process of photodamaged reaction centers [5]. Since the messenger for D1 protein (*psb*A mRNA) present in association with thylakoid membranes is abundant and stable even in the dark, during which accumulation of the protein is hardly detectable [6,7], the synthesis is assumed to be regulated by light at the translational and/or post-translational stage(s). The mechanism of light regulation at the stage of translation of this protein has been extensively analyzed during the last decade [8]. To date, however, no consistent picture has emerged. A previous study suggested that an activator protein(s) regulates translation of the D1 protein at the stage of initiation in *Chlamydomonas* [9]. On the other hand, it was reported that newly synthesized chlorophyll (Chl) post-translationally regulates accumulation of the D1 protein by increasing the stability of apoproteins in barley etioplasts in the process of greening [10]. In the previous analysis it was demonstrated that the light-induced stromal ATP level is one of determinants for translational regulation of the D1 protein in isolated spinach chloroplasts [11]. In the present study, the mechanism of light regulation was further analyzed using isolated pea chloroplasts. The result clearly demonstrated that accumulation of the D1 protein requires factor(s) caused by illumination, in addition to ATP, at the specific step(s) of polypeptide elongation and/or for post-translational stabilization.

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2. MATERIALS AND METHODS

2.1. *Plant materials*

Pea (*Pisum sativum*) seeds were planted in vermiculite and watered with half-strength Hoagland's nutrient solution. The seedlings were grown in a controlled environment chamber (Eyelatron FLI301N, Hitachi, Tokyo) at 25°C under continuous white light from fluorescent lamps (10,000 lux) for 10 days.

2.2. *Chloroplasts isolation*

Intact chloroplasts were isolated from 10-day-old pea leaves by stepwise Percoll gradient centrifugation and then resuspended in chilled 50 mM Tricine/KOH (pH 8.4) buffer containing 330 mM sorbitol as described in [11]. To minimize contamination by exogenous RNases and bacteria, all glassware was baked at 210°C for 5 h and all plastics and almost all solutions were sterilized by autoclaving at 120°C for 30 min.

2.3. *Protein synthesis in isolated chloroplasts*

The reaction mixture (30 μl) for analyzing protein synthesis in isolated chloroplasts contained 50 mM Tricine-KOH (pH 8.4), 330 mM sorbitol, pea chloroplasts equivalent to 500 μg Chl · ml^{-1} and 250 μCi · ml^{-1} L-[^{35}S]methionine. Pulse-labelling was carried out at 25°C for 10 min in the presence or absence of white light (50 W · m^{-2}) provided by a tungsten–halogen lamp (1,000 W). After pulse-labelling, chloroplasts were burst by hypotonic treatment with 25 mM MOPS/NaOH (pH 6.5) buffer containing 50 mM NaCl, and the membrane

fraction was collected by microcentrifugation and resuspended in the same buffer at 500 μg Chl · ml^{-1}. The isolated membranes were solubilized with an equal amount of sample buffer for SDS-PAGE containing 125 mM Tris-HCl (pH 6.8), 5% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol and 5% (w/v) SDS.

2.4. *SDS-PAGE and detection of radiolabelled proteins*

SDS-urea-PAGE conducted as described in [12] was used to analyze newly synthesized proteins. The separation gel contained 15% polyacrylamide and 6 M urea. In the case of fluorography, gels were fixed, treated with Enlightning (Du Pont Chem. Co., Wilmington, DE), dried and exposed to X-ray film (type NEW RX, Fuji Film Co., Tokyo) for 5 days, in a typical experiment. The fluorogram was quantified by densitometric scanning of exposed films at 540 nm with subsequent integration with a dual-wavelength TLC scanner (model CS-930; Shimadzu, Kyoto).

2.5. *Immunoprecipitation using antisera against D1 protein*

Immunoprecipitation was carried out as described in [13] with minor modifications. Polyclonal antibodies raised against spinach D1 proteins were provided by Dr. Ikeuchi (Riken) [14]. For immunoprecipitation, thylakoid membranes from pulse-labelled chloroplasts were solubilized in 2% SDS and boiled for 2 min and then diluted 10-fold with 50 mM Tris-HCl (pH 7.5) buffer containing 0.15 M NaCl, 2 mM EDTA and 1% Nonidet P-40. After removing unsolubilized materials by microcentrifugation, antiserum was added to the sample and then the mixture was incubated on ice for 12 h. After the incubation, protein A–Sepharose was added to the mixture and the antigen–antibody complex was collected by centrifugation.

2.6. *Chemicals*

Chloramphenicol, protein A–Sepharose (CL4B), polyoxyethylene 10-tridecylether and adenosine 5′-triphosphate (magnesium salt) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and lysylendopeptidase was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). L-[^{35}S]Methionine (specific activity, ca. 10 mCi/ml, 1,000 Ci/mmol) was obtained from ICN Biochemicals Inc. (Irvine, CA, USA).

3. RESULTS AND DISCUSSION

3.1. *Protein synthesis by externally added ATP*

Fig. 1A shows fluorograms of radiolabelled proteins in thylakoids of pea chloroplasts incubated with [^{35}S]methionine under various conditions shown in the figure legend. By pulse labelling for 10 min in the light

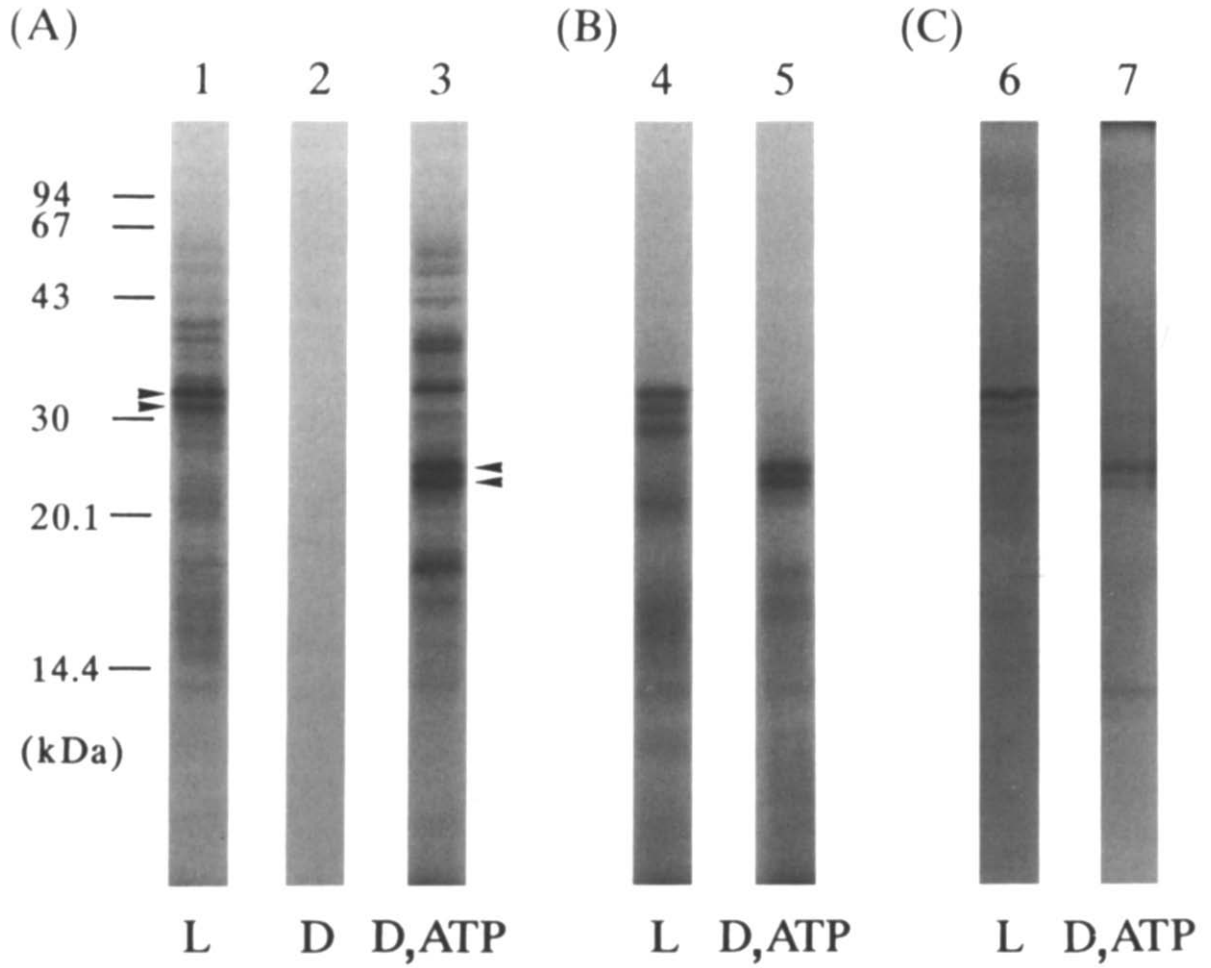

Fig. 1. Protein synthesis in isolated pea chloroplasts. Chloroplasts were pulse-labelled with L-[^{35}S]methionine for 10 min at 25°C in the light (lanes 1, 4 and 6) or in the dark (lanes 2, 3, 5 and 7). The reaction mixture contained MgATP in lanes 3, 5 and 7. In (B), thylakoid membranes from pulse-labelled chloroplasts were treated with lysylendopeptidase (300 μg · ml^{-1}) for 1 h at 25°C. Panel (C) indicates the immunological analysis of radiolabelled membrane proteins using antiserum against D1 protein after incubation with [^{35}S]methionine in the light (lane 6) or in the dark in the presence of ATP. Each lane contained 4 μg Chl except for lanes 6 and 7. Numbers on the left side stand for the molecular weight of marker proteins in kDa. See text for further explanations.

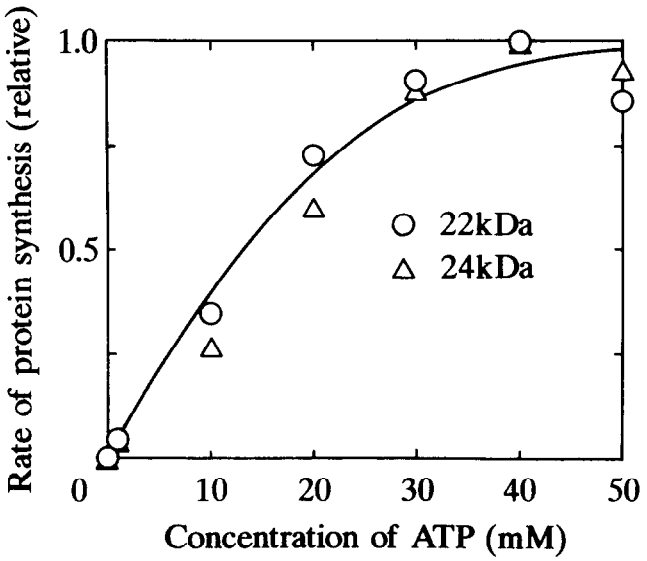

Fig. 2. Dependence on externally added ATP concentration of the accumulation of 22- and 24-kDa proteins. Isolated pea chloroplasts were pulse-labelled as described in Fig. 1 in the dark in the presence of various concentrations of MgATP (0–50 mM).

(lane 1), the precursor- and mature-sized D1 proteins of about 34 and 32 kDa, respectively, appeared as major bands (arrowheads), but not in the dark (lane 2), confirming that synthesis of the D1 protein in isolated pea chloroplasts is light dependent. Since our previous study indicated that translation of the D1 protein in isolated chloroplasts is regulated by the level of ATP in the stroma [11], MgATP, which is known to be imported into pea chloroplasts via translocators on the inner envelope [15,16], was added to the reaction mixture in the dark in the experiment shown in lane 3. Under this condition, however, neither the precursor- nor the mature-sized D1 protein was detected, although incorporation of [^{35}S]methionine to some chloroplastic proteins was observed as expected from [17]; a radiolabelled band on the fluorogram with slightly lower mobility than that of the precursor is not D1 protein since this band is sensitive to lysylendopeptidase (see later and lane 4 of Fig. 1B). Instead, under this condition, two distinct radiolabelled proteins of about 22 and 24 kDa (arrowheads) were observed as major bands. The accumulation of these two protein bands depended almost linearly on the amount of externally added MgATP at lower concentrations (0–20 mM) and then saturated at about 30 mM (Fig. 2). However, even at the saturating concentration of ATP (50 mM), no trace amount of D1 proteins appeared on the fluorogram. The formation of radioactive 22- and 24-kDa proteins just described was specific for ATP, but other nucleotides such as AMP, GTP, CTP, UTP or dTTP were ineffective in promoting the accumulation of these two bands (data not shown). On the other hand, externally added ADP supported formation of these two protein bands, although the efficiency was quite low.

The 22- and 24-kDa proteins accumulated by the addition of ATP in the dark were resistant to lysylendopeptidase, as in the case of precursor- and mature-sized D1 proteins synthesized in the light (Fig. 1B). This result may indicate that the 22- and 24-kDa proteins are translational intermediates and/or degradation products of the D1 protein, since the amino acid sequence of the protein deduced from the *psb*A gene of pea contains no lysine residue [18]. The evidence that these two proteins immunoprecipitated with specific antibodies raised against the D1 protein finally confirmed this interpretation (Fig. 1C). A partial proteolytic fingerprinting experiment using *Staphylococcus aureus* V8 protease further concluded that these two proteins correspond to the fragment containing the N-terminal half of the D1 protein based on the analysis described by Greenberg et al. [19] (data not shown).

3.2. *Pulse-chase experiment and polysome analysis*

In the experiment shown in Fig. 3, isolated pea chlo-

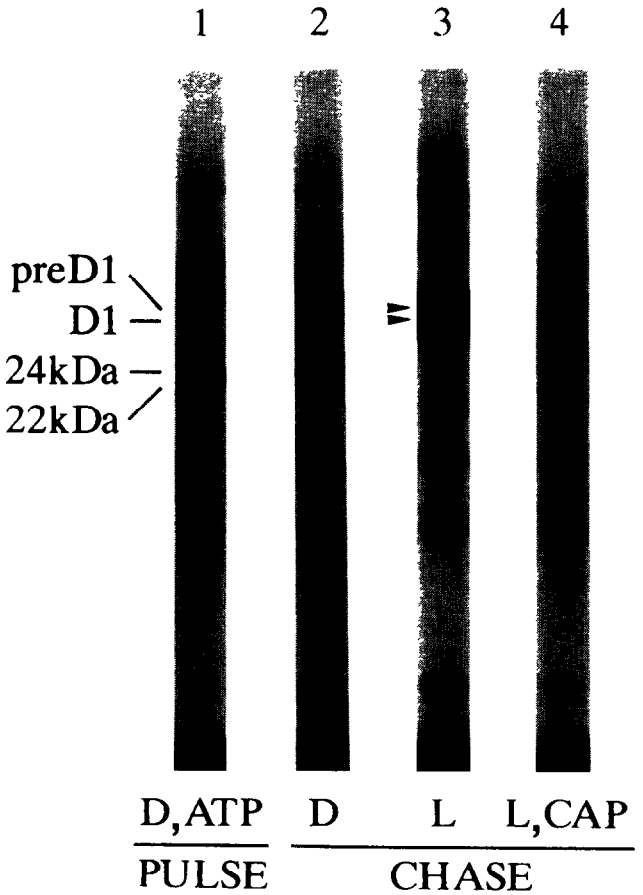

Fig. 3. Pulse-chase analysis of ATP-driven protein synthesis in isolated pea chloroplasts. Chloroplasts were pulse-labelled as described in Fig. 1 in the dark in the presence of 30 mM ATP (lane 1), and then chased in the presence of excess amounts of unlabelled methionine (40 mM) in the dark (lane 2) or in the light (lane 3), or in the presence of chloramphenicol (500 $\mu g \cdot ml^{-1}$) in the light (lane 4). Arrowheads indicate positions of the precursor- and mature-sized D1 protein. Each lane contained 4 μg Chl.

roplasts were pulse-labelled by incubating with L-[^{35}S]methionine at 25°C for 10 min in the presence of MgATP (30 mM), in the dark, and then chasing either in the presence of excess non-radiolabelled methionine (40 mM) or of chloramphenicol (500 $\mu g \cdot ml^{-1}$). By chasing in the presence of unlabelled methionine in the light (lane 3), but not in the dark (lane 2), the 22- and 24-kDa proteins which accumulated during the pulse-labelling period totally disappeared, concomitant with the appearance of two major bands of the precursor- and mature-sized D1 proteins (arrowheads); two prominent bands on the fluorogram of 40–60 kDa were sensitive to lysylendopeptidase and were thus attributed to components other than D1 protein (data not shown). The phenomenon just described can be interpreted to indicate that the 22- and 24-kDa components were converted into full-length D1 protein(s) during the light period, although there is a large discrepancy in the intensity of protein bands. However, this discrepancy can be explained by assuming formation of paused intermediates of the D1 protein of varying molecular size, in addition to the 22- and 24-kDa components, in the dark in the presence of ATP, which are 'read out' to full-length proteins during the chase in the light. This interpretation is supported by the experiment using chloramphenicol, a potent inhibitor of prokaryotic translation. By chasing in the light in the presence of chloramphenicol at 500 $\mu g \cdot ml^{-1}$, the appearance of the D1 protein band(s) was completely prevented (lane 4), although, under this condition, the 22- and 24-kDa proteins slightly decreased in amount, accompanied with the appearance of 26–28-kDa bands, possibly due to the incompleteness of the inhibition.

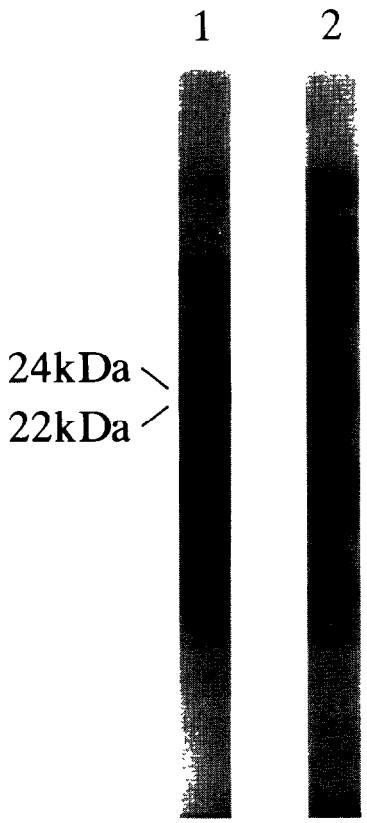

Fig. 4. Association of translational intermediate(s) with membrane polysomes. Membrane-bound polysomes were extracted from pea chloroplasts pulse-labelled in the presence of ATP in the dark as described in Fig. 1, and then pelleted through a 1.5 M sucrose cushion as described in [18]. Lane 1, thylakoid membranes; lane 2, polysome fraction.

In order to confirm the interpretation that the two proteins of 22 and 24 kDa that accumulated in the presence of ATP are polysome-bound paused translational intermediates of the D1 protein, thylakoid-bound polysomes were collected after pulse-labelling and then radiolabelled proteins in this fraction were analyzed by SDS-PAGE (Fig. 4). In the analysis, thylakoids were prepared by hypotonic treatment of chloroplasts and then partially solubilized with buffer containing 2% polyoxyethylene 10-tridecylether, as described in [20]. The polysomes thus solubilized from thylakoids, were collected by pelleting through a 1.5 M sucrose cushion, as in [20]. This experiment clearly demonstrated that proteins precipitated with ribosomes contain the 22-kDa component together with some other proteins (lane 2), supporting the above interpretation that the component is a translational intermediate. The interpretation, however, is not valid for the 24-kDa component since this protein was not found in the polysome fraction. One possible explanation of this result might be that, in case of the 24-kDa component, the association of the polypeptide to ribosomes is weak, and thus the component dissociates during preparation. However, another possibility is that the component is not a translational intermediate but a degradation product produced after synthesis due to instability of the nascent protein, as discussed by Mullet et al. [13].

The accumulation of translational intermediates of D1 protein has been reported by several authors [13,20,21]. Mullet and co-workers observed transient accumulation of several intermediates in barley chloroplasts in the process of greening [13,20]. They proposed that stable accumulation of full-length D1 protein requires newly synthesized Chl molecules [13], and pausing of ribosome facilitates co-translational binding of the pigment to D1 protein and aids integration of the protein into thylakoids [20]. On the other hand, Inagaki and Satoh [21] observed accumulation of an intermediate D1 protein of about 17.5 kDa which appears upon light–dark transition in spinach chloroplasts. Higher concentrations of ATP in stroma were proposed to be important for polypeptide elongation at the specific site on *psb*A mRNA. The 22-kDa intermediate observed in the present study, however, is clearly different in size, as well as in ATP dependency, from the 17.5-kDa component reported in [21]. The size of the translational intermediate reported here is rather similar to that reported by Kim et al. [20].

The immediate conclusion from this study is that the synthesis and/or the stable accumulation of the D1 protein requires factor(s) caused by illumination, in addition to ATP supply, in isolated pea chloroplasts. One possible consequence of illumination might be to supply Chl or other cofactors, as suggested by Kim et al. [20]. Another possibility, however, is that translation of the D1 protein is regulated at elongation steps by some protein factor(s) caused by illumination, which interacts with mRNA at the specific site, as in case of the synthesis of secretory proteins [22]. Formation of redox or energized state(s) in cloroplasts caused by illumination may also have relevance to the regulation. Evidently further analysis is needed to clarify the molecular mechanism of translational regulation of the D1 protein.

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