Evidence for SecA- and ΔpH-independent insertion of D1 into thylakoids

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Received 20 May 1995; revised version received 2 June 1995

Abstract Many nuclear-encoded proteins are targeted into chloroplast thylakoids by an azide sensitive Sec-related mechanism or by a ΔpH-driven mechanism. In this report, the requirements for the integration of chloroplast-encoded thylakoid proteins have been analysed in pulse-labeled intact chloroplasts. We show that the integration of the photosystem II reaction centre protein, D1, continues in the absence of a ΔpH and in the presence of azide. A range of other proteins are similarly targeted to thylakoids in the presence of azide, suggesting that the SecA-related mechanism is not widely used for the targeting of chloroplast-encoded proteins.

Key words: Chloroplast; Thylakoid; Protein transport; Photosynthesis; D1 protein

1. Introduction

The biogenesis of thylakoid proteins has emerged as a complex topic involving intraorganellar protein sorting on a large scale. Approximately 50% of the known thylakoid proteins are synthesised in the cytosol with N-terminal targeting signals, and the available evidence suggests that they are imported across the chloroplast envelope by a common mechanism. However, recent studies have shown that at least four pathways operate for the subsequent targeting of these proteins into or across the thylakoid membrane (reviewed in [1]). Plastocyanin (PC), the 33 kDa lumenal photosystem II protein (33K), and photosystem I subunit F are imported by means of bipartite presequences containing envelope transit and thylakoid transfer signals in tandem. After removal of the envelope transit signal in the stroma, the transfer signal directs transport across the thylakoid membrane by a *sec*-related mechanism which was probably inherited from the cyanobacterial progenitor of the chloroplast. This mechanism relies on ATP for the functioning of a stromal component of the transport machinery, SecA [2,3]. The 23 kDa and 16 kDa PSII proteins, PSII-T and PSI-N are likewise imported into the lumen by means of bipartite presequences, but after removal of the envelope transit signal these proteins are transported across the thylakoid membrane by a completely different pathway. The transport mechanism does not require either stromal factors or ATP, but is totally dependent on the thylakoidal ΔpH [4,5]. The integral membrane protein, LHCP, is synthesised with an envelope transit signal only, and hence integrates into the thylakoid membrane by means of information contained in the mature protein. The integration process requires nucleoside triphosphates and a stromal protein factor [6]. Finally, CF_0II, an integral component of the thylakoidal ATP synthase, is synthesised with a bipartite presequence yet appears to integrate into the thylakoid membrane by a spontaneous mechanism [7].

About 50% of the thylakoid membrane proteins in chloroplasts of green algae and higher plants are encoded by the chloroplast genome, but virtually nothing is known of the insertion mechanisms for these proteins. A large percentage of chloroplast-encoded proteins are synthesized on thylakoid-bound polysomes and are presumably cotranslationally inserted into the membrane (reviewed in [8]). However, little is known of the targeting of ribosomes to the thylakoid membrane, the binding site of the ribosomes on the thylakoid membrane or the components involved in translocation of the nascent chains. The synthesis of chloroplasts encoded proteins, on the other hand, has received a great deal of attention for many years (reviewed in [9]). Translation has been studied in intact cells of the green alga, *Chlamydomonas reinhardtii*, in intact leaves and in isolated chloroplasts. The multiple membrane spanning D1 reaction center protein of photosystem II has been central in many of these studies because it has a relatively short life time and is therefore one of the most strongly labelled proteins in vivo and in vitro [10–13]. The D1 protein, like many other membrane bound thylakoid proteins, is synthesized by membrane-bound polysomes [14,15]. It was shown that the apoprotein stability increased dramatically in presence of chlorophyll [16] but that protein translation initiation, elongation or termination of D1 and other chlorophyll binding proteins was independent upon the presence of chlorophyll [17]. Ribosomal pausing during translation of the D1 protein leading to distinct translation intermediates has been observed by several groups [13,18] and has been shown to be influenced by ATP levels [19]. This ribosomal pausing might be indicative of a stepwise translocation, possibly related to ATP binding and hydrolysis.

In this paper we have studied the effects of azide and uncouplers on the cotranslational insertion of chloroplast-encoded proteins, using an optimised procedure for translation in isolated chloroplasts which gives substantial incorporation rates over a reasonable period of time. The results suggest that D1, and other chloroplast-encoded proteins, are inserted by a mechanism distinct from those utilised for the translocation of proteins across the thylakoid membrane.

2. Materials and methods

Intact chloroplasts were isolated from seedlings of *Pisum sativum*, var. Feltham First. The age of seedling depended on the purpose of the experiment (see text). The basic protocol for pulse-labeling of chloroplast proteins was modified from that of Mullet et al. [20]. Incubations (600 μl) contained chloroplasts (200 μg/ml chlorophyll in HS buffer: 50 mM HEPES-KOH, pH 8.4; 330 mM sorbitol), 10 mM MgATP, 10 mM DTT and 40 μM amino acids minus methionine, and one of the following inhibitors: 5 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 10 mM Na azide, or 2 μM gramicidin. After preincubation for

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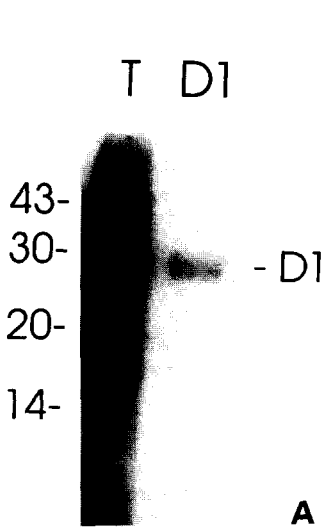
SSDI 0014-5793(95)00668-0

10 min at 25 °C, [^{35}S]methionine was added (6 μl containing 91 μCi). Incubation was for 5 min, after which cold methionine was added to 8.5 mM. Samples were then analysed at time-points given in the figures; 50 μl aliquots were mixed with 1 ml ice-cold HS buffer, the organelles were pelleted at 4000 × *g* for 1 min and lysed in 50 μl 10 mM HEPES-KOH, pH 8.0, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$. Thylakoid membranes were pelleted (5 min in a microcentrifuge), washed once and analysed by SDS-polyacrylamide gel electrophoresis.

3. Results and discussion

The synthesis and insertion of chloroplast-encoded thylakoid proteins can be studied by pulse-labeling intact chloroplasts with radiolabeled amino acids [15,20]. Chloroplasts from young seedlings synthesise a wide range of proteins, whereas those from relatively old seedlings synthesise predominantly D1, in order to continually replace this rapidly-turned over component of the PSII reaction centre (e.g. [12,13]). Little is known of the D1 insertion mechanism, and we therefore examined the possibility that the insertion process was mediated by components involved in the translocation/insertion of nuclear-encoded thylakoid proteins. Two of these pathways can be blocked by diagnostic inhibitors: the ΔpH-dependent translocase is totally inhibited by proton ionophores such as nigericin and CCCP, whereas the Sec-dependent pathway is inhibited by azide, which blocks the action of SecA [21,22]. The effects of these compounds on the insertion of chloroplast-encoded proteins were studied using in organello chloroplast translation assays, in which intact pea chloroplasts were preincubated with inhibitor for 10 min before addition of [^{35}S]methionine. The aim in this study was to examine the synthesis and insertion of nascent D1 chains, and a 10 min preincubation period should allow sufficient time for the completion of D1 chains which were already initiated before addition of inhibitor; in preliminary studies we have found that 95% of initiated D1 chains are completed within a 5 min chase (not shown). This is not surprising given that protein synthesis in *Escherichia coli* takes place at a rate of up to 900 residues per minute. In order to specifically examine the insertion of D1, our initial studies used chloroplasts from relatively old peas (12 day) in which most other chloroplast-encoded proteins are synthesised at low rates. Fig. 1A shows that D1 is the only significant labeled protein to be inserted into the thylakoid membrane following pulse-labeling with [^{35}S]methionine; the protein is specifically immunoprecipitated with antisera to spinach D1. At early time-points in pulse-chase experiments (Fig. 1B) the precursor protein is apparent, with the mature protein becoming the predominant form at later points. The processing of the C-terminus of pre-D1 takes place after termination of translation [23]. Fig. 1B also shows that 10 mM azide has little apparent effect on the efficiency of insertion of D1, although maturation is slowed down to some extent, perhaps due to a non-specific effect on the lumenal processing peptidase. We have quantitated the data from this (and other) assays and found no reduction in the amount of D1 associated with the thylakoids in the presence of azide. Furthermore, both the precursor and mature proteins are resistant to carbonate washing of the thylakoids, indicating that integration has taken place (data not shown). These results strongly suggest that SecA is not involved in the D1 insertion reaction.

Does azide affect the insertion of other proteins in the chloroplast? This question wasaddressed by pulse-labeling chloroplasts from young pea leaves, which actively synthesise a wider range of photosynthetic proteins. The data are shown in Fig. 2. In both the presence and absence of 10 mM azide, the key point is that the overall polypeptide profiles are very similar indeed. On the basis of published work [18], the bands indicated by arrowheads (which correspond to polypeptides of 24, 21, 19 and 15 kDa) are D1 synthesis intermediates which are paused during insertion, and the similarites of these profiles +/− azide is further evidence that SecA is not involved in the biogenesis of this protein. The faint band just above the pre-D1/D1 bands is D2 (K.J. van Wijk, in preparation) which is also inserted in the azide samples. The band of 55 kDa is believed to be the



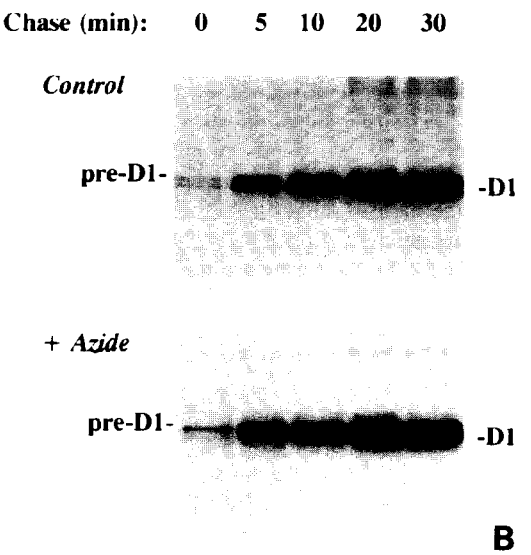


Fig. 1. Azide does not affect the insertion of D1 protein in pulse-labeled pea chloroplasts. (A) newly-synthesised proteins were pulse-labeled in pea chloroplasts from 12-day-old seedlings as detailed in section 2, and samples of labeled thylakoid membrane proteins analysed (lane T). A second sample was immunoprecipitated with antisera to spinach D1 (lane D1). Mobilities of molecular weight markers (in kDa) are given on the left. (B) chloroplasts were labeled for 5 min with [^{35}S]methionine in the presence or absence of 10 mM azide, and samples of thylakoid membranes analysed at the time points indicated. The precursor and mature forms of D1 are indicated.

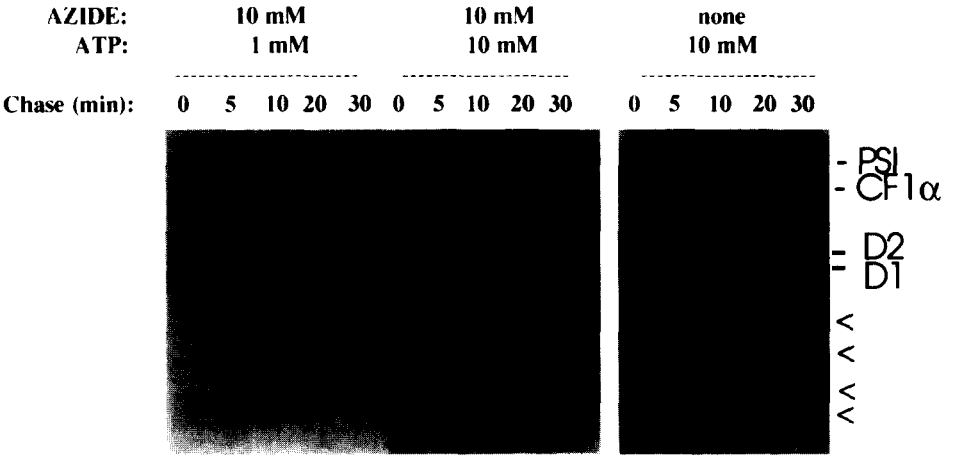


Fig. 2. Azide has no evident effects on the insertion of a range of thylakoid proteins. Chloroplasts were isolated from 8-day-old seedlings and pulse-labeled as in Fig. 1 in the presence of 10 mM MgATP and the presence/absence of 10 mM azide. An additional sample contained azide and 1 mM MgATP. Thylakoids were analysed after the labeling period at time points indicated. PSI, photosystem I proteins of 82–83 kDa; CF_1, α-subunit of the CF_1CF_0 ATPase. Arrowheads denote D1 synthesis intermediates (see text).

$CF_1\alpha$ subunit (K.J. van Wijk, in preparation) and the diffuse bands of 82–83 kDa represent the reaction center polypeptides of the PSI core [24]; all of these proteins are likewise inserted with high efficiency in the presence of azide.

One problem associated with the use of azide is that its effectiveness as a SecA inhibitor depends to some extent on the prevailing ATP concentration, possibly because azide andATP compete for the same site. In the presence of 1 mM ATP, inhibition is virtually complete whereas SecA is not fully blocked in the presence of 10 mM ATP [22]. Scanning of the labeled bands in the '10 mM ATP' panels in Fig. 2 shows no significant decline in the intensities of the major bands in the presence of azide (not shown), but we also carried out protein synthesis assays in the presence of 1 mM ATP/10 mM azide as a safeguard (left panel). The rate of protein synthesis drops dramatically at this concentration of ATP, but it is nevertheless evident that the overall polypeptide pattern shows no obvious changes.

Finally, we tested the possibility that insertion of D1 is mediated by the ΔpH-driven thylakoidal protein translocase, by carrying out assays in the presence of gramicidin or CCCP. The results (Fig. 3) are less clear because we found that both compounds drastically reduce the overall rate of protein synthesis in isolated chloroplasts (the synthesis of Rubisco large subunit in the stroma is similarly affected; not shown) and furthermore lead to the appearance of new bands of unknown origin. Nevertheless, D1 continues to be synthesised and inserted in the presence of both gramicidin and CCCP, and we conclude that insertion is not mediated by the ΔpH-driven translocase in the thylakoidmembrane. We can not determine whether the thylakoidal ΔpH stimulates D1 insertion, because a moderate inhibition of insertion rate would be difficult to detect under these conditions.

In summary, our data strongly suggest that D1 is not inserted by either the SecA- or ΔpH-dependent mechanisms involved in the translocation of numerous cytosolically-synthesised proteins into or across the thylakoid membrane. Furthermore, we can find no evidence for the involvement of SecA in the insertion of several other proteins, although we stress that only a minority of thylakoid proteins have been examined in this study, and others may well utilise the SecA- or ΔpH-dependent pathways. The data suggest that the development of in vitro insertion assays will be required to elucidate the integration mechanisms for the majority of chloroplast-encoded thylakoid proteins, because most of the other proteins are synthesised at prohibitively low rates.

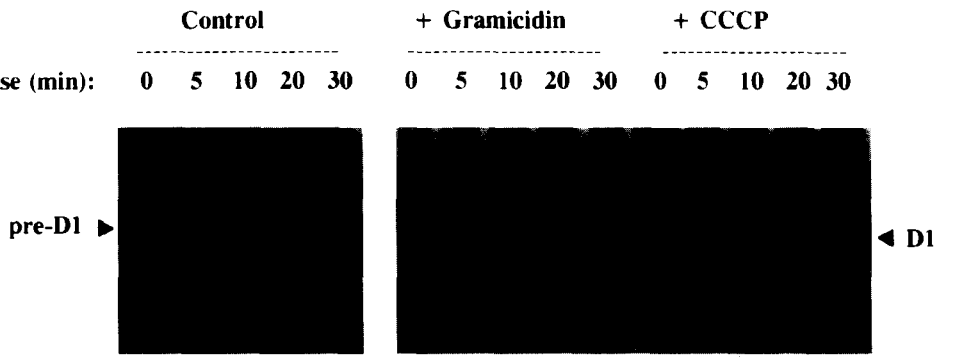


Fig. 3. A ΔpH is not essential for the insertion of D1. Chloroplasts from 12-day seedlings were labeled as in Fig. 1 in the absence of inhibitors (control) or in the presence of gramicidin or CCCP. Samples of thylakoid membranes were analysed; symbols as in Fig. 1.

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