Kinetic analysis of translocation into isolated chloroplasts of the purified ferredoxin precursor

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Time courses of the import into isolated chloroplasts of the purified ferredoxin precursor were measured at different protein concentrations. Analysis of the initial import rates indicates the presence of one saturable import system with an apparent K_m value of approximately 100 nM and a V_{max} corresponding to the uptake of approximately $2.5 \cdot 10^4$ precursor proteins per minute per chloroplast. We conclude that the in vitro observed activity of the chloroplast protein import machinery, functioning independently from cytosolic factors, would be enough to allow chloroplast development at at physiological rate.

Chloroplast protein import; Ferredoxin; Michaelis-Menten kinetics

1. INTRODUCTION

The majority of the proteins found in higher plant chloroplasts are encoded in the nucleus [1]. These proteins are synthesized in the cytosol as precursors. In the precursor the information for the post-translational chloroplast targeting and import is present in the transit sequence [2] that is cleaved off after translocation across the chloroplast envelope membranes [3]. Chloroplast import is proposed to be initiated by binding of the transit sequence to components of the outer membrane [4]. Binding [5] and complete translocation [6–8] require ATP hydrolysis. A limited amount of 1,500–3,500 binding sites on the outer envelope was found to be present [9]. Attempts to identify protein receptors of the import machinery [10–13] have so far yielded conflicting results [14]. In addition to protein receptors, envelope-bound heat-shock proteins [15] and envelope membrane lipids [16] are proposed to be involved in the translocation process.

At present, still very little is known at the molecular level about the import pathway of chloroplast precursor proteins. The determination of important characteristics like the affinity for precursors and uptake capacity of the import system requires the availability of purified radiolabelled precursors in sufficient amounts. We have previously reported the purification of the precursor of

the chloroplast protein ferredoxin (prefd) [17]. We have shown that this precursor is fully translocation-competent by itself and requires no cytosolic factors for its targeting and ATP-dependent transport into chloroplast [18]. We have used the purified ferredoxin precursor to analyze the kinetics of import in vitro. We give evidence for the presence of a saturable uptake system. Our results allow an evaluation of the in vitro uptake system. The results are discussed in the light of possible models of protein translocation.

2. MATERIALS AND METHODS

The purification of [35S]-labelled and unlabelled prefd, from an *Escherichia coli* strain overexpressing the precursor [17], and the modification of prefd by convalent attachment of [14C]acetamide to its five cysteine residues were performed as described [18]. The specific activity of the purified [35S]-labelled prefd was 500,000 dpm/μg and of the [14C]-labelled prefd 25,000 dpm/μg.

Intact chloroplasts were isolated from 9- to 11-day-old pea seedlings c.v. Feltham First [18,19]. Chlorophyll was assayed according to Bruinsma [20], protein was assayed according to Bradford [21] and chloroplasts were counted by phase-contrast microscopy. Surfacil (Pierce) and BSA were used to precoat Eppendorf cups used for the import experiments. Import incubations (final volume 150 μl) were prepared on ice. The import buffer consisted of 50 mM HEPES/KOH pH 8.0, 330 mM Sorbitol, 2 mM MgCl₂, 0.5 mM DTT, 200 μg/ml antipain and 2 mM MgATP. Precursors were added from concentrated stock solutions in 8 M urea. The final urea concentration of all reactions was adjusted to 200 mM. Urea was previously shown not to influence the import below 400 mM [17]. For each time point of a time course experiment a separate incubation was prepared. After addition of chloroplasts, 30 μg chlorophyll per incubation (which corresponds to 0.36 mg protein or $4.5 \cdot 10^7$ chloroplasts), the tubes were placed in a 25°C waterbath in the light. At indicated time points the incubations were transferred to an ice-water bath and 5 volumes of ice-cold 50 mM HEPES/KOH pH 8.0, 330 mM sorbitol containing 0.2

μM nigericin was added. The chloroplasts were then washed, protease-treated and re-isolated over 40% (v/v) Percoll step gradients essentially as described by Smeekens et al. [19]. The recovery of the chloroplasts from the incubations was determined by a protein assay [19,21]. The average recovery was found to be 74 ± 10%. Samples were analyzed by SDS- PAGE according to Laemmli [24] followed by fluorography. To obtain quantative data radioactive bands, corresponding to added precursor and imported mature sized protein, were excised, rehydrated and prepared for liquid scintillation counting in a 1:9 (v/v) mixture of Lumasolve and Lipoluma (Lumac, Belgium). The import efficiencies were calculated as described [17] and corrected for the chloroplast losses during the washing steps following incubation.

Time course data were fitted to the following first order reaction equation: $I = L \cdot (1 - e^{-kt})$. I is the percentage of added precursor that is imported and t is the time in minutes. The parameters L, the maximum amount of precursor that is imported, expressed by percentage of the added precursor, and k, the reaction constant, are obtained from the fitting procedure for each protein concentration. These parameters are used to calculate the initial uptake rates according to: $V = (P/C) \cdot k \cdot (L/100)$. In this equation V is the initial uptake rate (pmol·mg chloroplast protein^{-1}·min^{-1}). P is the amount of precursor added (pmol) to an import assay containing an amount of chloroplast protein equal to C (mg). Initial uptake rates, V, as a function of the concentration of added precursor, S, were fitted to the Michaelis–Menten equation $V = (V_{max} \cdot S)/(S + K_m)$ where V_{max} is the maximal uptake rate and K_m the Michaelis–Menten constant. Data were fitted by non-linear, least squares regression analysis using the program Enzfitter (Elsevier, Biosoft, Cambridge, UK).

3. RESULTS

Purified (^{35}S-labelled) prefd is fully translocation competent by itself and requires no cytosolic factors [18]. A modified precursor ferredoxin that was labelled by a modification of the cysteine residues with [^{14}C]acetamide in the mature sequence is in addition imported independently from co-factor assembly in the stroma [18]. At the low precursor concentrations ($\sim$ 40 nM) used and with a 20 min incubation time no difference was observed in the import efficiency between the parent precursor and this modified precursor [18]. However, it cannot be excluded that, at higher concentrations of added precursor, some process, perhaps related to the co-factor insertion, becomes limiting for the import rate. Therefore we used both labelling procedures of precursor ferredoxin for our analysis and the kinetics of translocation across the envelope were determined for the two types of preferredoxin independently.

Fig. 1 shows, as an example, the time courses of import of the acetamide modified precursor at four different concentrations. The curves show the expected saturation kinetics. A steep increase of the imported fraction with time is seen during the first minutes of the incubation, after a while a levelling off takes place. As is expected for saturation kinetics the initial increase of the imported fraction of precursor is much less at the higher precursor concentrations. Similar results were obtained with mixtures of ^{35}S-labelled prefd (65 nM) and increasing amount of unlabelled prefd (results not shown).

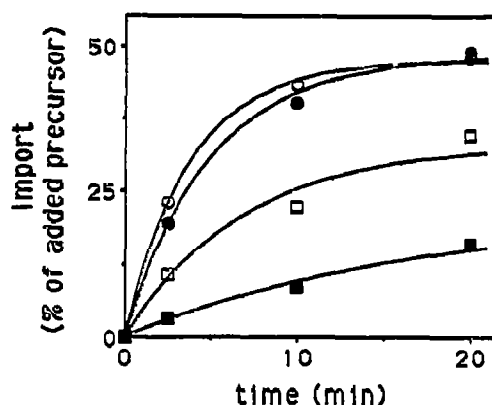For both precursors approximately 50% of the added

Fig. 1. Time course of the import of [^{14}C]acetamide-labelled prefd into isolated chloroplasts. The initial concentrations of added precursor were: ○, 10 nM; ●, 39 nM; □, 156 nM; ■, 778 nM. Data are expressed as the percentage of the added precursor that was imported by the chloroplasts. Each point is the average of two independent experiments. The curves drawn are obtained by fitting to a first-order rate equation.

prefd is imported after 20 min of incubation at the lower precursor concentrations. A similar result is obtained with in vitro (in a wheat-germ system) synthesized prefd (data not shown). Control experiments were performed where the import was blocked by nigericin treatment in the absence of ATP. Under these conditions the added precursors were nearly quantitatively recovered from the supernatant at all concentrations (not shown).

The time courses of import could be fitted to a first order rate equation (see Fig. 1). From the fitted parameters (L, the maximum percentage of added precursor that is imported and k, the reaction constant) the initial import rates for each concentration of added precursor were calculated (see section 2). Plots of the calculated initial import rates as a function of concentration for both precursors used are shown in Fig. 2. The lines drawn in Fig. 2 are determined by the parameters obtained by fitting the data points to the Michaelis–Menten rate equation. The obtained parameters for both precursors are given in Table I.

The Eadie–Hofstee plots (insets in Fig. 2) indicate the presence of a single saturable uptake system for both data sets. The kinetic parameters obtained for both precursors are very similar. The data obtained with the

Table I

Kinetic parameters of chloroplast import

Data set	K_m (nM)	V_{max} (pmol/mg protein/min)
Parent prefd	130 (± 70)	5.1 (± 0.9)
Acetylated prefd	70 (± 20)	3.9 (± 0.5)

The Michaelis–Menten equation was fitted to the data shown in Fig. 2. Standard errors are given between brackets.

A

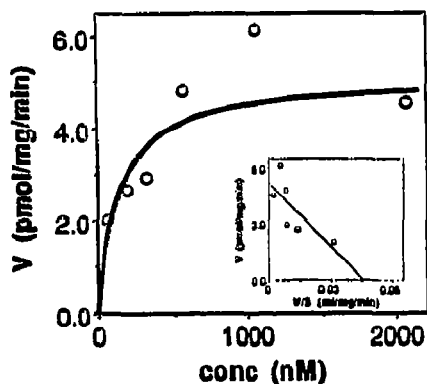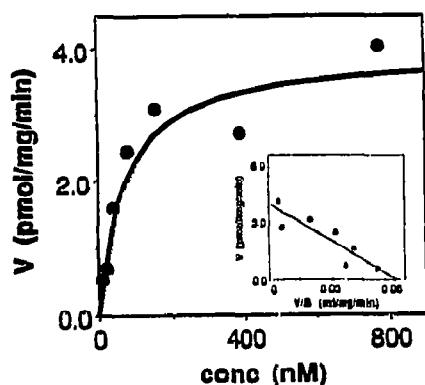

B

Fig. 2. Initial import rates as a function of concentration of added precursor. Panel A: parent precursor. Panel B: acetamide-modified precursor. Import rates (V) are expressed as pmol imported precursor per mg chloroplast protein per minute. The insets show an Eadie–Hofstee plot of the same data. The curves correspond to the fitted parameters shown in Table I.

acetylated prefd are more reliable, probably because the data points are better distributed. Kinetic parameters are preferably estimated from data obtained at concentrations ranging up to 5 × lower and 5 × higher than the K_m value [22].

From the average of the two data sets an apparent K_m (affinity constant) of 100 nM is obtained. In our preparations 1 mg of chloroplast protein corresponds to $1.25 \cdot 10^8$ chloroplasts. The average V_{max} thus corresponds to the uptake of approximately 22,000 precursor molecules per chloroplast per minute.

4. DISCUSSION

Our results are the first analysis of the kinetic parameters of chloroplast precursor protein import. At present, most of our knowledge on chloroplast import still comes from in vitro systems and therefore an evaluation of the kinetic parameters of in vitro protein import is valuable.

We have found a K_m value of approximately 100 nM.

A comparison with other data on chloroplast protein import and other transport systems can now be made. The K_m values of metabolite translocators of the chloroplast envelope for their substrates is clearly several orders of magnitude higher [25] and most likely completely different mechanisms are involved. Short (20 amino acids long) fragments of transit peptides exhibit half-maximum inhibition of the import into chloroplasts of in vitro synthesized precursors at 40 μM [26]. Larger peptides (30 amino acids long) already have a higher affinity, they exhibit half maximum inhibition of import at 0.5–1.0 μM peptide [27]. Our data are consistent with a precursor binding assay using in vitro synthesized precursors, where an association constant of 10 nM was obtained [9].

It is of interest to compare our data to those of the import of precursors into mitochondria. The import of several mitochondrial precursors was inhibited for 50% by a purified precursor form of the mitochondrial porin at concentrations of 50–90 nM [28]. The affinity that we observe in this paper for chloroplast import is thus in the same order of magnitude. We have used a purified precursor protein and therefore we can conclude that the affinity results from a direct recognition of the precursor by the translocation machinery at the chloroplast envelope. The import of a chloroplast protein that needs cytosolic factors to obtain import competence [32] could be mediated by a very similar mechanism. This we infer from an analogy of the mitochondrial protein import process, which can also take place independently from cytosolic factors [29,30]. In some cases cytosolic 'anti-folding' proteins are needed but these apparently have no role in targeting [31].

In this study we obtained a V_{max} of approximately 4.5 pmol per mg chloroplast protein per minute. Chloroplasts import 60–70% of their polypeptides [1]. When we assume that all proteins are imported with similar efficiency and we take as an average precursor size 50 kDa, then we calculate that at the V_{max} it would take ~2 days for the chloroplast to import the proteins needed for a division cycle. An indication for the division time of a chloroplast in growing pea leaves is the chlorophyll doubling time which is approximately 2 days [33]. We therefore conclude that the in vitro uptake rates are in reasonable proportion to what can be expected in vivo. This is an important conclusion because it validates the use of in vitro chloroplast import system.

In a previous study Friedman and Keegstra [9] have analyzed the number of binding sites for precursors present on the chloroplast envelope, using the in vitro synthesized precursor of the small subunit of Rubisco enzyme, and came to a number of approximately 2,000 binding sites per chloroplast. From competition studies employing synthetic peptides that represented parts of transit sequences [26,27], it can be concluded that prefd and the precursor of the small subunit of Rubisco use the same transport machinery. The number of prefd-

binding sites should therefore be comparable to those for the small subunit precursor. The maximum amount of purified prefd specifically bound to chloroplasts was approximately 2,000 per chloroplast at 100 μM ATP, which was found to be the optimal ATP concentration for binding [18]. Assuming that the number of actual translocation sites in the same as the number of binding sites on the outside of the envelope, we calculate a turn-over number of ~10 precursors translocated per binding site per minute. The rate of elongation during translocation on eukaryotic ribosomes is approximately 10 amino acids per second [23], prefd is 145 amino acids in size and therefore the times required for translation and translocation are in the same order of magnitude.

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