Import inhibition of poly(His) containing chloroplast precursor proteins by Ni^{2+} ions

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Abstract The precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase (pSS) and a modified pSS containing a C-terminal hexahistidyl tail (pSS(His)$_6$) were imported into isolated *Chlamydomonas* chloroplasts with comparable efficiency. In the presence of Ni^{2+} ions the import of pSS(His)$_6$ was inhibited and the precursor bound to the envelope remained protease sensitive, while import of pSS was not affected. Addition of an excess of L-histidine suppressed the inhibition demonstrating that the hexahistidyl-Ni^{2+} complex was responsible for import inhibition. Inhibition could be observed between about 0.5 and 10 mM Ni^{2+}, depending on the total protein content in the assay. Import incompetent Ni^{2+}-precursor complexes can be used to study early events in chloroplast protein import.

Key words: Chloroplast protein import; Inhibition by nickel ions; *Chlamydomonas reinhardii*

1. Introduction

Most proteins of chloroplasts as well as of mitochondria are imported into the organelle from the cytoplasm where they are synthesized as precursor proteins containing an N-terminal transit peptide [1]. Proteins of the envelope membrane are involved in import as receptors and components of the translocation apparatus [2,3]. Our knowledge of the chloroplast import apparatus is based mainly on the biochemical analysis of so called translocation complexes. As import is a rather fast process such analysis will be most successful when importation of in vitro synthesized, labeled precursor proteins into isolated intact chloroplasts is arrested at an intermediate stage, thereby accumulating translocation complexes. Such an accumulation might be induced by precursor proteins bearing a C-terminal domain that can be transformed into a rigid or bulky structure which is sterically hindered to pass the membrane pore. Import of precursors into mitochondria is blocked e.g. by binding of antibodies to the C-terminal domain [4], or by binding of the substrate analogue methotrexate to a DHFR domain attached to the C-terminus of the precursor protein. This inhibition is probably due to hindered unfolding of the import protein, i.e. stabilization of a rigid conformation [5,6].

Similar attempts have been made for the analysis of components of the import apparatus in chloroplast envelopes, however, with contradictory results. On the one hand the import of a transit peptide-DHFR fusion protein is not blocked by the presence of methotrexate in higher plants, pointing to a strong unfolding activity associated with chloroplast envelope membranes [7,8]. Also the protease sensitivity of a chimeric precursor protein containing the ricin A chain, when bound to chloroplasts, points to an unfolding activity of the chloroplast envelope [9]. On the other hand binding of specific antibodies to the DHFR moiety or to the protein A moiety of a precursor-protein A fusion protein arrested import [10,11]. Also consistent with an unfolding/refolding model is the inhibition by glyphosate of the import of the precursor for 5-enolpyruvylshikimate-3-phosphate synthase in the presence of its substrate [12].

Not much attention has so far been paid to the protein import into chloroplasts of *Chlamydomonas reinhardii*, although this unicellular green alga is widely used as a model organism to investigate various cell biological problems of green plants [13]. This neglect is probably due to the tedious isolation procedure for chloroplasts and to the fact that precursor proteins from higher plants are not imported or not correctly processed by *Chlamydomonas* chloroplasts, and vice versa [14].

A very common affinity tag introduced into fusion proteins is the hexahistidyl sequence simplifying the isolation of proteins via its high affinity to nickel ions bound to a chelating resin [15]. This rather small sequence, however, was never used for formation of a rigid structure to arrest translocation. Here, we present evidence that the import into chloroplasts of *Chlamydomonas* of a fusion precursor protein containing a hexahistidyl tail at the C-terminus is hindered by the addition of nickel ions.

2. Materials and methods

2.1. Construction of plasmid

The plasmid pSSpSP64 containing the cDNA of the gene *rbcS2*, which codes for one of the two forms of pSS present in *Chlamydomonas reinhardii*, was a generous gift of Dr. M.L. Mishkind. The two pSS in *Chlamydomonas* differ only in a few amino acids in the mature protein but not in the transit sequence [16].

The 0.8 kb *Pst*I fragment of plasmid pSSpSP64 was cloned into the *Pst*I site of the commercial expression vector pQE-3 (Qiagen GmbH, Hilden, Germany). The recombinant plasmid was cut with *Eco*47III and *Eco*RI and the 0.8 kb fragment was isolated from an agarose gel. The plasmid pQE-12 (Qiagen GmbH) which contained the hexahistidyl-coding sequence was cut with *Bgl*II, blunted with Klenow enzyme and cut with *Eco*RI. The 3.4 kb fragment was ligated with the 0.8 kb fragment of the *Eco*47III-*Eco*RI restriction. The 0.8 kb *Pvu*II-*Pst*I fragment of the resulting plasmid was ligated with the 2.9 kb *Pvu*II-*Pst*I fragment of plasmid pSSpSP64, finally leading to plasmid pSSHispSP64. The in vitro transcript obtained from this plasmid served to in vitro synthesize precursor proteins of which the last

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Abbreviations: DHFR, dihydrofolate reductase; EDTA, ethylenediamine tetraacetate; (His)$_6$, hexahistidyl peptide; hsp70, 70 kD heat shock protein; pSS, precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase; pSS(His)$_6$, pSS with C-terminal hexahistidyl tail

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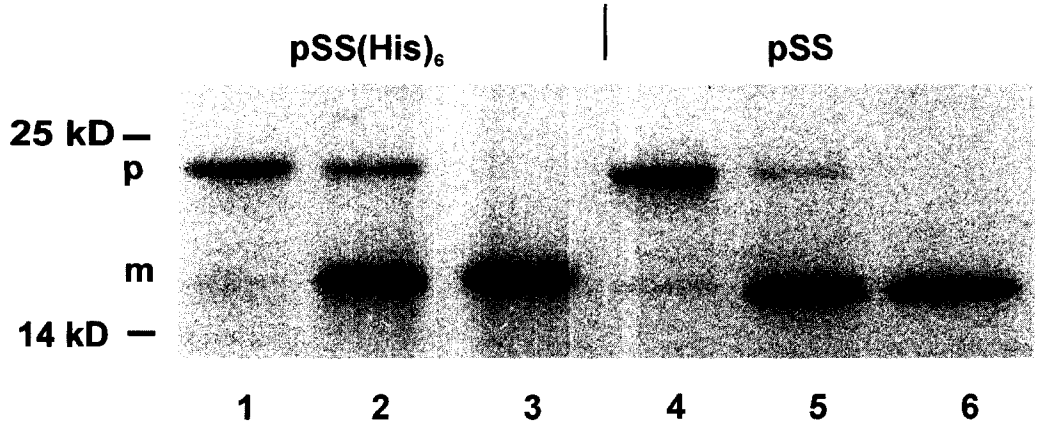

Fig. 1. Import of in vitro synthesized precursor protein into isolated chloroplasts. Fluorogram of a 10–20% SDS-polyacrylamide gel with electrophoretically separated incubation mixtures. Each lane was loaded with an equal amount of chlorophyll a+b. Isolated chloroplasts were incubated with in vitro synthesized, radiolabeled pSS (lanes 1–3) or pSS(His)$_6$ (lanes 4–6). After incubation the chloroplasts were centrifuged through Percoll and washed. Lanes 1 and 4: Incubation in the dark without addition of ATP. Lanes 2 and 5: Incubation in the light with externally added ATP. Lanes 3 and 6: Chloroplasts were treated with thermolysin after incubation. p: precursor protein; m: mature protein.

amino acid (val) was replaced by the hexahistidyl tail [17]. All operations were essentially done according to Maniatis et al. [18].

2.2. Preparation of precursor proteins

The in vitro transcription of the genes was performed in 50 μl assays as described earlier [19]. For the in vitro translation a wheat germ extract was prepared according to Mishkind et al. [20]. To synthesize radiolabeled precursor proteins each 150 μl of translation mixture contained 30 μl wheat germ extract; 2 mM ATP; 0.4 mM GTP; 25 μM nonradioactive amino acids (all but methionine); 60 μCi [^{35}S]methionine, 2 mM dithiothreitol; 40 μM spermine; 24 mM HEPES buffer, pH 7.5; 1 mM Mg-acetate; 60 mM K-acetate and 5 μl mRNA (about 2 μg). The translation was carried out during 40 min at 30°C.

2.3. Preparation of chloroplasts

Chloroplasts were isolated from synchronized cultures of *Chlamydomonas reinhardii cw-15* (stock CC-277 from the Chlamydomonas Genetics Center, Duke University, Durham, NC, USA) as described previously [21].

2.4. Import assay

Chloroplasts (20 μl, 2–4×10^8/ml) and 10 μl translation mixture containing the radiolabeled precursor proteins were incubated in the final volume of 100 μl import buffer (250 mM sorbitol; 35 mM HEPES buffer, pH 7.8) with an excess of 4 mM ATP for 30 min at 25°C in white light. The chloroplasts were reisolated by centrifugation through 20% Percoll in import buffer and carefully washed with import buffer. Blockage of import was achieved by addition of 1 mM $NiSO_4$ to the import assay. The reisolated chloroplasts were solubilized and the proteins analyzed by SDS-polyacrylamide gel electrophoresis [24]. For quantitative determination of the radioactivity in the protein bands the dried gel was scanned by use of a PhosphorImager (Molecular Dynamics).

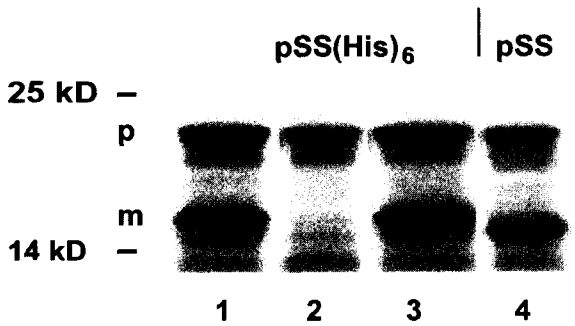

Fig. 2. Inhibition of pSS(His)$_6$ import by Ni^{2+} ions. Fluorogram of a 10–20% polyacrylamide gel with electrophoretically separated incubation mixtures. Isolated chloroplasts were incubated in the light and in the presence of ATP with in vitro synthesized, radiolabeled pSS(His)$_6$ (lanes 1–3) or pSS (lane 4). Additions: 1 mM Ni^{2+} to the samples of lanes 2–4 prior to incubation. Lane 3: After half the incubation time, i.e. 15 min, 5 mM L-histidine was added. Chloroplasts were reisolated by centrifugation through Percoll and washed. Each lane was loaded with an equal amount of chlorophyll a+b. p: precursor protein; m: mature protein.

3. Results and discussion

Starting with the cloned gene of the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase (pSS) an expression vector was constructed coding for a fusion protein composed of pSS with the last amino acid replaced by a C-terminal hexahistidyl tail (pSS(His)$_6$). The genes for the native protein pSS and for the fusion protein pSS(His)$_6$ were transcribed in vitro and the precursor proteins synthesized and labeled with [^{35}S]methionine in a wheat germ translation system. Driven by light, the labeled precursors were imported into isolated chloroplasts of *Chlamydomonas reinhardii* and the proteins electrophoretically analyzed. Fig. 1 demonstrates that the fusion protein pSS(His)$_6$ was processed in isolated chloroplasts to about the same degree as the non-modified pSS. With the very active chloroplast preparation of Fig. 1 the radioactivity of the mature proteins SS(His)$_6$ and SS were 83% (lane 2) and 92% (lane 5), respectively (100% = mature and precursor protein). With chloroplasts of average activity 55% and 57% (Fig. 2) or 72% and 63% (Fig. 3) of the precursors were processed. To prove that the processed proteins were internalized, the chloroplasts were treated with thermolysin after the import reaction (Fig. 1). This protease is known to degrade and remove the external precursor proteins, but not the mature processed proteins protected within the chloroplast. In spite of the poly(His)$_6$ tail the in vitro synthesized chimeric protein was imported and processed with high efficiency by isolated chloroplasts.

However, an important difference between the two precursor proteins with and without poly(His)$_6$ tail became obvious when the import reaction was performed in the presence of nickel ions (Ni^{2+}). At a concentration of 1 mM Ni^{2+} the import of pSS(His)$_6$ was inhibited while the import of pSS was not affected (Fig. 2, lanes 2, 4). The precursor protein

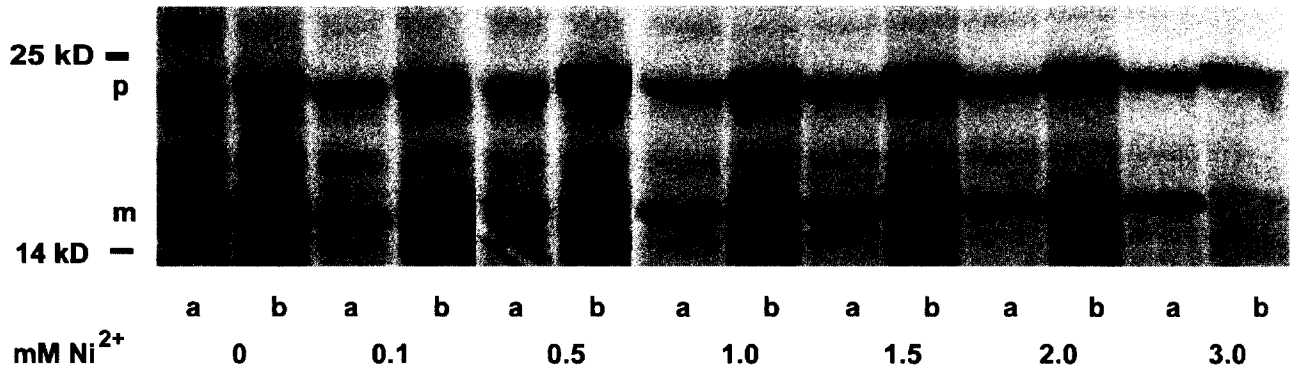

Fig. 3. Effect of increasing Ni^{2+} concentrations on the chloroplast import of pSS and pSS(His)$_6$. Composite picture of three fluorograms of a 10–20% polyacrylamide gel with electrophoretically separated incubation mixtures. Each lane was loaded with an equal amount of chlorophyll a+b. Isolated chloroplasts were incubated in the presence of the indicated Ni^{2+} concentrations with in vitro synthesized, radiolabeled pSS (lanes a) or pSS(His)$_6$ (lanes b). p: precursor protein; m: mature protein.

pSS(His)$_6$ blocked by Ni^{2+} and adsorbed at the envelope surface was completely thermolysin sensitive (data not shown). If indeed metal ions were responsible for the import inhibition, addition of Ni^{2+} binding agents, such as EDTA or histidine, to the reaction mixture should have an antagonistic effect. While addition of 5 mM L-histidine could indeed revert the import inhibition (Fig. 2, lane 3) the addition of 5 mM EDTA had only a slight effect (not shown). The import reactions per se of pSS and of pSS(His)$_6$ were not impaired, either by EDTA or by L-histidine. Therefore, this inhibition by Ni^{2+} is clearly different from the previously described effect of the oxidizing $CuCl_2$ on protein import [22].

The import inhibition of pSS(His)$_6$ by nickel ions could only be observed in a narrow concentration range between about 0.5 and 10 mM Ni^{2+}. The limits could not be determined precisely. As proteins generally have an inherent binding capacity for heavy metals, and as in different experiments the amount of protein in the assays varied depending on the quality of the chloroplast preparation and of the in vitro translation system, the Ni^{2+} concentration available for complex formation with pSS(His)$_6$ probably varied to a considerable degree from experiment to experiment.

Nevertheless, Fig. 3 shows the processing and import activity of intact chloroplasts for pSS and pSS(His)$_6$ as a function of low Ni^{2+} concentrations in the assay. Between 0.5 and 1 mM Ni^{2+} the import of pSS(His)$_6$ became increasingly inhibited as shown by the disappearance of the distinct band of mature SS(His)$_6$. In contrast, the import of pSS was much less affected by Ni^{2+}, demonstrating that the chloroplasts were still active in the presence of even 3 mM Ni^{2+}. The quantitative evaluation of this experiment by PhosphorImager is shown in Fig. 4. Even under Ni^{2+} inhibition a background level of mature SS(His)$_6$ was formed. It is not yet clear whether this reflects an incomplete import inhibition or more likely the processing of some precursor by a stromal peptidase released from broken chloroplasts during the incubation. In experiments with an additional thermolysin treatment of the assays this background level was less pronounced (data not shown).

To explain the inhibition of protein import by formation of complexes between the import protein and a ligand, the idea was proposed – as a working hypothesis – that an import protein with rigid conformation will mechanically not be able to pass through the import pore [5]. The envelope, however, equipped with a membrane bound chaperonin of the *hsp70* class [23,24], contains an unfolding capacity. Weak conformational forces in an import protein will be compensated when the precursor protein binds to the receptor, hereby facilitating the translocation of the protein [7]. Yet a rigid conformation stabilized by strong forces cannot be compensated. Such a protein remains adsorbed or stuck firmly as intermediate import complex in the envelope. Table 1 shows that the affinity of Ni^{2+} is highest to an excess of monomeric L-histidine and decreases from poly(His)$_6$ to EDTA. This could explain that in contrast to L-histidine EDTA was not able to suppress import inhibition. Furthermore, the binding constant of methotrexate to DHFR is lower than that of Ni^{2+} to (His)$_6$. Eventually the unfolding capacity of the chloroplast

Table 1
Binding constants related to complexes of the binding domain in chimeric import proteins and ligands

Ligand	Binding domain	Binding constant	Reference
Ni^{2+}	L-His	4.6×10^{8} M^{-1} (K_1)	[25]
		3.3×10^{15} M^{-1} (β_2)	[25]
Ni^{2+}	-(His)$_6$	1×10^{13} M^{-1}	[26]
Ni^{2+}	Histidylhistidine	3.1×10^{10} M^{-1} (β_2)	[27]
Ni^{2+}	$EDTA^{3-}$	3.6×10^{11} M^{-1}	[27]
Ni^{2+}	Imidazole	1×10^{3} M^{-1} (K_1)	[25]
		5×10^{10} M^{-1} (β_5)	[25]
Ni^{2+}	ATP	1×10^{5} M^{-1}	[27]
Methotrexate	DHFR	3.2×10^{10} M^{-1}	[27]

$K_1 = (Me{\cdot}L)/(Me)(L)$; $\beta_n = (Me{\cdot}L_n)/(Me)(L)^n$ and applies to the equation $Me+n{\cdot}L = Me{\cdot}L_n$, where Me = metal ion, L = ligand.

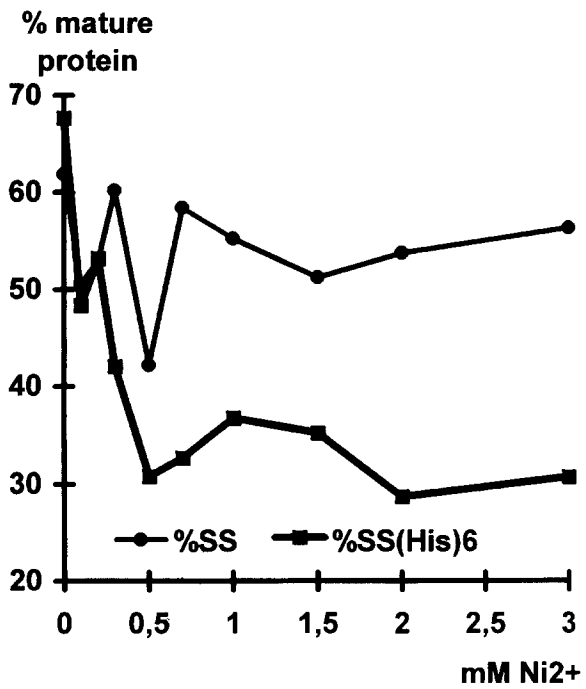

Fig. 4. Quantitative estimation of the inhibition of pSS(His)$_6$ import into isolated chloroplasts at increasing Ni^{2+} concentration. The amount of radioactivity in the mature and precursor protein band of Fig. 3 was measured using a PhosphorImager. The amount of mature protein is represented in percent of the sum of mature and precursor protein.

envelope membrane [8,9] is able to discriminate between the two binding constants, thereby explaining why the import of (His)$_6$-containing precursor protein is inhibited by Ni^{2+}, while DHFR fusion proteins are still imported in the presence of methotrexate. To further investigate this point we currently produce a precursor fusion protein with two C-terminal additions: a DHFR sequence followed by a hexahistidyl peptide.

This construct will allow us to concomitantly test the blocking capacities of methotrexate and Ni^{2+} ions in *Chlamydomonas reinhardii*.

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