Copper chloride, an inhibitor of protein import into chloroplasts

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Abstract **We have used the oxidant $CuCl_2$ to study its effect on precursor protein import into chloroplasts and on the components involved. $CuCl_2$ reversibly oxidizes thiol groups, which in turn, can form disulfide bridges. Concentrations of 40 μM $CuCl_2$ almost completely inhibit precursor protein binding and subsequent translocation into chloroplasts. This inhibitory effect is reversible by a dithiothreitol treatment. Disulfide bridges, which form upon oxidation by $CuCl_2$, are build up intramolecular and intermolecular, if the thiol groups are in close vicinity to each other. $CuCl_2$ can thus be used as a thiol cleavable crosslinker without an additional spacer distance between the two targets. When purified outer envelope membranes were treated with $CuCl_2$, a hetero oligomeric complex is detected, consisting of OEP86, OEP75 and OEP34, indicating the close vicinity and protein-protein interaction between polypeptides in situ, which are involved in protein translocation into chloroplasts.**

Key words: *Pisum sativum* L.; Precursor protein; Crosslinking; Copper chloride; Protein import; Chloroplast

1. Introduction

Most chloroplast proteins are nuclear coded, synthesized in the cytosol as higher molecular weight precursors and posttranslationally imported into the organelles [1,2]. The proteins of the outer chloroplast envelope involved in this process are organized as a membrane protein complex, which can be isolated as a functional protein import unit [3]. Three constituents of this import complex have recently been identified, namely OEP86, a putative part of the precursor receptor unit [4,5], OEP75, a component of the translocation pore [5,6] and OEP34, a new type of GTP binding protein with possible regulatory function [7,8,9].

The identification of components of the protein import machinery in chloroplasts now raises the questions, which components interact with each other and how might this interaction occur. An understanding of this problem will help to describe the mechanisms and series of events involved in precursor protein translocation. The sulfhydryl modifying agent *N*-ethylmaleimide (NEM) inhibits binding and translocation of preSSU into chloroplasts [10]. A stimulatory effect of DTT on precursor protein import into chloroplasts in vitro was also described [11], indicating, that thiol-groups are necessary for a productive import process. We choose the thiol oxidizing agent $CuCl_2$ [12,13] to study the effect of thiol groups in translocation. $CuCl_2$ can be used simultaneously as a crosslinking reagent, which can form intermolecular disulfide bridges during the course of thiol-oxidation without an additional spacer arm. Polypeptides crosslinked by this method should therefore be in very close proximity to each other. We report here, that $CuCl_2$ inhibits preSSU binding and hence import in a reversible manner. Furthermore, OEP86, OEP75 and OEP34 are covalently linked by disulfide bridges upon $CuCl_2$ treatment, indicating their close physical interaction in situ

2. Materials and methods

2.1. Precursor binding and translocation

PreSSU binding and import reactions were done using intact pea chloroplasts [14] equivalent to 15 μg chlorophyll in an 100 μl reaction volume in 330 mM sorbitol, 50 mM HEPES-KOH pH 7.6, 3 mM $MgSO_4$, 10 mM $NaHCO_3$, 20 mM K-gluconate, 2% BSA and reticulocyte lysate containing preSSU [15]. Conditions, which preferentially established binding or import conditions used 50 μM or 3 mM ATP, respectively. Reactions were carried out for 5 min at 25°C under dim green safety lights. Organelles were repurified after completion of the experiment by centrifugation through a 40% Percoll (Pharmacia, Sweden) cushion and washed once prior to SDS-PAGE and fluorography [14]. Intact chloroplasts were pretreated with the protease thermolysin at a protease concentration of 750 μg per mg chlorophyll [16,17] and repurified prior to further use.

2.2. Treatment with $CuCl_2$

Chloroplasts were treated with the indicated amounts of $CuCl_2$ for 5 min at 4°C in 330 mM sorbitol, 3 mM $MgSO_4$, 50 mM HEPES-KOH pH 7.6. In some cases $CuCl_2$ treated chloroplasts were subjected to an additional treatment with DTT (1 mM, 4°C, 5 min). $CuCl_2$ treated chloroplasts were recovered by centrifugation before further experimentation.

Purified pea chloroplasts outer envelope membranes [18] equivalent to 25 μg protein were treated with 1 mM $CuCl_2$ at 4°C for 15 min in 25 mM HEPES-KOH, pH 7.6, 3 mM $MgCl_2$ in a final volume of 100 μl. Membranes were recovered by centrifugation (250,000 × *g*, 10 min, 4°C) and analysed further.

2.3. SDS-PAGE and Western-blotting

SDS-PAGE was carried out as in [19] with the following modifications. SDS was omitted from the stacking and the separation gel. Mercaptoethanol (EtSH) was omitted from the sample buffer, when indicated (non reducing conditions). Samples were not heated prior to electrophoresis. In some cases polypeptides were eluted from polyacrylamide gel slices after staining in Coomassie brilliant blue. Gel slices were incubated in sample buffer in the presence of EtSH, heated at 95°C for 3 min and introduced into a well of a second polyacrylamide gel. The well was filled with sample buffer and electrophoresis carried out as above. To obtain good elution rates it is useful to omit acetic acid from the staining solution.

Polypeptides were transferred to nitrocellulose filters using a semi-dry blotting procedure (Pharmacia, according to the manufacturer's recommendations). The filters were treated and immunodecorated with antisera as described [20]. An alkaline phosphatase stain in the presence of 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue-tetrazolium was used for detection.

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Abbreviations: LSU, SSU: large, small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase; preSSU: precursor form of SSU; EtSH: mercaptoethanol; NEM, *N*-ethylmaleimide; OEP: outer envelope protein.

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3. Results and discussion

A couple of reports in recent years have indicated, that the redox-status of thiol groups might be important for a productive precursor protein import process into chloroplasts. It was demonstrated [10], that the thiol modifying agent NEM lowered the affinity for preSSU binding to pea chloroplasts and inhibited import. Pilon et al. [11] showed, that a pretreatment of chloroplasts with the reductant DTT stimulated import efficiency of purified preferredoxin two- to threefold, indicating that thiol groups have an essential function in this process. $CuCl_2$ can be used to oxidize thiol groups [12,13], which can result in the formation of disulfide bridges, if the thiol groups are in close vicinity to each other. We thus wanted to know, whether $CuCl_2$ inhibits import of preSSU, a typical chloroplast destined precursor protein. As shown in Fig. 1 import of preSSU into $CuCl_2$ pretreated chloroplasts is completely inhibited under conditions, which favour translocation, i.e. in the presence of 3 mM ATP(Fig. 1, lanes 9–12). The inhibitory effect of $CuCl_2$ can be reversed, if chloroplasts are incubated with 1 mM DTT after completion of the treatment with 0.1 mM $CuCl_2$ (see section 2). The import yield is comparable to control levels (compare Fig. 1, lanes 9, 11). A DTT treatment of intact chloroplasts had no obvious effect on the yield of preSSU import (Fig. 1, lane 12). Together these data indicate, that $CuCl_2$ exerts a specific and reversible inhibitory effect on preSSU import into chloroplasts.

To further analyse the inhibitory effect of $CuCl_2$ preSSU import was carried out at 50 μM ATP, conditions, which in our hands allow to observe preferentially binding but also some translocation. Binding of preSSU is greatly reduced in $CuCl_2$ pretreated chloroplasts and complete translocation is again not detected (Fig. 1, lanes 1, 2). A treatment with DTT brings binding of preSSU back to control levels (Fig. 1, lanes 2, 3). The chloroplasts surface bound preSSU is detected under μM ATP concentration in an environment, which partially protects the precursor from protease degradation by exogenous protease. These translocation intermediates [14] are termed deg 1–4 and can be used to monitor the movement of the precursor through the translocation apparatus of chloroplasts [3,21]. Therefore chloroplasts were treated with the protease thermolysin after binding of preSSU to the organelles to determine the exact

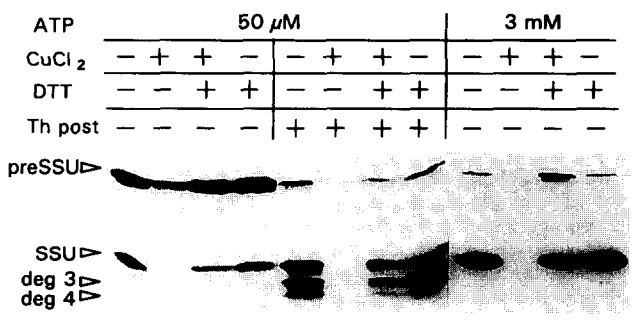

Fig. 1. Precursor protein import into chloroplasts is inhibited by $CuCl_2$. Intact pea chloroplasts were either not treated or treated with 100 μM $CuCl_2$, repurified and subsequently incubated with ^{35}S-labelled preSSU in the presence of 50 μM or 3 mM ATP, respectively. When indicated $CuCl_2$ treated chloroplasts where subsequently subjected to 1 mM DTT, prior to incubation with preSSU. After completion of the translocation experiments organelles were treated with the protease thermolysin (Th_{post}), when indicated. A fluorogram is shown.

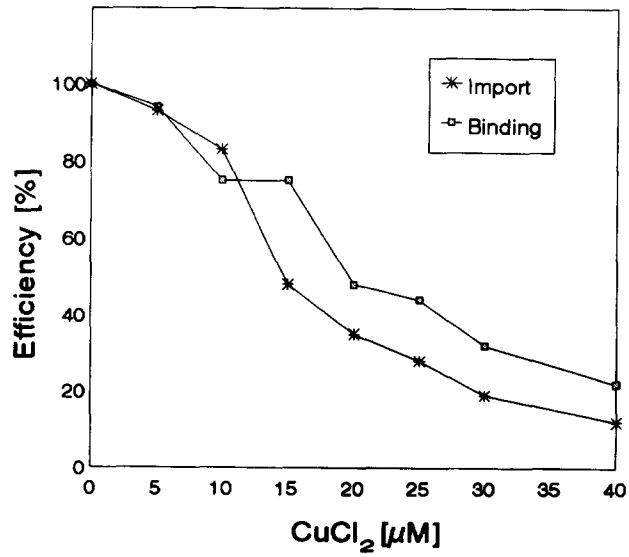

Fig. 2. Concentration dependency of the inhibition of preSSU import into pea chloroplasts by $CuCl_2$. Intact chloroplasts were incubated for 5 min with various $CuCl_2$ concentrations, reisolated and preSSU translocation and binding measured at 3 mM ATP and at 50 μM ATP, respectively. The yield of import (mature SSU in the stroma) and binding (total preSSU interacting with plastids) was determined after SDS-PAGE and fluorography by laser densitometry of the exposed X-ray films.

localization of preSSU in the import process. In the presence of $CuCl_2$ the translocation intermediates deg 3 and deg 4 are no longer detected in contrast to control translocation reactions (Fig. 1, lanes 5, 6), demonstrating that chloroplast bound preSSU remained in a protease accessible localization, i.e. before the insertion of preSSU into the translocation machinery occured. $CuCl_2$ treated chloroplasts always bound less preSSU than non treated organelles (see Fig. 1, lanes 2, 6, 10) indicating the inhibition of a component of the translocation apparatus by $CuCl_2$, which operates very early in the event, i.e. binding or recognition. DTT can reverse the inhibitory effect of $CuCl_2$, but has no additional effect by its own on early steps in import (Fig. 1, compare lanes 6–8), while others [11] have described an import stimulation by DTT. We feel, that this is not a contradiction to the results presented here but most likely due to different chloroplasts isolation procedures. Buffer conditions, isolation time and duration of the complete experimentation might be critical for the redox status of thiol groups, which might be partially oxidized under certain conditions. Thus, a treatment with DTT will recover full import activity [11].

$CuCl_2$ inhibits binding and translocation already at very low effector concentration (Fig. 2). Half maximal inhibition of both processes is reached at around 15 μM $CuCl_2$ (Fig. 2). The residual binding (20–25%), we observed, was always slightly higher than the amount of residual translocation (10–15%) (Fig. 2). This difference could either be due to non productive interaction of preSSU with chloroplast surface proteins or to an interaction of preSSU with chloroplast surface lipids [22], which is not inhibited by $CuCl_2$. Together $CuCl_2$ seems to be a very specific and potent inhibitor of precursor import in pea chloroplasts.

Recently several outer envelope membrane proteins were identified in the protein translocation machinery of pea chloroplasts. OEP86 was shown to be a receptor for preSSU [4], while

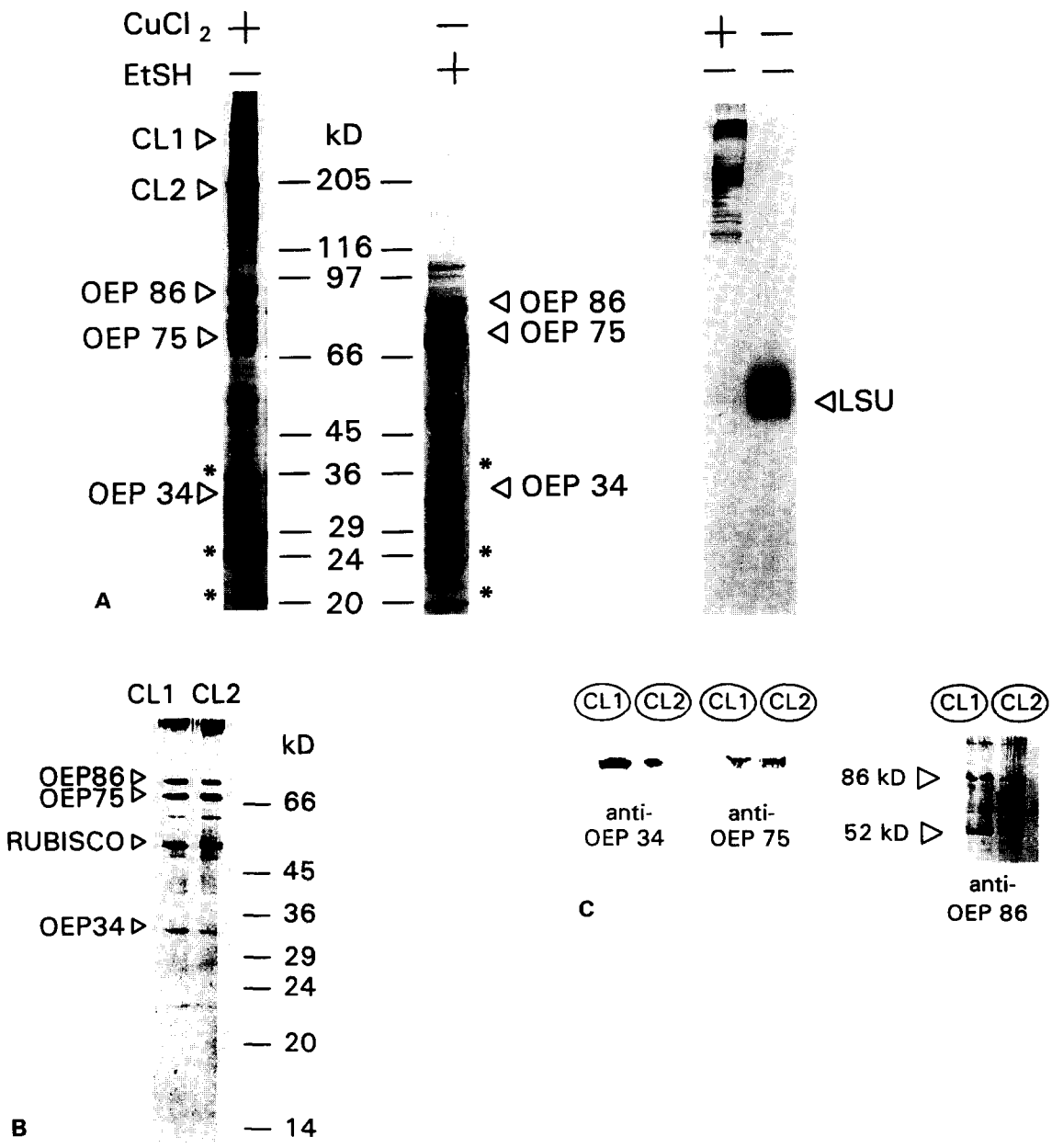

Fig. 3. OEP86, OEP75 and OEP34 form a major crosslink product in purified outer envelope membranes upon $CuCl_2$ treatment. (A) Outer envelope membranes from pea chloroplasts were treated with 1 mM $CuCl_2$ for 5 min and subjected to SDS-PAGE under non reducing conditions. Lane 1 and 2 show a Coomassie brilliant blue protein pattern of $CuCl_2$ treated membranes (lane 1) or control membranes (lane 2). The position of OEP86, OEP75 and OEP34 is indicated by an arrow, while the position of envelope proteins, which are not affected by $CuCl_2$ are indicated by stars. CL1, CL2 denote major crosslink products, which were analysed further. Lanes 3 and 4 show an immunoblot analysis of outer envelope membranes using an antiserum against the large subunit of Rubisco either treated (lane 3) or not treated (lane 4) with $CuCl_2$ and separated by SDS-PAGE under non denaturing conditions. (B) The crosslink products CL1 and CL2 were cut from the polyacrylamide gel, shown in (A) and subjected to a second round of electrophoresis under reducing conditions. A silver stained [27] polyacrylamide gel is shown. (C) CL1 and CL2 were produced as in (A). The crosslinked products were subjected to a second round of electrophoresis under reducing conditions and subsequently transferred to nitrocellulose filters. The upper segment of the blot was immunodecorated with an antiserum specific against OEP75, the lower segment with an antiserum against OEP34. Lanes 4 and 5 show a parallel experiment, which was probed with an OEP86 antiserum.

OEP75 seems to function at a later stage maybe as part of the translocation pore [5,6]. OEP34 represents a new type of GTP binding protein, which could have a regulatory role in the translocation process [7–9]. It is not known today how these proteins are arranged in the import machinery, if and how they might interact with each other. We therefore wanted to know whether the import complex proteins OEP86, OEP75 and OEP34 could be linked together by disulfide bridges in the presence of $CuCl_2$. Chloroplasts outer envelope membranes were treated with 1 mM $CuCl_2$, analysed by SDS-PAGE in the absence of EtSH (Fig. 3, lane 1) and compared to non treated outer envelope membranes (Fig. 3, lane 2). The amount of OEP86, OEP75 and OEP34 was greatly reduced in $CuCl_2$ treated membranes, while other OEP's, like OEP36, OEP24 and OEP20 (indicated by an asterisk) were not affected. Two high molecular weight crosslink products detected in a

Coomassie brilliant blue stained gel, namely CL1 and CL2 (see Fig. 3, lane 1) were studied further. The stained bands, corresponding to CL1 and CL2, were cut out off the polyacrylamide slab gel, incubated with sample buffer [19] in the presence of EtSH and subjected to a second round of SDS-PAGE. Upon silver staining four prominent polypeptides of an apparent mass of 86 kDa, 75 kDa, 54 kDa and 34 kDa were detected (Fig. 3B). Furthermore two minor polypeptides of 64 kDa and 22 kDa were visible which could be further components of the import machinery [23,24]. These data indicate, that OEP86, OEP75 and OEP34 are in very close proximity to each other in the import complex. The prominent band present at 54 kDa represents most likely the large subunit of ribulose-1,5-bisphosphate carboxylase (LSU), which is known to contaminate envelope preparations. The large subunit of Rubisco is very susceptible to $CuCl_2$ treatment and forms multiple high molecular weight oligomers (Fig. 3A, lanes 3, 4). These oligomers are also present in the areas in the polyacrylamide gel were CL1 and CL2 were eluted from (compare lane 1 and lane 3 of Fig. 3A). Immunoprecipitation of the aggregates CL1 and CL2 from solubilizised envelope membranes after $CuCl_2$ treatment using antibodies against components of the import machinery to exclude LSU as a crosslinked constituent of CL1 and L2 were not possible, because LSU and the IgG heavy chain have an identical molecule size and cannot be seperated on SDS- PAGE. We conclude, that LSU is a non specific contaminant in CL1 and CL2 and that it is not covalently crosslinked to the OEP's.

To clearly identify the proteins present in CL1 and CL2 these were subjected in a parallel experiment to a second round of SDS-PAGE in the presence of EtSH, transferred to nitrocellulose and immunodecorated with antisera specific for OEP86, OEP75 and OEP34. The immunoblot analysis establishes the presence of OEP86, OEP75 and OEP34 in CL1 and CL2. OEP86 is labile to degradation and yields a 52 kDa proteolytic fragment [4,25]. We conclude, that both proteins stained at 52 kDa and 86 kDa represent indeed OEP86. The overall composition of CL1 and CL2 seems identical, only the stoichiometry might be different. The connection of components of the protein import complex of outer envelope membranes by disulfide bridges indicates their close vicinity and interaction in situ.

4. Conclusions

The recent identification of components of the chloroplast outer envelope protein translocation machinery, namely OEP86, OEP75 and OEP34 [4–8,14,26] allowed us to look for specific targets, which might be influenced by the $CuCl_2$ treatment. We found, that OEP86, which forms an initial crosslink product with preSSU after binding to chloroplasts [5] and which was identified as a receptor for preSSU [4] is indeed affected by $CuCl_2$ in a way, that it forms a crosslinked hetero oligomer with OEP75 and OEP34. This hetero oligomer is formed by disulfide bridges as shown by its reversibility in the presence of DTT. Thiol groups of each of the three import components must be in close vicinity to each other in situ, otherwise the $CuCl_2$ treatment is unlikely to yield intermolecular crosslink products. OEP34 is preferentially associated with OEP75 in situ. This interaction can be seen even without the addition of a crosslinker [8], but it becomes more pronounced upon $CuCl_2$ treatment. OEP34 contains only one cysteine at amino acid position 214. Therefore OEP34 can be attached only to one partner by $CuCl_2$, we therefore speculate, that this is OEP75. Consequently OEP86 should be coupled to OEP75, both of which contain several cysteines. Our data would therefore indicate a close interaction between OEP34 with OEP75 and OEP75 with OEP86. The results add further evidence to the findings, which postulate the function of OEP86, OEP75 and OEP34 in protein translocation. The formation of intra- and intermolecular disulfide bridges might change the conformation and or mobility of the components of the import complex and thus lead to an inactivation of their biological function.

$CuCl_2$ can also be used to crosslink preSSU to polypeptide components, which are involved in translocation and contain an exposed thiol group (Seedorf and Soll, unpublished). Crosslinking with $CuCl_2$ seems therefore a valid method to identify further constituents of the protein translocation machinery of chloroplasts.

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