The Early Stage of Chloroplast Protein Import Involves Com70*

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The chloroplast envelope protein Com70 is a hsp70 homolog identified recently as a component of the protein translocation apparatus. The stage of protein import involving Com70 was determined by examining the nature of the association of Com70 with the envelope and its interaction with translocating proteins. Com70 is accessible to thermolysin, but its association with the envelope could not be disrupted by stringent washes. In light of the external membrane-bound location, the involvement of Com70 at the early stage of protein translocation was investigated using a combination of in vitro binding assays, chemical cross-linking, and immunoprecipitation. The results provide evidence that Com70 is in close physical proximity to different types of chloroplast protein precursors under conditions supporting binding rather than complete translocation. The formation of cross-linked complexes is dependent on the presence of a typical plastid transit signal and protease-accessible outer envelope components. The close proximity of Com70 and the translocating protein occurs while the protein is still exposed to the cytosol.

The mechanism for importing chloroplast protein precursors into the compartment requires energy and is facilitated by specific envelope proteins (1). Recent studies revealed that envelope proteins with sizes of 34, 36, 44, 70 (at least two such related proteins), 75, 86, and 97 kDa are in close physical proximity to translocating proteins (1, 2). Although the role of each component remains to be elucidated, the outer envelope location of the 34-, 70-, 75-, and 86-kDa proteins places their involvement in the earlier part of the import pathway. The combination of GTP-binding domains in the 34- and 86-kDa proteins and the inhibition of precursor binding with nonhydrolyzable GTP analogs suggests that these two components may be involved in the energy-requiring aspect of the binding step (3–5). The 75-kDa protein is hypothesized, based on its predicted characteristics, to be a candidate for the import channel (6, 7). The 70-kDa components are related forms of the heat shock proteins (hsp70s) and may thus act similarly as chaperones/unfoldases (6, 8, 9).

The importance of hsp70 in protein transport is exemplified by their widespread involvement in translocation mechanisms of the cytosol and/or the interior of different organelles (10–12). Chloroplast protein import also appears to involve multiple 70-kD components, which are related forms of the heat shock protein (hsp70). The 75-kDa component is hypothesized to be involved in the energy-requiring aspect of the binding step (3–5). The 70-kDa protein is hypothesized, based on its predicted characteristics, to be a candidate for the import channel (6, 7). The 70-kDa components are related forms of the heat shock proteins (hsp70s) and may thus act similarly as chaperones/unfoldases (6, 8, 9).
Com70 Participation

RESULTS

Com70 Is Strongly Associated with the Outer Envelope—Com70 is an externally located outer envelope polypeptide and is in close proximity to a partially translocated protein (8, 14, 31). Although an uncleavable signal is required for Com70 association with the envelope, the absence of membrane-anchoring domains necessitates an assessment of this interaction (31). The assessment of Com70 association with the outer envelope is important for predicting its functional locale, especially with the presence of different hsp70-containing sites in the envelope. The anti-Com70 IgGs used were generated against the divergent COOH terminus (8, 14, 31) and did not cross-react with stromal or thylakoid proteins (Fig. 1A).

Com70 interaction occurs largely on the cytosolic face since the majority of the proteins are thermolysin-accessible, a protease that is unable to penetrate the outer envelope (Fig. 1A, B). The largely thermolysin-sensitive nature corroborates previous import data showing the external location of Com70 (31), distinguishing Com70 from the protease-resistant internal hsp70s, e.g. Iap70. The Com70 interaction was assessed further using agents known to disrupt the membranous association of peripheral or apparent cytosolic proteins (3, 32–34). Total envelopes were washed to ensure that all possible locations where Com70 may reside were tested. Blots were reprobed with IgGs against the abundant integral inner membrane protein Cim37 (35), an internal normalization control. Com70 association could not be disrupted effectively by EGTA or EDTA, which disrupt Mg$^{2+}$/Ca$^{2+}$-enhanced electrostatic bonds (Fig. 1C) or by NaCl concentrations capable of disrupting electrostatic interactions (Fig. 1C). Although it is not known at present why NaCl causes an apparent decrease in Cim37, NaCl clearly does not reduce the tenacity of the Com70 interaction with the membrane. Alkaline washes consisting of 0–200 mM Na$_2$CO$_3$, pH 11.5 (Fig. 1C), were also found not to be disruptive. Peripheral proteins, such as the hsp70-related protein associated with the mitochondrial outer membrane, are removed by 100 mM Na$_2$CO$_3$, pH 11.5 (36). Based on the same criteria, the Com70 interaction with the outer envelope is different from typical peripheral proteins and must be assigned a membrane-bound external location.

Com70 Is in Close Physical Proximity to Translocating Protein Precursors at the Early Stage of Import—Chloroplast protein precursors bind to the cytosolic face of the outer membrane prior to translocation, hence Com70 is most likely near precursors at this early external stage. The proteins chosen to test for early stage associations were pLhcb, pRbcs, pOee1, and pOee1-Dhfr, representing precursors destined for different suborganellar compartments (thylakoid, stroma, and lumen) and a fusion containing a non-plant protein Dhfr. Associations were enhanced quantitatively using nigericin and the highest radio-labeled precursors to chloroplasts ratio. Chemical cross-linking was employed to stabilize the associations further and identified by immunoprecipitation with anti-Com70 IgGs. Anti-Com70 IgGs were unable to immunoprecipitate any of the precursors tested or to immunoreact with the authentic proteins in the corresponding chloroplast subfraction (Fig. 1A, lanes 3–5; Fig. 2, lane 8).

The membrane-permeable chemical cross-linker DSP and its impermeable analog DTSSP were shown previously to stabilize pOee1-Dhfr-Com70 complexes that were immunoprecipitated by anti-Com70 IgGs (8). Similar results were obtained with DSP and DTSSP in this study when nigericin was used to enhance the level of precursors collecting at the binding step (data not shown for DSP and Fig. 2). The formation of cross-linked complexes at an externally exposed part of the import pathway, namely binding, was assessed using DTSSP due to its membrane-impermeable nature, as evidenced by previous studies. DTSSP, unlike DSP, is unable to form cross-links between a translocating protein and inner envelope proteins.

FIG. 1. Association of Com70 with the outer envelope. Panel A, immunoblot of chloroplast subfractions. Inner (IE), outer (OE), and total envelopes (TE), stroma (STR), and thylakoids (THYL) were probed with anti-Com70 IgGs. Inner and outer envelope fractions were reprobed with anti-Cim37 IgGs. Panel B, Com70 sensitivity to thermolysin. Lanes 1–3 contain total envelopes isolated from intact thermolysin-pretreated chloroplasts. Immunoreactive bands were quantified by laser densitometry and normalized as Com70/Cim37 ratios. The ratio in the control was set to 100%. Panel C, envelopes were washed in increasing concentrations of disruptive agents as indicated and analyzed by immunoblotting with anti-Com70 IgGs and reprobed with anti-Cim37 IgGs as an internal control.
such as Cim97 (8). The amount of radiolabeled precursors immunoprecipitated as part of cross-linked complexes with Com70 was enhanced by DTSSP (Fig. 2, lanes 5–7). Preimmune and anti-Cim37 IgGs did not immunoprecipitate any of the precursors (data not shown for preimmune IgGs and Fig. 2, lanes 2–4). These results indicate that Com70 can form complexes with at least four different types of chloroplast protein precursors. Interestingly, anti-Com70 IgGs did not immunoprecipitate pLhcb or pRbcs-containing complexes in the absence of DTSSP, whereas complexes with pOee1 and pOee1-Dhfr were observed, albeit at relatively lower levels (Fig. 2, lane 5). The two latter precursors appear to form a more stable complex with Com70 when compared with pLhcb and pRbcs under the tested conditions. It is not known at present why DTSSP significantly reduces the level of pOee1 associations with Com70 even though DTSSP stabilizes the association of its intermediate form. Nevertheless, the results obtained with the membrane-impermeable DTSSP clearly indicate that associations occur with externally exposed pOee1 intermediates. The increased stability observed without DTSSP may be reflective of features of the pOee1 transit peptide such as the length (85 residues) and its relative efficiency. The formation of complexes with both the full-length precursor and a smaller sized import intermediate form in the pOee1 and pOee1-Dhfr experiments (Fig. 2, lanes 5–7) can be attributed to these characteristics. Although the long Oee1 transit peptide has already advanced inward becoming exposed to internal “processing” events such as that reported earlier (8), part of the translocating protein remains external and accessible to cross-linking. The predominant formation of complexes with pOee1-Dhfr precursors versus the intermediate form of pOee1 is due to the higher ATP level added via the higher volumes of Oee1 translation mixture, used to maximize the precursor to chloroplast ratio, necessitated by the lower translation efficiency of pOee1 (50% lower than the other precursors). Higher levels of ATP gradually overcome the inhibitory effects of nigericin resulting in the generation of import intermediates by advancing precursors further inward and exposing pOee1 to processing events as discussed above. It is important to note that the prominent smaller sized pOee1 intermediate form is observed only with nigericin and/or when a high volume of translation mixture is used, evidence for an import intermediate (see Figs. 2 and 3). Another contributing factor is that pOee1-Dhfr requires more ATP than pOee1 (100 μM versus 25 μM) to advance the precursors to the same stage of import (8), therefore a high portion of pOee1-Dhfr will be more externally located than pOee1 and protected from internal processing events. The smaller bands appearing in the pRbcs experiments represent prematurely terminated translation products since they are present in the translations\(^2\) and thus can still engage, albeit at lower efficiencies, the import machinery. These events are especially prominent with high precursor to chloroplast ratios.

Com70-protein precursor complex formation was also observed under conditions supporting import (Fig. 3A) and a high precursor to chloroplast ratio. Com70 is in close proximity to unprocessed precursor proteins, but not with imported mature proteins. Because of the high precursor to plastid ratio employed, approximately half of the presented precursor proteins were imported and processed to their mature size (Fig. 3A, lanes 1 and 3), while the other half remained as bound full-length precursors. Lower precursor to plastid ratios used in a previous study showed that 1 mM ATP promotes dissociation from components of the protein import apparatus and protein translocation, resulting in a lack of cross-linking (8). This aspect was evident in the pOee1 experiments where the translocation intermediate that coimmunoprecipitated with Com70 using nigericin no longer coimmunoprecipitates with Com70 in the presence of 1 mM ATP (Fig. 3A, lane 4 versus 3). Very low levels of the full-length precursor were instead complexed with Com70 (Fig. 3A, lane 3). A similar but less pronounced trend was observed with pLhcb (Fig. 3A, lanes 3 versus 4). The

\(^2\) L. Kourtz, unpublished data.
smaller pOee1 intermediate form complexed with Com70 is no more than 500 Da larger than mature Oee1 and forms complexes only under conditions promoting import intermediates, i.e. nigericin and high volumes of translation mixture. This smaller pOee1 intermediate form was also observed in another recent study (37). Differences in transit peptides and translocation competence of pOee1 and pLhcb thus appears to modify the level of ATP-induced disassociation from Com70. These combined data demonstrate the tendency of Com70 to associate with externally exposed precursor proteins.

Thermolysin pretreatment of chloroplasts impairs binding/import and is reflected in the failure to form cross-linked complexes (Fig. 3B). These results confirm that external thermolysin-accessible factors on the cytosolic face of the outer envelope, including Com70, are necessary for the formation of Com70-protein precursor complexes and that Com70 participates in the early stage of importation.

Com70-Protein Precursor Complex Formation Requires the Presence of a Typical Chloroplast Transit Peptide—The necessity of a chloroplast transit peptide in the formation of Com70-protein precursor complexes was examined to determine whether Com70 was actively participating in import or was acting in a general capacity. Mature Lhcb and Oee1 and a non-plant protein, mouse cytosolic Dhf, were unable to bind to or import (Fig. 4A, lanes 2 and 3) and failed to form complexes (Fig. 4A, lanes 4 and 5), indicating that the presence of a transit peptide is required for complex formation and that the association is not due to the generic binding of Com70 to proteins. Although Com70 appears unable to come into close contact to form cross-links with proteins lacking transit peptides, it does not necessarily imply that the transit peptide functions directly in the association with Com70.

The formation of complexes was assessed as above using precursors with atypical import characteristics. pCoxIV-Dhf, associates with chloroplasts at a level comparable to that of pLhcb, pRbcs, and pOee1 (Figs. 2 and 4B). The same sized product as the in vitro translation was observed under all conditions (Fig. 4B, lane 1 versus 4), and the associated forms were protease-sensitive (Fig. 4B, lanes 1–6). Unlike typical chloroplast protein precursors, pCoxIV-Dhf is capable of binding to thermolysin-pretreated chloroplasts and hence does not appear to require thermolysin-sensitive binding receptors (Fig. 4B, lanes 9–11). As a consequence, pCoxIV-Dhf does not form cross-linked complexes with Com70 under either import condition (Fig. 4B, lanes 7 and 8). These results suggest that association with the envelope alone is not sufficient for the formation of cross-linked complexes with Com70 but that this association requires the presence of a plastid transit peptide.

The necessity of a typical chloroplast transit peptide for the formation of complexes was assessed using the leukoplast protein precursor pPka. pPka displays atypical import characteristics in that import occurs at levels comparable to precursors and proteins in the chloroplast outer envelope (internal and external) and one in the stroma. The outer envelope and cytosolic hsp70s have been implicated in the chloroplast protein translocation process, but the nature of each locale’s participation requires further individual examination (1, 6, 8, 9, 13, 15). This study focuses on the externally located outer envelope hsp70, Com70.

Despite the overall hydrophilic characteristic (14) and the thermolysin-sensitive profile of the externally located Com70 is not imported into a protease-protected location (Fig. 4B, lanes 1–6). The smaller sized bands in the pRbcs2 reactions represent prematurely terminated translation products such as those described for pRbcs. Portions of the bound precursors were fully or partly protected from thermolysin but not from trypsin, indicating that these forms are translocation intermediates. Despite the altered import characteristics, pRbcs2 can still form cross-linked complexes as in the case of unaltered pRbcs (Fig. 2; Fig. 4B, lanes 7 and 8). Although pRbcs2 associates with the chloroplast more readily with nigericin than ATP, it appears that ATP promotes more Com70-pRbcs2 complex formation. The reason(s) for this observation is currently not known.

**DISCUSSION**

Recent studies clearly indicate the presence of two hsp70-related proteins in the chloroplast outer envelope (internal and external) and one in the stroma. The outer envelope and cytosolic hsp70s have been implicated in the chloroplast protein translocation process, but the nature of each locale’s participation requires further individual examination (1, 6, 8, 9, 13, 15). This study focuses on the externally located outer envelope hsp70, Com70.
in intact organelles, Com70 membrane association could not be effectively disrupted based on the criteria used to classify peripheral or apparent cytosolic proteins (32, 33, 36). This suggests that Com70 is strongly associated with the cytosolic face of the outer chloroplast envelope, differentiating Com70 from the protease-inaccessible outer envelope hsp70 forms, from the cytosolic hsp70s, and from hsp70s known to possess transient membrane association states (6, 9, 38–41). Although the Com70 association appears difficult to disrupt, the mechanism of interaction remains unclear. This is intriguing since the predicted Com70 sequence does not contain an obvious membrane anchoring domain despite the presence of a noncleavable hydrophilic envelope targeting signal (31). The involvement of the signal, whether it is fully or partly responsible for the strong membrane association of Com70, still remains to be elucidated. The prenylation CaaX-type domain present in the COOH terminus may be a contributing factor, but this possibility requires further experimentation (14). Nevertheless, the present data place the potential involvement of Com70 at an early external stage of importation by virtue of its locale in the outer envelope.

Involvement at an early external stage was confirmed by the close association of Com70 with different chloroplast protein precursors under binding conditions rather than import. Close associations did not appear to be specific to a precursor but occurred with proteins destined for different subcompartments. Interestingly, in the case of pOee1, Com70-precursor protein complexes were immunoprecipitated even in the absence of chemical cross-linkers, albeit at lower levels. The behavior observed with pOee1 suggests that the translocating protein comes into close contact with Com70 at an early external stage and that this association is strong enough to withstand subsequent manipulations. In addition, the strength of the interaction can vary depending on the requirements of the protein precursor. The higher yields of Com70-precursor complexes observed for all cases with DTSSP can therefore be attributed to the stabilization of existing interactions rather than to the establishment of new links due to the use of chemical cross-linkers. The early external location of the Com70 involvement is exemplified further by the following observations: (i) the membrane-impermeable nature of DTSSP, which suggests that the complexes are formed externally; (ii) the sensitivity of complex formation to thermolysin pretreatment of the chloroplast; (iii) the failure to form complexes with fully translocated and processed forms; (iv) in the case of pOee1, that complex formation occurs with a translocation intermediate that is still externally accessible (8); and (v) that complex formation can be abolished or substantially reduced upon the addition of ATP and the concomitant internalization of the intermediate.

The formation of Com70-precursor protein complexes requires a typical chloroplast transit peptide. Close associations were generated with different types of precursors even though they are destined to various subcompartments, but not with the corresponding mature forms or with the foreign cytosolic protein Dhfr. The typical chloroplast transit peptide requirement is demonstrated further by the inability to form complexes with precursors such as pCoxIV-Dhfr and pPkα, which are capable of associating but lack typical chloroplast targeting signals and exhibit atypical import characteristics. Even though the mitochondrial targeting signal in pCoxIV-Dhfr allows binding to the plastid, this association is not dependent on protease-sensitive outer envelope components, nor is the precursor imported into a protease-protected site. These characteristics suggest that the mitochondrial transit peptide directs Dhfr to a binding component distinct from that employed by typical chloroplast precursor proteins and is reflected in the inability to form Com70-pCoxIV-Dhfr complexes. In the case of pPkα, alternate components at the early stages of import may be employed despite its ability to import in a typical fashion, and this is reflected in the inability to form Com70-pPkα complexes. These conclusions are corroborated by the data regarding RbcS2, which, in direct contrast to pPkα, binds, but is no longer imported. Even in the absence of import, the typical chloroplast transit peptide of RbcS2 facilitates complex formation with Com70.

Despite the demonstration that Com70 associates with precursors engaging typical translocation machinery components, the actual role of Com70 in protein import remains to be determined. Although the data collectively show a requirement for a typical chloroplast transit peptide, they do not reveal whether Com70 complexes directly with the transit peptide or with the mature part of the protein or with both parts. Although it is evident that Com70 works at an early external stage, Com70 most likely acts in cooperation with other outer envelope components (1). The ATP binding ability of Com70 suggests that it may play a role parallel to the relay/pulling type mechanism ascribed to mitochondrial matrix-hsp70s (mt-hsp70), with the exception of being external (42). Mt-hsp70 binds to the emerging NH2 terminus of translocating precursors, preventing the precursor from slipping out of the import channel and back into the cytosol. Subsequent cycles of ATP hydrolysis and re-binding to Mim44 by mt-hsp70 facilitate inward movement of the precursor. In contrast to a pulling mechanism, Com70 may prevent precursors from diffusing back into the cytosol, facilitating precursor unfolding and entry into the import channel. The strong unfoldase activities associated with envelopes may represent such a mechanism (43). Com70 may also facilitate protein import as part of a relay of hsp70s of an unfoldase/translocase machinery. Cytosolic hsp70s have been predicted to participate in import, perhaps by maintaining the nascent precursor proteins in an import competent state prior to their interaction with Com70 and other outer envelope components (13). Com70 could then facilitate a similar role and pass precursors to other hsp70s such as the outer envelope thermolysin-insensitive hsp70s and the stromal hsp70s (6, 9, 16–17, 44).

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