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Functional cooperation between BiP and calreticulin in the folding maturation of a glycoprotein in *Trypanosoma cruzi*

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Abstract

Proteins may adopt diverse conformations during their folding *in vivo*, ranging from extended chains when they emerge from the ribosome to compact intermediates near the end of the folding process. Accordingly, a variety of chaperones and folding assisting enzymes have evolved to deal with this diversity. Chaperone selection by a particular substrate depends on the structural features of its folding intermediates. In addition, this process may be modulated by competitive effects between chaperones. Here we address this issue by using TcrCATL as model substrate. TcrCATL is an abundant *Trypanosoma cruzi* lysosomal protease and it was the first identified endogenous UDP-Glc:glycoprotein glucosyltransferase (UGGT) substrate. We found that TcrCATL associated sequentially with BiP and calreticulin (CRT) during its folding process. Early, extended conformations were bound to BiP, while more advanced and compact folding intermediates associated to CRT. The interaction between TcrCATL and CRT was impeded by deletion of the UGGT-encoding gene but, similarly to what was observed in wild type cells, in mutant cells TcrCATL associated to BiP only when displaying extended conformations. The absence of TcrCATL–CRT interactions in UGGT null cells resulted in a drastic reduction of TcrCATL folding efficiency and triggered the aggregation of TcrCATL through intermolecular disulfide bonds. These observations show that BiP and CRT activities complement each other to supervise a complete and efficient TcrCATL folding process. The present report provides further evidence on the early evolutionary acquisition of the basic tenets of the *N*-glycan dependent quality control mechanism of glycoprotein folding.

Keywords

BiP; Calreticulin; *Trypanosoma cruzi*; Glycoprotein folding quality control

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1. Introduction

Proteins may adopt different intermediate structures during their conformational maturation *in vivo*, ranging from extended conformations when they exit the ribosomes to more compact structures at advanced folding stages. Chaperone specificity has evolved to deal with this structural heterogeneity, and for this reason a protein may be assisted by several chaperones during its folding process [1–3]. The endoplasmic reticulum (ER) is the folding subcellular location for nearly one third of proteins synthesized in eukaryotic cells and, accordingly, it is an environment rich in chaperones and folding assisting enzymes. The most abundant chaperones in the ER are BiP (the homologue of HSP70), GRP94 (the homologue of HSP90), calreticulin (CRT) and calnexin (CNX). The last two proteins are lectins that base their activities on recognition of monoglucosylated *N*-glycan moieties displayed by glycoproteins [4]. In addition, the ER has several protein disulfide isomerases that help in the formation and isomerization of disulfide bridges.

Proteins unable to attain a stable conformation are retained in the ER by folding quality control systems (QC). For glycoproteins, the so-called *N*-glycan-dependent QC involves the concerted action of CRT and CNX with the enzymes glucosidase II and UDP-Glc:glycoprotein glucosyltransferase (UGGT) [5]. Briefly, in most eukaryotic cells *N*-glycosylation starts with the *en bloc* transfer of glycan Glc₃Man₉GlcNAc₂. The two external glucoses are removed by the sequential activity of glucosidases I and II, thus generating monoglucosylated glycoproteins. These intermediates are retained in the ER by CRT and/or CNX, which are lectins that specifically bind monoglucosylated glycans. This interaction enhances folding efficiency by inhibiting protein aggregation and allowing the action of ERp57, a protein disulfide isomerase associated with the lectins [6]. Eventually, the remaining glucose residue is removed by GII and glycoproteins are released from the lectin anchors. At this point, proteins displaying native conformations proceed to their final destinations. On the contrary, proteins displaying non-native structures are reglucosylated by UGGT, thus re-establishing their association with CRT and/or CNX [7]. Cycles of deglucosylation by GII and reglucosylation by UGGT persist until proteins fold properly or, alternatively, until they are targeted for proteasomal degradation. Interestingly, UGGT is a unique protein that combines features of a glucosyltransferase and of a chaperone sensing the degree of exposition of the protein moiety hydrophobic core [8–11], as it only glucosylates glycoproteins or glycoprotein complexes not displaying their native tertiary or quaternary structures [12].

Chaperone selection in the ER depends on the structural features of the folding intermediates, which favors their interaction with particular sets of chaperones. In addition, competitive effects between chaperones may also play an important role in this process. In this sense, the association of a chaperone with a substrate displaying suitable binding sites may be prevented by a previous and/or stronger association with a different chaperone. Many proteins entering the ER interact first with BiP, which receives its substrates from Sec63, a HSP40 homologue associated to the Sec61 translocation [13]. The following steps are varied. While some proteins fold without further help, others interact with CRT/CNX [14,15] or GRP94 [16]. On the other hand, some proteins associate first with CRT/CNX instead of BiP, a process triggered by the presence of *N*-glycans near the N-terminus of the translocating polypeptide [17]. Nevertheless, the rules governing chaperone selection in the ER remain unclear.

Almost all components of the *N*-glycan-dependent QC mechanism described in higher eukaryotes are also present in trypanosomatids as UGGT, glucosidase II, CRT, but not CNX occur in them [18–23]. In fact UGGT-mediated protein glucosylation was first detected in *Trypanosoma cruzi* due to the fact that *N*-glycosylation in this protozoon starts, as in all

trypanosomatids studied so far, upon transfer of a glycan lacking glucose residues (Man₉GlcNAc₂ in this species) [24,25]. Thus, the transient presence of protein-linked Glc₁Man_{7,8,9}GlcNAc₂ in cells pulsed with [¹⁴C]Glc could only be ascribed to a glucosylation of protein-linked *N*-glycan and not, as in almost all other eukaryotes, to a partial deglucosylation of the compound transferred in them (Glc₃Man₉GlcNAc₂). Therefore, the only available pathway for glycoprotein association to CRT in *T. cruzi* is through UGGT activity. This is at variance with most eukaryotic cells, in which partial trimming of the transferred glycan is an alternative source of monoglucosylated species. For this reason *T. cruzi* is an advantageous model to study certain aspects of glycoprotein folding in the ER as, for instance, characterization of protein conformations recognized by UGGT *in vivo*. TcrCATL (previously called cruzipain or cruzain, see Ref. [26] for nomenclature) is an abundant *T. cruzi* lysosomal protease and it was the first identified endogenous, (that is chromosome- and not expression vector- or virus-encoded) UGGT substrate [21] as well as a virulence factor [20]. BiP has also been described to occur in trypanosomatids [20,22,27], although in *Trypanosoma brucei* the chaperone shows a unique feature as disruption of the UGGT encoding gene did not lead to an upregulation of the protein as it occurs in mammalian, yeast and even in *T. cruzi* cells [20]. We have previously shown that during its folding in the ER, TcrCATL interacts with BiP and CRT [20]. To gain more information on the *N*-glycan dependent QC mechanism occurring in trypanosomatids we study here the conformational maturation of TcrCATL *in vivo*, focusing the attention on the structural features recognized by BiP, CRT and UGGT.

2. Materials and methods

2.1. Reagents

Iodoacetamide (IAM), Geneticin (G-418), dithiothreitol (DTT) and Protein A-Sepharose were purchased from Sigma (St. Louis, MO). Hygromycin B, 4-acetamido 4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) were from Invitrogen. TcrCATL antiserum was generated in rabbits as described previously [21], whereas *T. brucei* Grp78/BiP antiserum was a generous gift of Dr. J.D. Bangs (University of Wisconsin-Madison Medical School). This antibody cross-reacts with *T. cruzi* BiP [20].

2.2. Cells and culture media

T. cruzi CL Brener clone epimastigotes were grown in BHT medium as described before [28]. UGGT null mutants (*uggt*^{-/-}) of the same clone were obtained in our laboratory as previously described [20] and grown in the indicated medium supplemented with G418 and Hygromycin B, both at a final concentration of 250 µg/ml.

2.3. Cell disruption by freezing and thawing

The method described in [29] was followed for cell disruption by freezing and thawing. Briefly, cells were twice washed with 0.25 M sucrose, 5 mM KCl and the pellet obtained upon a low speed centrifugation was kept frozen at -20°C for 48 h, after which cells were thawed at 4°C and resuspended in 50 mM Tris-HCl, pH 7.6, 0.15 M NaCl (TBS). The suspension was centrifuged for 10 min at 15,000 × *g*. The supernatant was removed (lysosomal fraction) and the pellet was resuspended in the same buffer supplemented with 1% NP-40. The suspension was centrifuged as above and the supernatant was removed (ER fraction). This procedure yielded about 17–20% of soluble ER lumenal and 80–84% of soluble lysosomal enzymatic contents in the first supernatant and 80–83% of soluble ER lumenal and 14–20% of soluble enzymatic contents in the second one [29].

2.4. Pulse-chase labeling

T. cruzi epimastigotes (1 g wet weight, exponential growth phase) were twice washed with DME/F-12 Base medium (Met, Gln, Leu, and Lys free, Sigma) supplemented with 365 mg/l Gln, 59.1 mg/l Leu, 91.3 mg/l Lys, 61.2 mg/l MgCl₂, 154.5 mg/l CaCl₂, and 1.2 g/l NaHCO₃. Parasites were resuspended in 8 ml of the same medium and incubated at 28 °C, for 5 min with [³⁵S]-Met and [³⁵S]-Cys (1 mCi, 1000 Ci/mmol; EasyTag protein labeling mix; New England Nuclear). Suspensions were then subjected to low-speed centrifugations, and the pellets were resuspended in 4 ml of *T. cruzi* growth medium supplemented with 3 mM Met and 3 mM Cys. Aliquots (0.4 ml) were withdrawn after the indicated times at 28 °C and were kept frozen at -20°C for 48 h.

2.5. Alkylation procedure

Lysosomal and ER fractions were treated with 5 mM IAM for 5 min at room temperature in the presence of 8 M urea. Next, 10 mM DTT was added and the samples were incubated for 30 min at 37 °C. Finally, 30 mM AMS was added and the samples were further incubated for 10 min at 37 °C. To study the conformation of TcrCATL associated with BiP and CRT, immunocomplexes from the ER fraction isolated with anti-CRT or anti-BiP sera were alkylated following a similar procedure.

2.6. Immunoprecipitations

Cytosolic and ER fractions from labeled cells were prepared as described above. For non-reducing conditions, supernatants (200 µl) were incubated with 1:50 diluted anti-CRT, anti-TcrCATL or anti-BiP serum for 2 h at 4 °C in a shaker incubator. Protein A-Sepharose (40 µl) was added, and the suspensions were further incubated overnight. Beads were then twice washed with 400 µl of TBS, resuspended in cracking buffer, and submitted to 10% SDS-PAGE. Sequential immunoprecipitations were first performed under nondenaturing conditions using anti-BiP or anti-CRT sera as described above. The complexes were resuspended in TBS buffer containing 0.5% SDS and heated for 5 min at 95 °C. Next, suspensions were threefold diluted with TBS and supernatants obtained upon centrifugation were submitted to immunoprecipitations with anti-TcrCATL serum. Beads were then twice washed with 400 µl of TBS, resuspended in cracking buffer and submitted to 10% SDS-PAGE.

3. Results

3.1. TcrCATL folding assessed by disulfide bridge formation

The study of protein folding *in vivo* is limited by the scarcity of suitable experimental tools. One useful approach is to follow the oxidation of protein displaying disulfide bridges. The rationale behind this methodology is that more oxidized species correspond to more advanced folding stages. TcrCATL displays an N-terminal signal peptide, followed by a pro-domain. Presumably, as happens in other eukaryotic cells, the pro-domain remains covalently bound to the enzyme, thus inhibiting its activity, until arrival of the protease to lysosomes, where the acidic environment would promote its proteolytical cleavage. TcrCATL has 17 cysteine residues and three *N*-glycosylation consensus sites at positions 169, 292 and 377. *N*-glycosylation sites at positions 169 and 377 are used, while the occupation of the site at position 292 is uncertain yet. In addition, site at position 377 may display high mannose or complex type *N*-glycans [30,31]. It should be mentioned that TcrCATL is a highly polymorphic protein that is encoded by numerous genes (about 120 in certain strains) [32]. This fact, together with the above mentioned presence of both high mannose and complex *N*-glycans determines a broad running in SDS-PAGE (one of the available sequences is depicted in Fig. S1) [33].

To follow TcrCATL oxidation *in vivo*, parasites were labeled for 5 min followed by a chase with unlabeled medium. At indicated times, 5 mM IAM was added, the parasites were frozen for 48 h at -20°C and the ER and lysosomal fractions were prepared as described under Material and methods [29]. Samples were further treated with 5 mM IAM in the presence of 8 M urea to block the remaining free cysteines. Samples were then treated with 10 mM DTT in order to quench the excess of IAM and reduce disulfide bridges formed during pulse and chase. Finally, the newly reduced cysteines were blocked with 30 mM AMS, TcrCATL was immunoprecipitated using a polyclonal serum and analyzed by SDS-PAGE and autoradiography. The sequential alkylation procedure increases the molecular mass of TcrCATL by 490 Da for each cysteine residue modified with AMS, thus decreasing the electrophoretic mobility of the protein (Fig. S2). Since AMS-modified residues originate from those involved in disulfide bridges, the mobility of a protein will decrease in more oxidized species, which can be ascribed to more advanced folding stages [34].

In wild type parasites TcrCATL became more compact at a rather steady rate during the first 2 h after stopping protein labeling, as assessed from its mobility on SDS-PAGE (Fig. 1A). Although several folding intermediates are expected, the broad TcrCATL bands precluded their resolution at the level of single disulfide bridges. By contrast to wild type cells, in *uggt*^{-/-} parasites [20] TcrCATL remained in a mostly reduced state all along the chase (Fig. 1A). A similar procedure was employed to assess the folding status of TcrCATL in its final destination. In wild type parasites completely oxidized TcrCATL started to arrive to lysosomes after a 30 min chase (Fig. 1B). It should be noted that the difference in mobility between purified TcrCATL samples treated with IAM or AMS was higher than that predicted from their respective molecular weights (Fig. 1B, right panel). This anomalous behavior was also observed using purified lysozyme (data not shown) and could be due to an altered SDS-protein interaction upon treatment with AMS. The fact that partially oxidized forms of TcrCATL were totally retained in the ER illustrates the capacity of the QC system to recognize immature species. By contrast, in *uggt*^{-/-} cells the arrival of TcrCATL to lysosomes was seriously impaired. Here, most of TcrCATL was retained in the ER in a mostly reduced state (Fig. 1A), while a minor fraction of completely oxidized TcrCATL started to arrive to the lysosome after a 60 min chase (Fig. 1B).

3.2. Chaperone association to TcrCATL depends on its folding status

The association of BiP and CRT with TcrCATL was studied by pulse-chase experiments and co-immunoprecipitations with anti-BiP or anti-CRT sera. Next, TcrCATL in complexes with chaperones was isolated by a second immunoprecipitation using anti-TcrCATL polyclonal serum. Samples were analyzed by SDS-PAGE and autoradiography. In wild type parasites TcrCATL only associated with BiP during the first 10 min of chase (Fig. 2A). On the other hand, CRT-TcrCATL association persisted through all the chase period (Fig. 2B). A different result was obtained in *uggt*^{-/-} parasites: BiP-TcrCATL association persisted though all the chase period (Fig. 2C), whereas CRT-TcrCATL interaction was completely abolished (not shown, see Ref. [20]). A comparison of time dependence of chaperone association with TcrCATL oxidation kinetics (Fig. 1) suggested that BiP interacts with early and less compact folding intermediates, while CRT does so with more advanced folding stages. This was confirmed by analyzing the oxidation state of TcrCATL associated with CRT or BiP. To this end, ER fractions from wild type or *uggt*^{-/-} parasites lysed in the presence of IAM were prepared and complexes containing CRT or BiP were immunoprecipitated. Next the immunocomplexes were treated with DTT and AMS, subjected to SDS-PAGE and TcrCATL was revealed by western blot using TcrCATL antiserum. In wild type parasites TcrCATL associated with CRT was more oxidized than that associated with BiP (Fig. 2D). Although in the pulse and chase experiment TcrCATL started to associate with CRT at very early chasing times (Fig. 2B), results shown in Fig. 2D

indicated that most TcrCATL does so during advanced folding stages. CRT-associated TcrCATL molecules detected after 5–10 min chase (Fig. 2B) conceivably represent rapidly folding species as folding is an asynchronous process. In addition, in *uggt*^{-/-} parasites BiP-bound TcrCATL also displayed an extended conformation, thus showing that BiP could not replace CRT during the whole folding process (Fig. 2D). Finally, we studied the effect of UGGT deletion on the aggregation state of TcrCATL. To this end, ER and lysosomal fractions of wild type or *uggt*^{-/-} parasites were treated with 5 mM IAM and the samples were boiled in the presence of 1% SDS with or without the addition of DTT. The samples were submitted to SDS-PAGE and TcrCATL was detected by western blot. Under reducing conditions the behavior of TcrCATL was similar in both parasites. By contrast, the non-reducing gel of the ER fractions of the *uggt*^{-/-} parasites displayed a slow migrating form that did not enter the resolving gel (Fig. 3). Results shown indicate that this band originates from disulfide-bonded aggregated forms of TcrCATL, presumably produced by the absence of TcrCATL–CRT interaction.

4. Discussion

During their maturation *in vivo*, proteins may adopt diverse conformations, ranging from extended structures at the first stages to more collapsed chains near the end of the folding process. Accordingly, a significant number of chaperones and folding assisting enzymes have evolved to deal with this structural diversity [1–3]. Since *N*-glycosylation in *T. cruzi* starts with the transfer of a glycan devoid of glucose residues, the only pathway to generate monoglucosylated structures is through the glucosylation reaction mediated by UGGT. For this reason, sugar-mediated association with CRT is completely abolished in *uggt*^{-/-} *T. cruzi* [20]. In this vein, a comparative study of wild type parasites with their UGGT KO counterparts produces valuable information regarding the molecular determinants recognized by UGGT and the complementation between BiP and CRT functions.

Chaperone selection by a particular substrate depends on the exposition of suitable chaperone binding sites, which will favor its interaction with some chaperones. In addition, some folding intermediates might be recognized by more than one chaperone, thus establishing a competition between chaperones for their access to the substrate. Therefore, both effects, conformational selection by the substrate and competition between chaperones, should be taken into account when analyzing substrate–chaperone interactions *in vivo*. Regarding chaperone competition, it has been shown that the presence of *N*-glycans near the N-terminus of proteins favor their initial interaction with CRT and/or CNX [17]. When this association was abolished by inhibiting glucose trimming glycoproteins associated instead to BiP, thus showing that for N-terminus glycan rich glycoproteins, the initial chaperone selection had been determined by a competition in which CNX/CRT had prevailed. Fig. 4 summarizes the folding process of TcrCATL in the parasite ER. Conformational maturation of the protease in wild type *T. cruzi* took a maximum of about 2 h, as assessed by disulfide bridge formation. This value agrees with that reported for the kinetics of TcrCATL arrival to lysosomes [29]. Partially folded forms were retained in the ER, and only completely oxidized TcrCATL arrived to lysosomes. TcrCATL folding was sequentially assisted by BiP and CRT. The early folding stages were preferentially bound to BiP, while more advanced and compact intermediates were recognized by CRT. Given the particular features of protein *N*-glycosylation in *T. cruzi* mentioned above, this is the first *in vivo* confirmation that UGGT recognizes preferentially advanced folding stages of an endogenous glycoprotein. This agrees with previous *in vitro* experiments or *in vivo* ones but using heterologously expressed glycoproteins [8,9,11,35]. In *uggt*^{-/-} cells TcrCATL was unable to finish its folding process and was retained in the ER by BiP in a mostly extended conformation. A minor fraction of TcrCATL was able to reach the lysosomes in a compact conformation, presumably due to spontaneous folding without the assistance of CRT. These observations show that ER

retention can be accomplished either by BiP or by CRT, although at different stages of conformational maturation. Even though in *uggt*^{-/-} parasites BiP expression is induced approximately 3-fold [20], this chaperone cannot replace the specific functions played by CRT. Therefore, CRT not only retained immature TcrCATL, but also played an active role during the protease folding process. This role could be aggregation prevention and/or facilitation of correct disulfide bond formation via a CRT-associated protein disulfide isomerase as has been described for ERp57 in mammalian cells. We are currently exploring this possibility. In addition, lack of UGGT triggered the formation of heavy disulfidebonded forms of TcrCATL, illustrating the ability of CRT to prevent TcrCATL aggregation. On the other hand, we can no rule out that a protein of the PDI family could be trapped in these complexes. A novel endogenous UGGT substrate named prosaposin has been recently identified in mammalian cells [36]. Similarly to our observation, in *uggt*^{-/-} cells prosaposin secretion is severely impaired and the protein localizes to aggresome-like inclusions. In summary, the activities of BiP and CRT are finely tuned to complement each other in order to nurture the complete folding process of TcrCATL, from the access of the growing chain to the ER to the final acquisition of the native structure. The present report provides further evidence on early evolutionary acquisition of the basic tenets of the QC mechanism of glycoprotein folding represent [37].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AMS	4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid
BiP	immunoglobulin heavy chain binding protein
CNX	calnexin
CRT	calreticulin
DTT	dithiothreitol
ER	endoplasmic reticulum
IAM	iodoacetamide
QC	quality control
TcrCATL	stands for a protease previously called cruzipain or cruzain
UGGT	UDP-Glc:glycoprotein glucosyltransferase

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.molbiopara.2010.10.002.

Fig. 1.

TcrCATL oxidation *in vivo*. Wild type or UGGT null parasites were radio-labeled for 5 min with [³⁵S]-Met and [³⁵S]-Cys and chased with complete media for the indicated times. 5 mM IAM was added to block free cysteines and the ER (A) and lysosomal (B) fractions were obtained as described under Section 2. Samples were then reduced with 10 mM DTT and incubated with 30 mM AMS. TcrCATL was immunoprecipitated using a polyclonal antibody, analyzed by SDS-PAGE and detected by autoradiography. Lower right gel: TcrCATL obtained from the lysosomal fraction was reduced with 10 mM DTT in 8 M urea and alkylated either with 30 mM AMS, 30 mM IAM or a mixture of 30 mM AMS plus 5 mM IAM (central lane). The arrows indicate the migration of unmodified TcrCATL.

Fig. 2.

Chaperone association with TcrCATL in the ER. Wild type (A and B) or UGGT null parasites (C) were radiolabeled for 5 min with [³⁵S]-Met and [³⁵S]-Cys and chased with complete media for indicated times. The ER fractions were prepared and CRT (B) or BiP (A and C) containing complexes were immunoprecipitated. Precipitates were then subjected to a second immunoprecipitation with anti-TcrCATL serum. The presence of TcrCATL was analyzed by SDS-PAGE and autoradiography. Right panels: quantification of TcrCATL by optical densitometry. The values are normalized by the maximum value of each experiment. Bars represent the standard error of triplicate experiments. (D) Folding status of TcrCATL associated with BiP or CRT. ER fractions were prepared from wild type and UGGT null parasites. Protein complexes containing BiP or CRT were isolated by immunoprecipitation. The samples were alkylated (see legend to Fig. 1) and TcrCATL was analyzed by SDS-PAGE and revealed by western blot using anti TcrCATL serum. The arrow indicates the migration of unmodified TcrCATL. Right gel: TcrCATL obtained from the ER fraction was reduced with 5 mM DTT in 8 M urea and alkylated either with 30 mM AMS, 30 mM IAM or a mixture of 30 mM AMS plus 5 mM IAM (central lane).

Fig. 3.

Analysis of TcrCATL aggregation. ER and lysosomal fractions were prepared from wild type and UGGT null parasites. Samples were analyzed by reducing or non-reducing SDS-PAGE and TcrCATL was revealed by western blot.

Fig. 4.

Model for TcrCATL–chaperone interaction in the ER. In wild type parasites TcrCATL first interacts with BiP when displaying its Cys residues in a mostly reduced state. More oxidized stages interact with CRT after being glucosylated by UGGT. In *uggt*^{-/-} parasites TcrCATL interacts with BiP in a mostly reduced state. The absence of UGGT precludes TcrCATL interaction with CRT. A minor fraction of TcrCATL folds without further assistance and may reach lysosomes, while the protein retained in the ER forms disulfide bridge-bonded aggregates.