Calreticulin functions *in vitro* as a molecular chaperone for both glycosylated and non-glycosylated proteins

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Calreticulin (CRT) is thought to be a molecular chaperone that interacts with glycoproteins exclusively through a lectin site specific for monoglucosylated oligosaccharides. However, this chaperone function has never been directly demonstrated nor is it clear how lectin–oligosaccharide interactions facilitate glycoprotein folding. Using purified components, we show that CRT suppresses the aggregation not only of a glycoprotein bearing monoglucosylated oligosaccharides but also that of non-glycosylated proteins. Furthermore, CRT forms stable complexes with unfolded, non-glycosylated substrates but does not associate with native proteins. ATP and Zn²⁺ enhance CRT's ability to suppress aggregation of non-glycoproteins, whereas engagement of its lectin site with purified oligosaccharide attenuates this function. CRT also confers protection against thermal inactivation and maintains substrates in a folding-competent state. We conclude that in addition to being a lectin CRT possesses a polypeptide binding capacity capable of discriminating between protein conformational states and that it functions *in vitro* as a classical molecular chaperone.

Keywords: calreticulin/endoplasmic reticulum/molecular chaperone/protein folding

Introduction

Calreticulin (CRT) is an abundant 46 kDa luminal protein of the endoplasmic reticulum (ER) that participates in Ca²⁺ storage and intracellular Ca²⁺ signalling, cell adhesion, gene expression and glycoprotein folding (Krause and Michalak, 1997). CRT binds Ca²⁺, Zn²⁺, possibly ATP, and also the thiol oxidoreductases ERp57 and protein disulfide isomerase (Khanna *et al.*, 1986; Nigam *et al.*, 1994; Baksh *et al.*, 1995; Corbett *et al.*, 1999). It is 39% identical to the ER membrane protein calnexin (CNX) and, like CNX, it is a lectin that binds to the Asn-linked oligosaccharide processing intermediate Glc₁-Man₉GlcNAc₂ (Ware *et al.*, 1995; Spiro *et al.*, 1996). It is widely believed that CRT functions as a molecular chaperone for Asn-linked glycoproteins, since it associates predominantly with folding or assembly intermediates *in vivo* but not with fully folded glycoproteins.

Furthermore, when the binding of CRT and CNX are simultaneously prevented by interfering with the formation of their oligosaccharide ligand, the efficiency of folding of most glycoproteins is diminished and the formation of misfolded aggregates is frequently observed (reviewed in Helenius *et al.*, 1997; Leach and Williams, 1999). However, these *in vivo* experiments are indirect and it remains an open question whether CRT functions as a true molecular chaperone, i.e. a protein that binds to hydrophobic surfaces exposed in unfolded but not native protein conformers, thereby preventing incorrect interactions (such as aggregation) and increasing the yield of properly folded protein (Bukau and Horwich, 1998).

Two models have been proposed to describe the interactions of CRT (and CNX) with nascent glycoproteins. Following the cotranslational transfer of Glc₃Man₉GlcNAc₂ oligosaccharides to polypeptide, glucosidases I and II remove the two outermost glucose residues to form the monoglucosylated (Glc₁Man₉GlcNAc₂) oligosaccharide recognized by CRT. In the 'lectin-only model', CRT binds to glycoproteins exclusively through lectin–oligosaccharide interactions (Peterson *et al.*, 1995). It is thought that these complexes are dissociated by the further action of glucosidase II, which removes the single glucose residue, and that re-binding can occur if the single glucose is added back by UDP-glucose:glycoprotein glucosyltransferase, an ER enzyme that glucosylates unfolded but not native glycoproteins (Sousa and Parodi, 1995). The glucosyltransferase functions as the folding sensor and the cycle continues until a native conformation is attained. CRT does not function as a typical molecular chaperone in this model since it does not bind to non-native polypeptide segments and thereby lacks the capacity to discriminate between glycoprotein conformational states. Rather, its lectin function may help to prevent aggregation through steric interference (Helenius *et al.*, 1997) or it may promote folding by recruiting other ER chaperones and folding enzymes such as ERp57 to the unfolded glycoprotein (Elliott *et al.*, 1997). The lectin-only model is supported by the finding that glucosidase inhibitors block complex formation and, if added after complexes are formed, can block complex dissociation (reviewed in Helenius *et al.*, 1997). Furthermore, CRT was shown to be unable to discriminate between reduced and native forms of monoglucosylated RNase B, and complexes between CRT and RNase B could be dissociated *in vitro* solely by digestion with glucosidase II (Rodan *et al.*, 1996).

The 'dual-binding model' includes the main tenets of the lectin-only model but proposes that CRT (and CNX) possesses a second binding site that is able to recognize polypeptide segments of unfolded glycoproteins, i.e. it functions as a classical chaperone (Ware *et al.*, 1995; Leach and Williams, 1999). This model requires that the dissociation of CRT–glycoprotein complexes occurs

through the action of glucosidase II as well as a conformational change in the polypeptide binding site. Cycles of binding and release would continue until the glycoprotein folds into a conformation no longer recognized by CRT's polypeptide binding site or by the glucosyltransferase. Support for this model comes from the finding that complexes of CNX and a variety of glycoproteins cannot be dissociated simply by oligosaccharide removal (Arunachalam and Cresswell, 1995; Ware *et al.*, 1995). Furthermore, CRT binds to several non-glycosylated proteins including protein disulfide isomerase (Baksh *et al.*, 1995) and ERp57 (Corbett *et al.*, 1999), and also to non-glycosylated peptides both *in vitro* and *in vivo* (Basu and Srivastava, 1999). Finally, CRT has been shown to discriminate in its binding between native and non-native conformations of non-glycosylated proteins *in vitro* (Sværke and Houen, 1998).

To distinguish between these models, we took advantage of *in vitro* assays that have been used to characterize the functions of the HSP90, HSP70, HSP60 and small heat shock protein families of chaperones. We found that CRT possesses a polypeptide binding capability that distinguishes between unfolded and native protein conformers. Although its lectin site confers some preference for glycoproteins, CRT efficiently suppresses the aggregation of both non-glycosylated and glycosylated substrates, it inhibits thermal denaturation, and maintains unfolded proteins in a conformation competent for refolding. We conclude that CRT is a true molecular chaperone that utilizes both lectin and polypeptide binding sites to effect its functions.

Results

CRT prevents the thermal aggregation of glycosylated and non-glycosylated IgY

To investigate potential chaperone functions of CRT, we tested its ability to suppress the thermal aggregation of IgY, the predominant soluble antibody in birds. Ohta *et al.* (1991) determined that the IgY heavy chain (H chain) contains 27.1% monoglucosylated oligosaccharides ($\text{Glc}_1\text{Man}_{7-9}\text{GlcNAc}_2$) that are ligands for CRT's lectin site, 8.6% $\text{Man}_{5-9}\text{GlcNAc}_2$ oligosaccharides, and the remainder complex oligosaccharides. By treating IgY with endoglycosidase H (endo H) and peptide *N*-glycanase F (PNGase F), we determined that the H chain contains one high-mannose and one complex oligosaccharide. The L chain was not glycosylated. Furthermore, the predominance of the $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ species on the H chain was confirmed by fluorophore-assisted carbohydrate electrophoresis of oligosaccharides released by endo H digestion (data not shown).

To assess the effect of CRT on the aggregation of unfolded IgY, reduced and chemically denatured IgY was diluted rapidly into solutions containing various concentrations of CRT and heated at 44°C. This temperature was used because aggregation was minimal at 37°C. In these assays, molar ratios were calculated assuming monomeric IgY H and L chains, and a monomeric form of CRT. As shown in Figure 1A, IgY aggregated rapidly following the shift to 44°C and CRT was capable of suppressing its aggregation in a dose-dependent manner. Aggregation was almost completely prevented when CRT

was present at a 1:4 stoichiometry with IgY. Mouse IgG added at the same molar ratio did not affect aggregate formation.

The participation of CRT's lectin site in its aggregation-suppressing function was assessed by completely removing glucosylated oligosaccharides from IgY with endo H (eH-IgY). As shown in Figure 1B, CRT effectively suppressed the aggregation of eH-IgY, although maximal suppression required an equimolar concentration of CRT. Similar results were obtained when both high-mannose and complex glycans were removed by PNGase F digestion (Figure 1C, PF-IgY). Note that the latter experiment was performed at 31°C since the completely deglycosylated IgY was much more prone to aggregation. These results suggest that lectin-oligosaccharide interactions are involved in CRT's ability to suppress IgY aggregation but that CRT also has the capacity to suppress aggregation through protein-protein interactions in a manner similar to other molecular chaperones.

The ability of CRT to suppress aggregation was not an artefact arising from its instability at 44°C since CRT effectively suppressed the aggregation of PF-IgY at 31°C. Furthermore, when CRT was tested for its ability to compete for the binding of $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ oligosaccharide and ERp57 to immobilized glutathione *S*-transferase (GST)-CNX, it was found to be as effective a competitor at 44°C as it was at 22°C (Table I).

CRT suppresses aggregation of the non-glycosylated proteins citrate synthase and malate dehydrogenase

To confirm the capacity of CRT to suppress aggregation through interactions independent of its lectin site, we tested its ability to prevent the thermal aggregation of proteins that naturally lack Asn-linked oligosaccharides. Citrate synthase (CS) and malate dehydrogenase (MDH) are mitochondrial/cytosolic proteins that are homodimers of 50 and 35 kDa subunits, respectively. Both have been used extensively as substrates in aggregation and refolding assays with molecular chaperones of the HSP90, HSP60 and small heat shock protein families (Buchner *et al.*, 1991; Jakob *et al.*, 1995; Lee *et al.*, 1997). Upon heating at 43–45°C these enzymes denature and form large aggregates. As shown in Figure 2A and B, CRT effectively suppressed the thermal aggregation of both CS and MDH. Similar to other classes of chaperones, CRT was effective in stoichiometric amounts (expressed as CRT monomer:CS or MDH dimer). Essentially complete suppression of MDH aggregation was observed at a 1:1 molar ratio, whereas CS required a 2-fold molar excess of CRT. In contrast, equivalent concentrations of mouse IgG had only minimal effects. Therefore, CRT resembles other molecular chaperones in its capacity to suppress thermal aggregation and is capable of doing so in the absence of lectin-oligosaccharide interactions.

CRT suppresses protein aggregation through a site distinct from its ERp57 binding site

ERp57 is a thiol oxidoreductase that binds to both CRT and CNX (Zapun *et al.*, 1998; Corbett *et al.*, 1999). This association markedly enhances ERp57's activity toward substrates bound to CRT or CNX (Zapun *et al.*, 1998). To investigate the possibility that CRT may suppress

Fig. 1. Effect of CRT on the thermal aggregation of various glycoforms of IgY. (A–C) IgY, eH-IgY or PF-IgY were denatured in 6 M guanidine hydrochloride containing 40 mM dithiothreitol and then diluted 100-fold (2.4 μ M final concentration of monomeric H and L chains) in the absence or presence of various amounts of CRT (0.038–2.4 μ M) or mouse IgG (0.6 or 2.4 μ M). Protein aggregation at 44°C (for IgY and eH-IgY) or at 31°C (for PF-IgY) was monitored by measuring light scattering at 360 nm.

Table I. Calreticulin retains oligosaccharide and ERp57 binding capability at 44°C

		Oligosaccharide binding to GST–CNX			ERp57 binding to GST–CNX		
		No competitor (c.p.m.)	CRT (c.p.m.)	% inhibition	No competitor (c.p.m.)	CRT (c.p.m.)	% inhibition
22°C	experiment 1	191	43	78	236	69	71
	experiment 2	200	48	76	537	28	95
44°C	experiment 1	141	13	91	179	26	85
	experiment 2	114	16	86	681	97	86

GST fused to the ER luminal segment of calnexin (GST–CNX, 1 μ M) was immobilized on 20 μ l of glutathione–agarose beads and incubated with either [3 H]Glc₁Man₉GlcNAc₂ oligosaccharide or [14 C]ERp57 in the absence or presence of 10 μ M CRT essentially as described by Vassilakos *et al.* (1998). Results are reported as specific binding (radioactivity bound to GST–CNX minus radioactivity bound to identically treated GST).

protein aggregation through its ERp57 binding site, we tested whether the presence of ERp57 would affect CRT's ability to prevent the thermal aggregation of MDH. In control experiments, ERp57 alone showed no tendency to aggregate at 45°C nor did it exhibit any capacity to inhibit MDH aggregation (Figure 2C). When ERp57 was mixed with CRT and MDH, ERp57 did not interfere with the ability of CRT to suppress MDH aggregation. This was also the case if ERp57 and CRT were first pre-incubated for 30 min at 25°C under conditions that permit complex formation (Zapun *et al.*, 1998) and then mixed with MDH and shifted to 45°C (Figure 2C). The lack of effect of ERp57 was not due to its dissociation from CRT at the elevated temperature because direct binding experiments with radiolabelled ERp57 confirmed that it remained bound to CRT at 45°C (data not shown).

It has been reported that the ERp57–CRT association requires Zn²⁺ (Corbett *et al.*, 1999). Although we did not observe this requirement in our direct binding studies, we repeated the experiments in the presence of 100 μ M ZnCl₂ but the aggregation-suppressing capacity of CRT remained unaltered (data not shown). Collectively, these results indicate that CRT suppresses protein aggregation through a site that is distinct from its site of binding to ERp57.

Identification and characterization of CRT–substrate complexes

The interaction between CRT and substrate proteins was analysed by size exclusion chromatography (SEC). When CRT was incubated with either CS or MDH for 60 min at 25°C, CS and MDH eluted at positions consistent with their dimeric molecular weights (Figure 3A and B). However, CRT eluted as a 330 kDa species, which was considerably larger than its expected mol. wt of 46 kDa, suggesting that it may exist as an oligomer, possibly a heptamer. This was not an artefact of its high-level expression or the method of purification from bacterial extracts since gel filtration of a rat liver microsomal extract, followed by immunoblotting of fractions, revealed that the endogenous CRT also eluted as an ~300 kDa species (data not shown). Upon incubation of CRT and CS or MDH at 45°C, a much larger species was progressively formed with an apparent mol. wt of ~1900 kDa (MDH) up to 2000 kDa (CS), considerably less than the 10 000 kDa exclusion limit of the column (Figure 3A and B, 45°C 0–60 min). This was not an aggregate of either CRT or substrate since incubation of CRT alone at 45°C for 60 min did not alter its elution behaviour except for the formation of a minor component of ~700 kDa also seen

Fig. 2. (A and B) Effect of CRT on the thermal aggregation of non-glycosylated proteins. CS or MDH dimers (1 μ M) were incubated at 45°C in the absence or presence of various concentrations of CRT (0.125–2 μ M) or IgG (1 or 2 μ M) as indicated. Aggregation was monitored by measuring light scattering at 360 nm. (C) Effect of ERp57 on the aggregation-suppressing function of CRT. Various combinations of MDH (0.6 μ M), CRT (0.24 μ M) and ERp57 (0.24 μ M) were incubated at 45°C as indicated and aggregation was monitored. In one case (designated 'pre'), CRT and ERp57 were pre-incubated at 25°C for 30 min and then incubated with MDH at 45°C.

in Figure 3A. Incubation of MDH or CS alone at 45°C produced insoluble aggregates that were removed by the low-speed centrifugation step employed prior to SEC analysis (data not shown). That the 1900–2000 kDa species was in fact a complex between CRT and CS or MDH was confirmed by SDS–PAGE analysis, which showed that the column fractions eluting at 6.5–6.6 min contained CRT and either CS or MDH (Figure 3C). Based on densitometric analysis (Figure 3C legend), the molar ratios in the complexes were 1 CRT monomer:2 CS or MDH dimers. If CRT is indeed a heptamer, this would correspond to a complex of one CRT heptamer per 14 substrate dimers with mol. wts of 1300 and 1700 kDa for MDH and CS, respectively, fairly close to the apparent molecular weights of the complexes.

To evaluate conformational differences between free and CRT-bound substrate, the sensitivity of substrate to tryptic digestion in each case was compared. MDH was used for this experiment since it was possible to prepare MDH in either free or completely CRT-bound states, depending on incubation conditions (Figure 3B, compare the 25 and 45°C panels at 60 min). MDH was incubated with CRT for 60 min at either 25°C (completely unbound) or 45°C (completely bound) and the mixtures were digested with trypsin and analysed by SDS–PAGE (Figure 3D). Whereas unbound, native MDH was resistant to digestion, MDH bound to CRT was hypersensitive to digestion, suggesting that it was in a substantially unfolded conformation.

Complexes between CRT and MDH were further analysed by electron microscopy. CRT incubated alone at 45°C appeared as spherical particles ranging from 7 to 15 nm in diameter (Figure 4, CRT), which was not significantly different from its appearance at 25°C (data

not shown). This size suggests an oligomeric form of CRT, consistent with the SEC data, although no regular subunit structure could be detected at higher magnification. MDH incubated at 45°C produced an insoluble aggregate that appeared as an extremely large network by electron microscopy (Figure 4, MDH; note the 10-fold lower magnification). When CRT and MDH were incubated together at 45°C, aggregate formation was suppressed and CRT–MDH complexes appeared as irregularly shaped particles ranging in size from ~25 to 50 nm (Figure 4, CRT+MDH). No complexes were observed when CRT and MDH were incubated at 25°C and, owing to the small size of MDH dimers, the mixture appeared essentially the same as CRT alone (data not shown).

Regulatory effects of oligosaccharide, ATP and Zn^{2+} on the aggregation-suppressing function of CRT

As an alternative means to assess the participation of lectin–oligosaccharide versus protein–protein interactions in the ability of CRT to suppress the aggregation of glycoproteins, we tested the effects of adding purified oligosaccharides that are known to bind to CRT's lectin site. We showed previously that Glc α 1-3Man α 1-2Man α 1-2Man-OH (G1M3) is a potent competitor for the binding of Glc α 1-3Man α 1-2Man-OH (G1M2) to CRT, whereas Glc α 1-3Man α 1-2Man-OH (G1M2) is 5-fold less potent and Glc α 1-3Man-OH (G1M1) or Glc α 1-3Glc-OH (G2) is 500-fold less potent than G1M3 (Vassilakos *et al.*, 1998). These oligosaccharides were added to mixtures containing IgY and CRT and their effects on the extent of aggregation suppression were examined (Figure 5A). The low-affinity oligosaccharides G2 and G1M1 had no effect on aggregation suppression by CRT, but G1M2 and

Fig. 3. Characterization of CRT-substrate complexes. (A and B) CS or MDH (1 μ M) were incubated with 2 or 1 μ M CRT, respectively, at either 25 or 45°C for the indicated periods of time and analysed by SEC. The sizes of molecular weight standards (kDa) are indicated above the elution profiles. Vo, void volume. (C) SDS-PAGE analysis of isolated CRT-substrate complexes. CRT was incubated with CS or MDH at 45°C for 60 min and then CRT-substrate complexes (elution time: 6.5 min for CS and 6.6 min for MDH) were isolated by SEC. Samples were analysed by 10% SDS-PAGE and stained with Coomassie Blue. Staining ratios obtained by densitometry using standards of CRT (1 μ g) and MDH or CS (2 μ g) were: CRT:MDH = 1:0.53 and CRT:CS = 1:0.86. Using these values, the observed staining ratios of CRT to MDH (1:0.81) or CS (1:1.73) in the complexes were corrected and used to calculate molar ratios. Molar ratios in the complexes were determined to be 1 CRT monomer: 2.0 MDH or CS dimers (average of three independent experiments). (D) Trypsin sensitivity of free and CRT-bound MDH. MDH (1 μ M) was incubated with CRT (1 μ M) at 25 or 45°C for 60 min. Samples were digested with the indicated amounts of trypsin on ice for 10 min and then analysed by 10% SDS-PAGE followed by staining with Coomassie Blue.

G1M3 inhibited CRT's ability to prevent IgY aggregation. This effect was dose-dependent with maximal inhibition occurring at 10 μ M G1M3 oligosaccharide (Figure 5B). Remarkably, when the same experiment was performed with endo H-treated IgY, G1M3 caused a substantial inhibition of CRT's aggregation-suppressing function despite the removal of glucosylated oligosaccharides from IgY. Maximal effect was observed at a 4- to 10-fold higher concentration of G1M3 when compared with fully glycosylated IgY (Figure 5C). This was not an artefact of using a higher concentration of G1M3 since both G2 and G1M1 oligosaccharides were ineffective at the same concentrations (data not shown). These results suggest two distinct effects of added oligosaccharide: (i) competitive inhibition of lectin-oligosaccharide interactions, thereby decreasing the binding avidity of CRT for the fully glycosylated IgY substrate; and (ii) engagement of CRT's

Fig. 4. Electron microscopic analysis of CRT, MDH and CRT-MDH complexes. CRT and MDH (1 μ M) were incubated alone or together at 45°C for 60 min. Samples were diluted 10-fold and then negatively stained with uranyl acetate. Note the 10-fold lower magnification in the MDH panel (6600 versus 66 000 for CRT and CRT+MDH).

lectin site with oligosaccharide modulates a polypeptide binding site in CRT, leading to less effective aggregation suppression via polypeptide-based interactions. The latter effect was also observed using the non-glycosylated CS and MDH substrates (Figure 5D and E). MDH showed considerably greater sensitivity to the addition of oligosaccharide than CS.

Given that several classes of chaperones are regulated

Fig. 5. Effect of oligosaccharides on the aggregation-suppressing function of CRT. (A) Chemically denatured IgY (2.4 μ M) was incubated with CRT (0.15 μ M) in the presence or absence of various oligosaccharides at 44°C for 60 min. Protein aggregation was measured by monitoring light scattering at 360 nm. The aggregation of IgY alone after 60 min incubation was defined as 100%. (B–E) Denatured IgY (2.4 μ M), denatured eH-IgY (2.4 μ M), CS (1 μ M) or MDH (1 μ M) were incubated with CRT (0.15 μ M for IgY, 1.2 μ M for eH-IgY, 0.5 μ M for CS and 0.3 μ M for MDH) in the presence of various concentrations of G1M3 oligosaccharide at 44–45°C for 60 min. Protein aggregation was measured and expressed relative to the aggregation of substrate alone.

by ATP binding and/or hydrolysis (Bukau and Horwich, 1998; Buchner, 1999) and that CRT appears to bind ATP (Nigam *et al.*, 1994), we tested the effect of various nucleotides on the ability of CRT to suppress protein aggregation. In these and subsequent experiments, CRT was used at a lower molar ratio such that substrate aggregation was suppressed to 50–80% of control values. Contrary to what was observed with G1M3 oligosaccharide, ATP enhanced CRT's ability to suppress the aggregation of IgY (Figure 6A). The effect was dose-dependent with a detectable enhancement at 0.3 mM and maximal enhancement at 3 mM (Figure 6B). ATP did not act through modulation of CRT's lectin site since the enhancement was also observed using the eH-IgY, CS and MDH substrates (data not shown). Furthermore, 1 mM ATP has no effect on CRT's ability to bind Glc₁Man₉GlcNAc₂ oligosaccharide (Vassilakos *et al.*, 1998). CRT's ability to suppress IgY aggregation was also enhanced by the non-hydrolysable analogues ATP- γ -S and AMP-PCP, whereas ADP and AMP had only slight effects (Figure 6A). GTP also enhanced aggregation suppression but its effects were somewhat less potent than ATP at all concentrations tested. Similar nucleotide specificity was observed with the eH-IgY, CS and MDH substrates with the exception that GTP did not influence the suppression of CS aggregation (data

not shown). GTP as well as other nucleoside triphosphates have been reported to influence substrate interactions with the hsp70 chaperone DnaK (Liberek *et al.*, 1991).

CRT has been shown to bind up to 14 mol of Zn²⁺ and to undergo accompanying conformational changes that expose hydrophobic segments to solvent. Most of the conformational changes occur following the binding of only a single Zn²⁺ ion at a concentration of ~20 μ M ZnCl₂ (Khanna *et al.*, 1986). Consequently, we investigated the influence of Zn²⁺ on CRT's ability to suppress the aggregation of CS and MDH. It was not possible to use IgY or eH-IgY in these experiments because Zn²⁺ alone increased the aggregation of both substrates (data not shown). As shown in Figure 7A and B, the addition of ZnCl₂ enhanced aggregation suppression by CRT in a dose-dependent manner. The maximum effect was observed at 15 μ M ZnCl₂ in both cases. Higher concentrations of Zn²⁺ promoted substrate aggregation when assayed without CRT (data not shown).

To assess the relationship between the effects of G1M3 oligosaccharide, ATP and Zn²⁺, we examined these ligands in various combinations (Figure 7C). The effects of ATP and Zn²⁺ appeared to be additive since in combination they enhanced CRT's ability to suppress CS aggregation to 17% of control compared with 43% (Zn²⁺) and 51%

Fig. 6. Effect of nucleotides on aggregate suppression by CRT. Chemically denatured IgY (2.4 μ M) was incubated with CRT (0.0375 μ M) and 10 mM MgCl_2 in the absence or presence of various nucleotides (**A**) or various concentrations of ATP (**B**) as indicated. After incubation at 44°C for 60 min, protein aggregation was measured and expressed relative to the aggregation of IgY alone.

(ATP) when tested separately. Interestingly, the addition of G1M3 oligosaccharide to mixtures containing Zn^{2+} resulted in a complete loss of the enhancing effect of Zn^{2+} . In contrast, when G1M3 oligosaccharide was tested along with ATP, the enhancing effect of ATP was dominant and there was little difference in aggregation suppression compared with ATP alone. Likewise, when G1M3 oligosaccharide was added in combination with both ATP and Zn^{2+} , the combined enhancing effects of ATP and Zn^{2+} were dominant and no inhibitory effect of G1M3 was evident. Similar trends were observed when CRT and CS were incubated alone or along with ATP and/or Zn^{2+} for 15 min at 45°C under conditions where aggregation was almost completely suppressed and then G1M3 was added to the pre-formed CRT–CS complexes (Figure 7D). The addition of G1M3 to CRT–CS complexes resulted in a significant increase in aggregation, suggesting that the binding of oligosaccharide to CRT's lectin site results in a weakening of CRT–CS interactions, thereby releasing CS molecules for aggregation. The same effect was observed when G1M3 was added to complexes containing Zn^{2+} but not when G1M3 was added to complexes containing ATP or ATP plus Zn^{2+} .

Conformational changes of CRT induced by oligosaccharide, ATP and Zn^{2+}

To determine whether the various ligands shown to influence CRT's aggregation-suppressing function also induce conformational changes in the protein, we assessed the effects of ligand binding by measuring intrinsic (tryptophan) fluorescence. Changes in intrinsic fluorescence emission are largely due to changes in the exposure of tryptophan residues to solvent and they provide a sensitive means of detecting alterations in tertiary structure. Khanna *et al.* (1986) reported that Zn^{2+} binding to CRT results in a dose-dependent increase in the intensity of CRT's fluorescence emission spectrum as well as a red-shift of ~11 nm. We also detected this increase in fluorescence intensity at the 15 μ M concentration that provided maximal enhancement of aggregation suppression, although no shift in the emission spectrum could be detected (Figure 8A). The addition of 40 μ M G1M3 oligosaccharide was accompanied by an increase in fluorescence intensity as well, whereas 40 μ M G1M1 had no effect, consistent with the inability of the latter oligosaccharide to alter aggregation suppression by CRT. In contrast, ATP induced a substantial decrease in fluorescence intensity and ADP, which only slightly influenced aggregation suppression, had little effect. Although these experiments do not provide information on the nature of the conformational changes induced by ligand binding, they clearly demonstrate that the regulatory effects of G1M3, ATP and Zn^{2+} on aggregation suppression by CRT are accompanied by corresponding changes in tertiary structure.

1-anilino-8-naphthalenesulfonic acid (ANS) is frequently used as a probe to monitor the surface hydrophobicity of proteins. The fluorescence emission intensity of ANS increases and undergoes a spectral blue-shift when placed in a hydrophobic environment. As shown in Figure 8B, both ATP and Zn^{2+} enhanced the intensity of the ANS emission spectrum when compared with the spectrum of ANS bound to CRT alone. Zn^{2+} binding was also accompanied by a slight blue-shift in the emission spectrum. These findings suggest that both ligands increase the surface hydrophobicity of CRT, although the nature of the environment reported on by ANS appears to be different. An increase in surface hydrophobicity is consistent with the ability of these ligands to enhance CRT's aggregation-suppressing function. In contrast, the fluorescence intensity of ANS was decreased with G1M3 oligosaccharide, suggesting a less hydrophobic surface, in accordance with the inhibitory effect of this oligosaccharide on CRT's ability to suppress protein aggregation. Neither ADP nor G1M1 oligosaccharide altered the ANS emission spectrum significantly, consistent with their minimal effects on aggregation suppression by CRT.

Effect of CRT on the thermal inactivation and reactivation of CS

To investigate whether CRT is capable of protecting a protein against thermal denaturation, CS was inactivated at 43°C in the presence or absence of various concentrations of CRT (Figure 9A). When assayed alone, CS enzymatic activity decreased to ~25% of its initial value over 60 min. This thermal inactivation was suppressed by CRT in dose-dependent manner such that 85% of the activity could be retained in the presence of a 3-fold molar

Fig. 7. Effect of ZnCl₂ alone or in combination with ATP and oligosaccharide on aggregate suppression by CRT. (A and B) CS or MDH (1 μM) were incubated with CRT (0.5 μM for CS and 0.125 μM for MDH) in the presence of various concentrations of ZnCl₂ at 45°C for 60 min and protein aggregation measured. (C) CS (1 μM) was incubated at 45°C along with CRT (0.25 μM) and the indicated combinations of ZnCl₂ (15 μM), G1M3 (100 μM) and ATP (3 mM). CS aggregation was measured by monitoring light scattering at the indicated time points. (D) CS (1 μM) was incubated at 45°C with CRT (1 μM) and the indicated combinations of ZnCl₂ (15 μM) and ATP (3 mM). G1M3 (100 μM) was added after 15 min of incubation (arrow) and aggregation was measured at the time points indicated.

excess of CRT. In contrast, a 3-fold molar excess of mouse IgG provided only minimal protection. We could not assess the effect of regulatory factors in this assay because ATP (1 mM) and G1M3 (100 μM) modestly protected and ZnCl₂ (15 μM) accelerated the loss of the enzyme activity when they were tested with CS alone (data not shown).

To determine whether CRT is capable of reactivating the heat-denatured enzyme either alone or in cooperation with other molecular chaperones, CS was first inactivated at 43°C for 60 min in the presence of equimolar amounts of CRT or mouse IgG. For reactivation, samples were diluted into solutions containing either buffer or rabbit reticulocyte lysate (RRL) plus various combinations of ATP, G1M3 or ZnCl₂, and incubated at 25°C for 60 min. RRL is a rich source of ATP-dependent molecular chaperones and although its chaperone composition differs from that of the ER it provides a convenient means to detect properties of CRT that depend upon cooperation with other molecular chaperones. As shown in Figure 9B (+buffer), when CS was inactivated in the presence of IgG and then shifted to 25°C it exhibited no capacity to regain enzymatic activity on its own. However, a modest degree of refolding (~9%) was observed when RRL was

added during reactivation, although this was independent of the presence of ATP. When CRT alone was present during the inactivation and reactivation stages, no refolding of CS was observed (Figure 9C, +buffer). However, a substantial degree of refolding (23%) was observed when RRL was present during reactivation and this was largely dependent on the presence of ATP (Figure 9C, +RRL ± ATP). Neither G1M3 oligosaccharide nor Zn²⁺ had a significant impact on the ability of CRT to refold CS either alone or in cooperation with RRL. Importantly, no reactivation of CS was observed if CRT was omitted from the inactivation stage and only included during the reactivation with RRL and ATP (data not shown). These findings suggest that although CRT exhibits no capacity to refold CS directly, its presence during inactivation maintains CS molecules in a conformation competent for refolding. Productive refolding then requires the participation of additional ATP-dependent chaperones present in RRL.

Discussion

In general, molecular chaperones bind reversibly to hydrophobic segments that are exposed in unfolded but not

Fig. 8. Oligosaccharide, ATP and Zn^{2+} induce different conformational changes in CRT. (A) Intrinsic fluorescence. CRT (1 μM) was incubated with the indicated concentrations of G1M3 or G1M1 oligosaccharide, ATP or ADP (with 10 mM MgCl_2), or ZnCl_2 and the intrinsic emission spectra were monitored. (B) ANS fluorescence. CRT (1 μM) was mixed with ANS (10 μM) and the indicated concentrations of G1M3 or G1M1 oligosaccharide, ATP or ADP (with 10 mM MgCl_2), or ZnCl_2 and the ANS fluorescence emission spectra were monitored.

native proteins, thereby stabilizing folding intermediates and preventing aggregation. This function leads to an increased yield of correctly folded protein. In the case of the Hsp70, Hsp60 and possibly the Hsp90 chaperone families, cycles of binding and release are regulated by conformational changes accompanying ATP binding and hydrolysis (Bukau and Horwich, 1998; Buchner, 1999). In the present study, we determined that CRT possesses the essential features of a molecular chaperone. Functionally, it prevented the aggregation of both chemically and thermally denatured proteins and it did so in the near stoichiometric amounts characteristic of most other

molecular chaperones (Buchner *et al.*, 1991; Jakob *et al.*, 1995; Lee *et al.*, 1997; Bukau and Horwich, 1998). It also functioned like Hsp60 and Hsp90 to protect a substrate against thermal denaturation (Hartman *et al.*, 1993; Jakob *et al.*, 1995), and it maintained an unfolded substrate in a state competent for refolding, a property it shares with Hsp90, Hsp70 and small heat shock proteins (Freeman and Morimoto, 1996; Lee *et al.*, 1997). As expected for a molecular chaperone, CRT associated with the polypeptide portion of unfolded proteins as determined by its ability to suppress the aggregation of proteins lacking N-linked oligosaccharides (PF-IgY, CS and MDH), by the formation of stable complexes between CRT and non-glycosylated proteins as assessed by SEC, and by direct visualization of CRT-MDH complexes using electron microscopy. CRT was also capable of discriminating between unfolded and native conformational states since it formed complexes with MDH that was unfolded, as demonstrated by hypersensitivity to trypsin, but no association with native, enzymatically active MDH could be detected. Finally, CRT's aggregation-suppressing function with either glycosylated or non-glycosylated substrates was influenced by ATP. As with other chaperone families, ATP binding and hydrolysis may play a role in regulating cycles of substrate binding by CRT. CRT has only very weak ATPase activity (<0.1 pmol/min/ μg protein; data not shown) but it is conceivable that this activity may be stimulated by co-chaperones within the ER, similar to the action of the Hsp40 co-chaperone on Hsp70 (Bukau and Horwich, 1998). Collectively, the data indicate that CRT functions as a true molecular chaperone and not simply as a lectin that binds glycoprotein substrates for presentation to other folding enzymes and molecular chaperones of the ER.

Our findings are at odds with previous *in vitro* studies showing that CRT bound to monoglucosylated RNase B irrespective of its conformational state and that the association was mediated solely by lectin-oligosaccharide interactions (Rodan *et al.*, 1996). We assume that this discrepancy is due to differences in the substrates tested since we have observed variation in the efficiency with which CRT suppresses the aggregation of different substrates. Whereas equimolar concentrations of CRT were capable of completely suppressing the aggregation of MDH, a 2-fold excess of CRT was required for CS. Substrate selectivity has also been documented for Hsp90 and cytosolic chaperonins (Kubota and Willison, 1997; Buchner, 1999). Unfolded RNase B may lack the polypeptide elements recognized by CRT and thereby rely exclusively upon lectin-oligosaccharide interactions for its association with CRT.

Although we have focused largely upon CRT's capacity to bind to polypeptide segments of unfolded proteins, it is clear that its lectin site plays an important role in its functions. This is exemplified by a host of *in vivo* studies demonstrating that the formation of immunoprecipitable complexes of CRT and most of its glycoprotein substrates can be prevented by blocking N-linked glycosylation or by preventing the formation of monoglucosylated oligosaccharides (Helenius *et al.*, 1997; Leach and Williams, 1999). The *in vitro* experiments presented here also underscore the contribution of lectin-oligosaccharide interactions in CRT's aggregation-suppressing function.

Fig. 9. (A) The effect of CRT on the thermal inactivation of CS. CS (1 μ M) was incubated at 43°C with or without CRT (1–3 μ M) or 3 μ M IgG. CS activity was assayed at the indicated times. (B and C) Refolding of CS after thermal inactivation. CS (1 μ M) was heated at 43°C for 60 min in the presence of CRT or IgG (1 μ M). For refolding, CS was shifted to 25°C and diluted 3-fold in buffer alone or buffer containing the indicated combinations of RRL (13.3%), ATP (1 mM) plus an ATP regeneration system, G1M3 (40 μ M) or ZnCl₂ (15 μ M). CS activity was assayed at the times indicated.

The aggregation of monoglucosylated IgY was effectively suppressed at a 4- to 8-fold lower concentration of CRT when compared with eH-IgY and PF-IgY, both of which lack monoglucosylated oligosaccharides. Likewise, CRT's ability to suppress the aggregation of monoglucosylated IgY was inhibited at about a 4-fold lower concentration of G1M3 oligosaccharide when compared with eH-IgY, consistent with G1M3 acting in part as a direct competitor of lectin–oligosaccharide interactions with the IgY substrate. In our aggregation experiments with denatured IgY, both the H chain, which contains a glycosylation site largely occupied by monoglucosylated oligosaccharides, and the non-glycosylated L chain participate in aggregate formation as detected by SDS–PAGE (data not shown). It is likely that CRT would be even more effective with a homogeneous substrate containing exclusively monoglucosylated oligosaccharides. We propose that for glycoprotein substrates the lectin–oligosaccharide interaction acts in conjunction with polypeptide-based associations to increase overall binding avidity and thereby suppress aggregate formation more effectively.

We found three factors that modulated the aggregation-suppressing function of CRT. Surprisingly, G1M3 and G1M2 oligosaccharides inhibited the ability of CRT to suppress the aggregation of non-glycosylated substrates. Since engagement of CRT's lectin site is accompanied by conformational changes that reduce surface hydrophobicity, it is likely that these changes alter a polypeptide

binding site such that it binds less effectively to unfolded protein substrates. In contrast, both ATP and Zn²⁺ enhanced CRT's ability to suppress aggregation in an additive fashion. Binding of these ligands was accompanied by distinctly different conformational changes that led to an increase in surface hydrophobicity of CRT. Combined with the observation that G1M3 binding could reverse the effect of Zn²⁺ but not the effect of ATP, the data are consistent with the notion that ATP and Zn²⁺ expose hydrophobic polypeptide binding sites in different regions of the CRT molecule, a situation reminiscent of the two polypeptide binding sites in the Hsp90 monomer (Buchner, 1999). Interestingly, when ATP and Zn²⁺ were present simultaneously, G1M3 was unable to reverse any of their combined effects. This latter observation, combined with the fact that ATP and Zn²⁺ are present within the ER (Reddy *et al.*, 1989; Clairmont *et al.*, 1992), leads us to suggest that the ATP- and Zn²⁺-bound form of CRT may be the predominant form that associates with unfolded glycoprotein substrates. It is noteworthy that ATP has different effects on the interactions of various chaperone families with unfolded substrate. In contrast to CRT, ATP promotes substrate dissociation from Hsp70 and Hsp60 and increases aggregation (Buchner *et al.*, 1991; Jakob *et al.*, 1996). However, ATP has little effect on aggregation suppression by Hsp90 (Jakob *et al.*, 1996) and enhances suppression of substrate aggregation by the small Hsp α B crystallin (Muchowski and Clark, 1998).

We propose the following revised dual-binding model to describe CRT's association with nascent Asn-linked glycoproteins. Following the formation of monoglucosylated oligosaccharides by the action of glucosidases I and II, a newly synthesized glycoprotein associates initially with the Zn^{2+} - and ATP-bound form of CRT. This is a high-avidity interaction involving both the lectin site and possibly two polypeptide binding sites in CRT. In this form, CRT effectively suppresses glycoprotein aggregation, maintains folding competence, and promotes formation and rearrangement of disulfide bonds through its association with ERp57 [via a site distinct from its site(s) of polypeptide binding; Figure 2C]. Upon loss or hydrolysis of ATP, possibly aided by co-chaperones, the polypeptide site(s) shifts to a lower affinity state, which is further destabilized by the continued engagement of the lectin site with oligosaccharide. In conjunction with deglycosylation by glucosidase II, which has been shown to be important for complex dissociation (Helenius *et al.*, 1997), the unfolded glycoprotein is released to undergo folding. If folding does not occur rapidly, the glycoprotein is a substrate for reglucosylation and subsequent rebinding to the ATP and Zn^{2+} form of CRT.

We have recently found that the ER luminal domain of CNX efficiently suppresses the aggregation of both glycosylated and non-glycosylated proteins, maintains folding competence, and prevents thermal denaturation in a manner virtually indistinguishable from CRT (Ihara *et al.*, 1999). Thus, the ER contains two bona fide molecular chaperones that utilize both oligosaccharide and polypeptide binding to associate with glycoprotein substrates, raising interesting questions about how they divide the labour of chaperoning nascent glycoproteins. Their conservation of functions is somewhat surprising given that they are only 39% identical and that the majority of identical residues are concentrated in a central region upon which their lectin sites are centred (Vassilakos *et al.*, 1998). It will be of particular interest to determine whether their polypeptide binding sites are located in less conserved regions and to what extent this influences their selection of overlapping but distinctly different sets of glycoprotein substrates (Helenius *et al.*, 1997).

Materials and methods

Purification of CRT and IgY

A pGEX-3X plasmid (Pharmacia) encoding GST fused to the N-terminus of rabbit calreticulin (GST-CRT) via a factor Xa-cleavable linker was provided by Dr Marek Michalak, University of Alberta (Baksh and Michalak, 1991). *Escherichia coli* BL21 cells were transformed with this plasmid and the GST-CRT fusion protein was expressed by induction with 0.1 mM isopropylthio- β -D-galactopyranoside for 16 h at room temperature. GST-CRT was purified using glutathione-agarose and CRT was released by Factor Xa cleavage essentially as described by the manufacturer (Pharmacia). CRT was further purified using a Mono Q anion-exchange column (Pharmacia) equilibrated in 10 mM Tris-HCl, 50 mM NaCl, 5 mM CaCl_2 pH 7.4. The column was developed with a linear 0.05–1 M NaCl gradient in the same buffer. CRT eluted at 0.47 M NaCl and was judged to be >99% pure by SDS-PAGE. Approximately 7.5 mg of purified CRT were obtained from 2 l of bacterial culture.

IgY was purified essentially as described by Akita and Nakai (1992). Approximately 250 mg of IgY (>95% pure) were obtained from five chicken eggs.

Aggregation assays

IgY was prepared as described above and its digestion with endo H (eH-IgY) or with PNGase F (PF-IgY) was performed as described by

New England Biolabs. All three forms of IgY (10 mg/ml) were denatured with 0.1 M Tris-HCl, 6 M Gdn-HCl, 40 mM dithiothreitol pH 8.0 for 2 h at room temperature. The denatured proteins were diluted 100-fold (2.4 μM final) into buffer containing 10 mM Tris-HCl, 0.15 M NaCl, 5 mM CaCl_2 pH 7.2 (TSC buffer), and various concentrations of either CRT or mouse IgG. Protein aggregation was induced by incubating the sample at 44°C for IgY and eH-IgY or at 31°C for PF-IgY. Aggregation was monitored over a period of 60 min by measuring light scattering at 360 nm in a Shimadzu 1601 spectrophotometer equipped with a temperature-controlled cell holder.

The non-glycosylated proteins CS (Roche Biomedical) and MDH (Sigma) aggregate without a requirement for prior chemical denaturation. CS or MDH (1 μM) were mixed with various concentrations of CRT or mouse IgG in TSC buffer. The samples were shifted to 45°C and aggregation was monitored by measuring light scattering at 360 nm.

Analysis of CRT-substrate complexes

Following incubation of CRT alone or with CS or MDH at 25 or 45°C, the samples were centrifuged at 5000 r.p.m. for 1 min and the supernatant fractions subjected to size exclusion chromatography with a TSK G4000 SW_{XL} column (TosoH Corp.). The mobile phase consisted of TSC buffer running at 1.0 ml/min at 25°C. Proteins were detected by absorbance at 230 nm.

For electron microscopic analysis of complexes, CRT and MDH (1 μM) were incubated alone or together in TSC buffer at 25 or 45°C for 60 min. The samples were diluted 10-fold with TSC buffer and then applied to glow-discharged carbon-coated copper grids. Following a 3 min incubation, proteins were negatively stained with 2% uranyl acetate. A Hitachi H600 transmission electron microscope operated at 75 kV was used to record electron micrographs at a magnification of 6600 or 66 000.

To assess the protease sensitivity of MDH complexed with CRT, CRT (1.5 μM) was incubated with MDH (1.5 μM) in TSC buffer at 25 or 45°C for 60 min. After centrifugation at 5000 r.p.m. for 1 min, soluble supernatants were treated with 1–40 $\mu\text{g}/\text{ml}$ trypsin for 10 min on ice. Reactions were terminated by adding 60 $\mu\text{g}/\text{ml}$ 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 10 $\mu\text{g}/\text{ml}$ antipain and 10 $\mu\text{g}/\text{ml}$ leupeptin, and proteins were analysed by SDS-PAGE (10% gel) with visualization by Coomassie Blue staining.

Fluorescence measurements

Fluorescence measurements were carried out with 1 μM CRT in TSC buffer containing various concentrations of ZnCl_2 , ATP or ADP (with 10 mM MgCl_2), and G1M3 or G1M1 oligosaccharide. Samples were allowed to equilibrate for 15 min before fluorescence measurements were taken. For intrinsic (tryptophan) fluorescence, emission spectra were monitored using a FluoroMax spectrofluorometer (Spex) with an excitation wavelength of 290 nm. Changes in intrinsic fluorescence were corrected for the absorption of ATP or ADP (inner filter effect) as described by Jakob *et al.* (1996). The binding of the hydrophobic fluorescent probe ANS (Sigma) to CRT was assessed by adding ANS to samples at a final concentration of 10 μM and monitoring emission spectra using an excitation wavelength of 370 nm. For each analysis, background fluorescence of the solution without CRT was subtracted from the measured values with CRT.

Reactivation of thermally inactivated CS

CS (1 μM) was inactivated in 50 μl of 10 mM Tris, 50 mM NaCl, 5 mM CaCl_2 pH 7.2 at 43°C in the presence of 1 μM CRT or mouse IgG. After 60 min, reactivation was initiated by diluting the sample with 2 vols of 10 mM Tris, 50 mM NaCl, 37.5 mM KCl, 5 mM CaCl_2 , 15 mM MgCl_2 pH 7.2 and also by shifting the temperature to 25°C. The reactivation mixture was supplemented with various combinations of 13.3% RRL (Promega), 1 mM ATP and an ATP-regenerating system consisting of 10 mM phosphocreatine and 50 $\mu\text{g}/\text{ml}$ creatine phosphokinase, 40 μM G1M3 and 15 μM ZnCl_2 . Aliquots were taken at various times and CS activity was measured as described by Buchner *et al.* (1998).

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