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Conformation-Independent Binding of Monoglucosylated Ribonuclease B to Calnexin

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Summary

Calnexin is a membrane protein of the endoplasmic reticulum that associates transiently with newly synthesized N-linked glycoproteins in vivo. Using defined components, the binding of ribonuclease B (RNase B) Man_7-Man_9 glycoforms to the luminal domain of calnexin was observed in vitro only if RNase B was monoglucosylated. Binding was independent of the conformation of the glycoprotein. Calnexin protected monoglucosylated RNase B from the action of glucosidase II and PNGase F but not from that of Endo H, which completely released the protein from calnexin. These observations directly demonstrate that calnexin can act exclusively as a lectin.

Introduction

Folding intermediates of several N-linked glycoproteins have been found to associate transiently in the endoplasmic reticulum (ER) with the type I membrane protein calnexin (Ou et al., 1993; Hammond et al., 1994; Tatu et al., 1995). N-linked oligosaccharides are attached to asparagine residues in N-X-S/T motifs while the polypeptides are being translocated into the ER. Treatment of cells with tunicamycin, an inhibitor of N-linked glycosylation, abolishes the association of glycoproteins with calnexin. These observations suggested that calnexin is a specialized chaperone for N-linked glycoproteins (Ou et al., 1993). More recently, calnexin has been found to improve the yield of the folding and assembly of major histocompatibility complex (MHC) class I molecules in vivo (Vassilakos et al., 1996) and of influenza hemagglutinin in microsomes (Hebert et al., 1996).

N-linked glycans are synthesized as a core unit of 14 residues ($GlcNAc_2Man_9Glc_3$). Once attached to proteins, these oligosaccharides are trimmed in the ER by glucosidase I, which removes the most external glucose, and glucosidase II, which successively removes the two remaining glucose residues (Hubbard and Ivatt, 1981). In cells treated with the glucosidase inhibitors castanospermine or 1-deoxynojirimycin or in cells deficient in either of the two glucosidases, the association of substrates with calnexin is suppressed (Hammond et al., 1994; Kearse et al., 1994; Balow et al., 1995; Hebert et al., 1995; Ora and Helenius, 1995). A temperature-sensitive mutant of vesicular stomatitis virus protein G, which is retained in the ER in association with calnexin, has predominantly monoglucosylated oligosaccharides (Suh et al., 1989; Hammond et al., 1994). These observations led to the proposal that calnexin may act as a lectin, which binds only to monoglucosylated glycoproteins (Hammond et al., 1994). Earlier work had shown that bromoconduritol, which inhibits the removal of the innermost glucose residue by glucosidase II (Datema et al., 1982), hinders the secretion of some glycoproteins but not of albumin, which is not glycosylated (Yeo et al., 1989). Yeo and colleagues speculated that the "$GlcNAc_2Man_9Glc_1$ structure may be part of the recognition site that is recognized by specific membrane receptors whose binding prevent[s] export from the rough ER."

Discrimination between fully folded and incompletely folded glycoproteins in the ER is thought to result from the specificity of the UDP-glucose:glycoprotein glucosyltransferase (UGGT). It had been shown that UGGT catalyzes the monoglucosylation of high mannose oligosaccharides on unfolded substrates (Trombetta et al., 1989; Sousa et al., 1992; Sousa and Parodi, 1995). The model can be summarized as a cycle of deglucosylation and glucosylation in which the monoglucosylated protein binds to calnexin and UGGT acts as the folding sensor. Once mature, a protein is no longer reglucosylated, does not bind to calnexin, and escapes the cycle, eventually to exit the ER (Helenius, 1994).

Further observations have supported a direct interaction between calnexin and the polypeptide chain of substrate proteins (Rajagopalan et al., 1994; Arunachalam and Cresswell, 1995; Carreno et al., 1995; Kim and Arvan, 1995; Loo and Clarke, 1995; Ware et al., 1995; Zhang et al., 1995). Therefore, calnexin, in addition to acting as a lectin for substrate recognition, may act as a true molecular chaperone according to the consensual definition of Hendrick and Hartl (1993): "a protein that binds to and stabilizes an otherwise unstable conformer of another protein, and by controlled binding and release of the substrate protein, facilitates its correct fate in vivo." Such a mechanism for calnexin is depicted in Figure 1A. However, if calnexin were to act simply as a lectin, binding would be irrespective of the conformational state of the substrate, as shown in Figure 1B. A

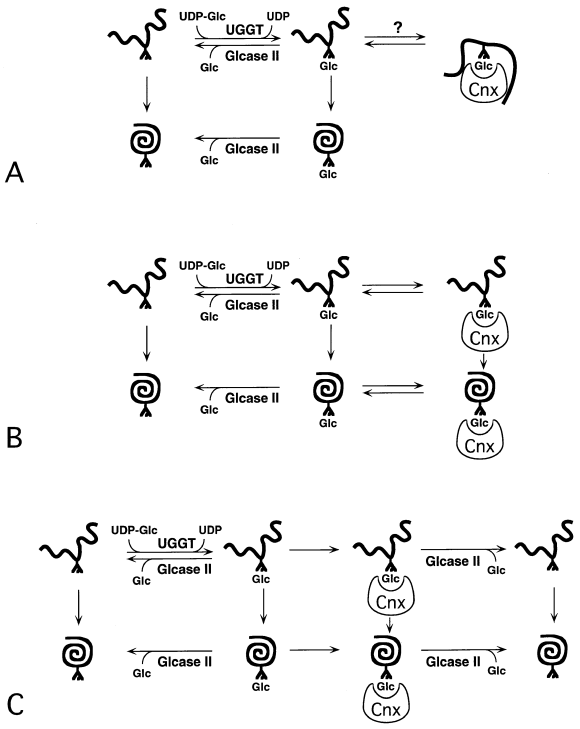


Figure 1. Models of the Interaction between Calnexin and Its Substrates

The serpentine and spiral structures represent unfolded and folded conformations of the substrate, respectively.

(A) In addition to recognizing monoglucosylated N-linked glycans, calnexin acts as a classic chaperone by binding and releasing substrates with nonnative conformations.

(B) Calnexin acts as a lectin binding monoglucosylated substrates, irrespective of their conformation. The interaction is an equilibrium and can be driven toward dissociation by the deglucosylation of the substrate by glucosidase II.

(C) Calnexin acts as a lectin as in (B), but release is triggered by deglucosylation of the substrate while it is bound.

Glcase II, glucosidase II.

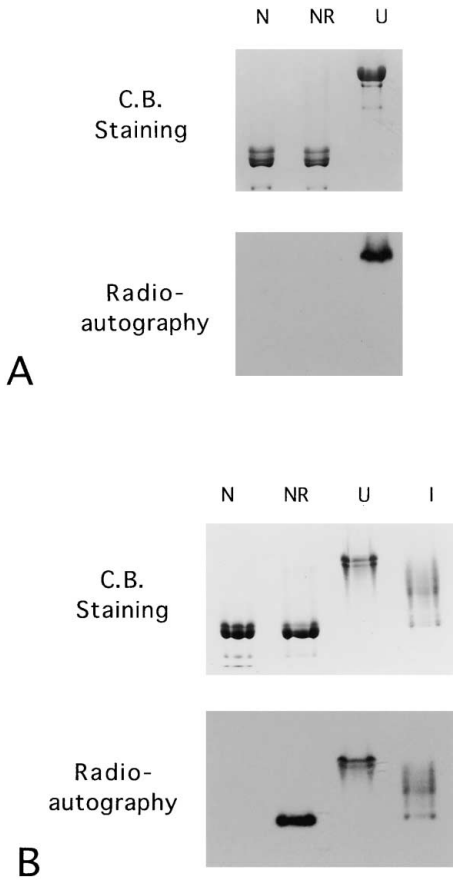


Figure 2. Glucosylation of Various Conformational States of RNase B Examined by Nondenaturing PAGE

(A) Specificity of UGGT for denatured RNase B. N-, NR-, and U-RNase B were incubated with UGGT and UDP-[^{3}H]glucose.

(B) Preparation of NR-G_1RNase B. Reduced denatured RNase B was incubated with UGGT and UDP-[^{3}H]glucose. Subsequently, the free thiol groups were blocked with iodoacetamide to generate U, or the protein was allowed to refold in the presence of PDI to form NR, or PDI was omitted and the folding reaction stopped with iodoacetamide to generate a mixture of intermediates (I). N-RNase B served as a control for the electrophoretic mobility of the other species.

C.B., Coomassie blue.

variety of alternative models also would be consistent with the available data. For example, calnexin may bind unfolded monoglucosylated substrate and release only folded substrate, with the possible involvement of releasing factors. As shown in Figure 1C, the release may result from the removal of the glucose residue of the substrate while it is bound to calnexin.

To distinguish among these models, we tested whether calnexin can discriminate among different conformational states of a protein. To reconstitute the binding reaction in vitro, the purified luminal domain (residues 1 to 462) of dog calnexin (Cnx ΔTMC; Ou et al., 1995) was used with bovine pancreatic ribonuclease B (RNase B) as a substrate, unfolded or refolded with recombinant protein disulfide isomerase (PDI), and monoglucosylated with UGGT purified from rat liver.

Results

Glucosylation of RNase B

Bovine pancreatic RNase B was selected to investigate the requirements for binding to calnexin because of its small size, unique site of N-glycosylation, and well-characterized conformation, which can easily be manipulated in vitro. RNase B has the sequence of RNase A but is N-glycosylated on Asn-34 with a glycan of the form $GlcNAc_2Man_{5-9}$ (Rudd et al., 1994). It was found to have essentially the same structure as the unglycosylated form (Williams et al., 1987) and comparable enzymatic activity (Rudd et al., 1994). The refolding of RNase A coupled to disulfide bond formation (oxidative folding) has been studied extensively (Creighton, 1979). The rate of folding with intact disulfide bonds and that of oxidative folding are the same for RNase A and RNase B (Wang and Hirs, 1977; Grafl et al., 1987).

Breakage, formation, and rearrangement of the disulfide bonds allows the preparation of various conformational species of RNase B, which were analyzed by nondenaturing polyacrylamide gel electrophoresis (PAGE) (Figure 2). The native form (N) contains four disulfide

bonds. When all of the disulfide bonds are broken by reduction with dithiothreitol under denaturing conditions and the free thiol groups are alkylated by reaction with iodoacetamide, the resulting unfolded species (U) has greatly reduced mobility.

Disulfide bonds were regenerated by incubation of the fully reduced protein with a mixture of reduced and oxidized glutathione (GSH and GSSG, respectively), which constitutes an oxidative redox buffer. Disulfide bond formation in RNase A is mostly random, and mixtures of species are observed from the one-disulfide to the four-disulfide stage. The native structure is acquired with the four native disulfide bonds (Creighton, 1979). Similar behavior was observed with RNase B. Rearrangements of the disulfide bonds to form the native set is slow, and when the reaction was quenched by alkylation of all free thiol groups, a mixture of intermediates (I) was obtained (seen as a smear in Figure 2B). The electrophoretic mobility of these species indicates that, though unfolded, they are more compact than the fully reduced form. The native refolded species (NR) was regenerated very efficiently when PDI was included in the reaction (Figure 2).

Heat-denatured RNase B is a substrate for UGGT (Sousa et al., 1992; Sousa and Parodi, 1995). N, NR, and a fully unfolded U species were incubated in the presence of UDP-[^{3}H]glucose and UGGT. The incorporation of the radiolabeled glucose was monitored by radioautography after nondenaturing PAGE. Figure 2A demonstrates that only the U-RNase B was glucosylated by UGGT. As observed previously (Sousa et al., 1992), N-RNase B was not a substrate for UGGT. NR-RNase B also was not glucosylated, indicating that it had achieved its native conformation and was not recognized by UGGT. Significant glucosylation of folding intermediates could not be obtained because their formation was always accompanied by the formation of small amounts of disulfide cross-linked oligomers that were better substrates and competed for glucosylation (data not shown). Unblocked, fully reduced RNase B, as well as U-RNase B with the thiol groups blocked with iodoacetate, could also be glucosylated (data not shown).

The objective was to assess the binding of calnexin to native RNase B when it is monoglucosylated. Since N-RNase B was not recognized as a substrate by UGGT (Figure 2A), the glucosylation reaction was performed on fully reduced RNase B, and the protein was refolded subsequently in a glutathione redox buffer in the presence of PDI. Analysis by nondenaturing PAGE and radioautography showed that the fraction of glucosylated RNase B was correctly refolded (Figure 2B).

Migration of proteins on nondenaturing PAGE is very sensitive to structural differences. The heterogeneity of the glycosylation of RNase B results in broad bands. When the radioautograms are superimposed on the Coomassie-stained gels (Figures 2A and 2B), it is apparent that the glucosylated forms, which contain the most mannose residues (see below), are observed only in the upper part of the broad Coomassie-stained band.

The RNase B used in this work was found to be heterogenously glycosylated (Figure 3A) with about 1.5% of Man_9, 6.5% of Man_8, and 11% of Man_7 containing glycans, but most of the molecules have either Man_6 (28%) or Man_5 (54%). After glucosylation with UGGT (Figure 3A, insets), changes in the proportion of glycans eluting at 7.86 glucose units (gu) (Man_7), at 8.82 gu (Man_7Glc_1 and Man_8), and at 9.5–9.6 gu (Man_8Glc_1 and Man_9) were consistent with monoglucosylation of Man_7 and Man_8 oligosaccharides. Traces of material eluting at 10.4 gu were indicative of Man_9Glc_1. Liquid scintillation counting based on the incorporation of [^{3}H]glucose indicated that about 1% of the RNase B molecules were glucosylated (data not shown), consistent with the HPLC results obtained with nonradiolabeled UDP-glucose. Such a low efficiency of glucose incorporation was not unexpected, owing to the large excess of unfolded RNase B with $GlcNAc_2Man_5$ and $GlcNAc_2Man_6$ oligosaccharides, which inhibits the glucosylation reaction (Sousa and Parodi, 1995) and the scarcity of the most appropriate substrate $GlcNAc_2Man_9$ (Parodi et al., 1984; Sousa et al., 1992).

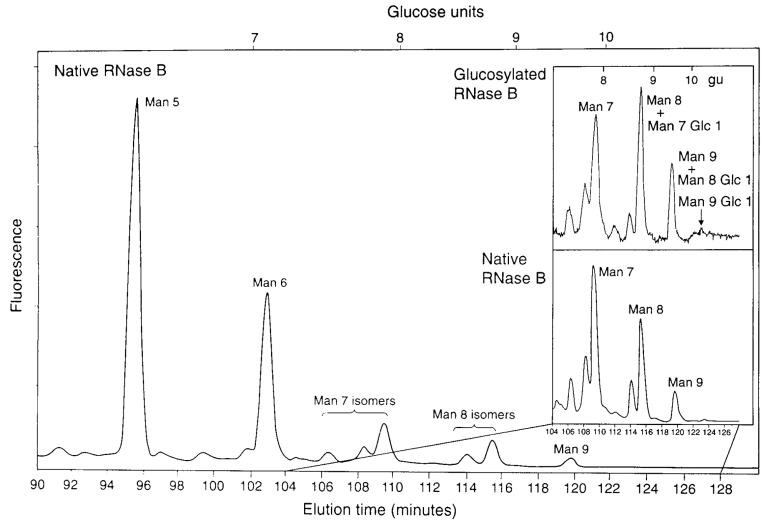
Binding of N-Linked Monoglucosylated Oligosaccharides to Calnexin

The luminal domain of Cnx ΔTMC was expressed in Sf9 insect cells with a baculovirus system and purified to near homogeneity from the medium (Figure 3B). Also shown in Figure 3B is the purity of the constituents of the in vitro reconstituted system, including RNase B and purified UGGT and glucosidase II.

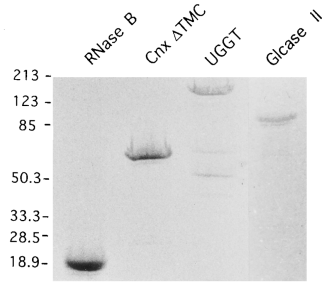
U-RNase B was monoglucosylated with radiolabeled glucose as described above. Oligosaccharides resulting from the digestion of monoglucosylated U-RNase B (U-G_1RNAse B) with PNGase F (peptide-*N*^{4}-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase F) were tested for binding to Cnx ΔTMC. Gel filtration chromatography was used to separate Cnx ΔTMC from RNase B (Figure 3C). The majority of the oligosaccharides containing radiolabeled glucose were found to coelute with Cnx ΔTMC. When the same samples were examined by SDS-PAGE and radioautography, no radiolabel was found associated with RNase B, in the Cnx ΔTMC peak or in the RNase B peak, indicating that the digestion was complete (data not shown). The same results were obtained with oligosaccharides resulting from treatment of U-G_1RNase B with Endo H (endo-β-*N*-acetylglucosaminidase H). These findings confirm earlier observations that monoglucosylated oligosaccharides alone can bind to calnexin (Ware et al., 1995). A similar calnexin domain was found to bind dolichol-linked $GlcNAc_2Man_9Glc_1$ oligosaccharides and to a lower extent $GlcNAc_2Man_{5-7}Glc_1$ glycans, suggesting that at least one of the mannosidase-sensitive residues on $GlcNAc_2Man_9Glc_1$ is important for optimal binding to calnexin (Ware et al., 1995). Since Endo H–digested monoglucosylated oligosaccharides bound equally well to calnexin, the innermost GlcNAc residue was not required for binding.

Binding of RNase B to Calnexin

The three forms of G_1RNase B, fully unfolded (U), unfolded but disulfide-bonded (I), or native refolded (NR) were tested for binding to calnexin by gel filtration chromatography. Collected fractions were analyzed by SDS-PAGE of TCA precipitates. NR-G_1RNase B coeluted with Cnx ΔTMC, whereas the majority of RNase B that was



A



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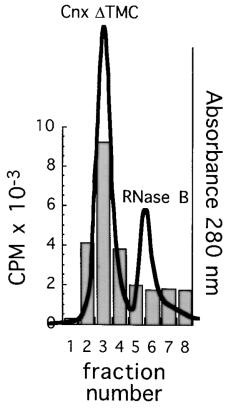

C

Figure 3. Glycan Composition of RNase B, Constituents of the In Vitro System, and Oligosaccharide Binding to Cnx ΔTMC

(A) Oligosaccharide composition of RNase B. The main graph shows the normal-phase HPLC elution profile of the glycan populations of RNase B before glucosylation. The insets show a comparison of the HPLC analyses of the RNase B glycans before and after glucosylation, normalized to the Man_6 peak.

(B) SDS-PAGE (8%–25% acrylamide; Phast-system, Pharmacia) of the purified proteins used in the reconstituted system. About 1 μg of RNase B and Cnx ΔTMC and 0.3 μg of UGGT and glucosidase II (Glcase II) were loaded. Coomassie blue stain.

(C) Binding of monoglucosylated oligosaccharides to Cnx ΔTMC. Radiolabeled U-G_1RNase B was treated with PNGase F prior to incubation with Cnx ΔTMC. The mixture was separated by gel filtration chromatography, and the radioactivity in the collected fractions was monitored by liquid scintillation counting.

observed by Coomassie staining and was nonradiolabeled was clearly resolved from the complex (Figure 4A). Because G_1RNase B represents only a small fraction (~1%) of the total RNase B present, it is not visible by Coomassie staining. The same results were observed with I-G_1RNase B and U-G_1RNase B (data not shown). Hence, there is binding of G_1RNase B to the luminal domain of calnexin irrespective of the conformation of the monoglucosylated glycoprotein.

In all binding experiments, the gel filtration column was refrigerated, and the temperature of the eluate was about 10°C. Incubation and loading were performed at room temperature (22°C). When the separation was performed at room temperature, no complex was isolated. This was likely due to the effective separation of unbound substrate from the complex during gel filtration, driving the equilibrium toward dissociation. Isolation of complexes at lower temperature was likely due to the reaction being slower. Calnexin has been reported to have greater affinity toward Man_9Glc_1-containing oligosaccharides than toward monoglucosylated glycans of lower mannose content (Ware et al., 1995). Our preparation of G_1RNase B contained mainly $GlcNAc_2Man_7Glc_1$ and $GlcNAc_2Man_8Glc_1$, which may explain the weak binding interaction. Also, a large excess of Cnx ΔTMC was required to separate entirely G_1RNase B from the nonglucosylated molecules, suggesting that the binding is very dynamic even at low temperature. Isolation of the complex requires the rate of binding to be greater than the rate at which RNase B and Cnx ΔTMC are separated in the column.

To test whether calnexin binds to different conformations of G_1RNase B with different affinities, a mixture of NR-, I-, and U-G_1RNase B was incubated with an amount of Cnx ΔTMC insufficient to allow observation of complete isolation of the complex by gel filtration. Collected fractions were analyzed by nondenaturing PAGE (Figure 4B). Unfolded RNase B eluted before the intermediates and the native protein eluted last. However, the fraction of G_1RNase B that coeluted with Cnx ΔTMC consisted of the three conformational species NR, I, and U, indicating that their affinities for calnexin are not different. The slightly faster elution of unbound G_1RNase B, compared to that of nonglucosylated RNase B, is due to the dissociation from Cnx ΔTMC during the gel filtration.

To test further that the binding was very dynamic, the binding of radiolabeled U-G_1RNase B was challenged with nonradioactive molecules. Radiolabeled U-G_1RNase

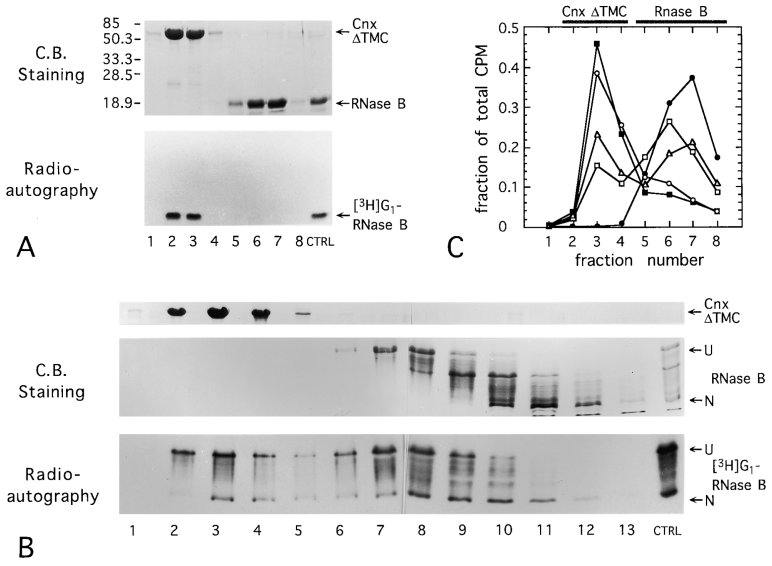

Figure 4. Binding of Monoglucosylated RNase B to the Luminal Domain of Calnexin

(A) NR-G_1RNase B was prepared as shown in Figure 2B and incubated with Cnx ΔTMC prior to gel filtration. Fractions were analyzed by SDS-PAGE. (B) (Bottom) A mixture of NR-, I-, and U-G_1RNase B was incubated with an amount of Cnx ΔTMC insufficient for complete binding. After gel filtration, eluted fractions were analyzed by nondenaturing PAGE. (Top) An SDS-PAGE analysis of Cnx ΔTMC, precipitated from fractions of a similar gel filtration.

(C) Radiolabeled U-G_1RNase B was incubated in the absence (closed circles) or the presence (closed squares) of an amount of Cnx ΔTMC sufficient for complete binding. Added to samples with Cnx ΔTMC, 5 min prior to gel filtration, was an equivalent amount of nonglucosylated U-RNase B (open circles), nonradioactive U-G_1RNase B (open squares), or nonradioactive U-G_1RNase B that had been digested with trypsin and proteinase K (open triangles). After gel filtration, the radioactivity in the collected fractions was counted by liquid scintillation.

C.B., Coomassie blue; CTRL, control.

B was incubated with an amount of Cnx ΔTMC slightly in excess of the amount required for observation of complete binding by gel filtration. Nonradioactive U-G_1RNase B was then added, and after further incubation the mixture was analyzed by gel filtration and liquid scintillation counting of the collected fractions (Figure 4C). The radioactivity was partially displaced from the Cnx ΔTMC peak, indicating that exchange of substrate had occurred. When the nonradioactive material was added immediately prior to the gel filtration, the same result was obtained, indicating that the reaction was rapid and taking place even in the column. The same displacement of radiolabeled U-G_1RNase B resulted from the addition of the same amount of nonradioactive G_1RNase B that had been digested with a mixture of trypsin and proteinase K. The smallest glycopeptide to result from tryptic digestion of RNase B is 6 residues long. These findings indicate that the affinity of G_1RNase B for Cnx ΔTMC does not result from extensive polypeptide binding.

A fragment of Cnx ΔTMC prepared by digestion with proteinase K, starting at Ser-36 and with additional cleavage at the C-terminus (as the shift in mobility on SDS-PAGE was greater than expected for the lack of 35 residues), was also found to bind U-G_1RNAse B (data not shown). Thus, 35 residues at the N-terminus of calnexin were not required for its binding activity as well as a few residues at its C-terminus.

Cnx ΔTMC has been shown to bind ATP (Ou et al., 1995), although no ATPase activity could be detected (W. J. Ou, personal communication). When U-G_1RNase B was incubated with Cnx ΔTMC in the presence of 1 mM ATP or 1 mM ATP and 10 mM $MgCl_2$, the binding of G_1RNase B was unaffected (data not shown). However, because the nucleotide is rapidly separated from the proteins during the gel filtration, the possibility cannot be excluded that the complex was formed in the column only after removal of the ATP.

Binding of U-G_1RNase B to Cnx ΔTMC was unaffected by a combined mixture of 100 mM α-methyl-D-glucopyranoside and 100 mM α-methyl-D-mannopyranoside. These two sugars did not affect binding even when present during the gel filtration (data not shown). Hence, the lectin interaction of calnexin must extend along the oligosaccharide chain.

Glycanase Protection

It has been proposed that trimming of the last glucose residue from $GlcNAc_2Man_9Glc_1$ by glucosidase II results in release of substrates from calnexin (Hebert et al., 1995, 1996). Two possibilities were considered. Glucosidase II could act in a ternary complex with the substrate bound to calnexin and trigger the release kinetically (Figure 1C). Alternatively, glucosidase II could act on the free substrate, resulting in release of bound substrate by driving the equilibrium toward dissociation (Figure 1B). Glucosidase II was active on the three conformational forms of G_1RNase B in the absence of Cnx ΔTMC. However, the presence of calnexin prevented the removal of the glucose residue by glucosidase II (Figure 5A). In a control experiment with the chromogenic substrate p-nitrophenyl α-D-glucopyranoside, the activity of glucosidase II was not inhibited by the presence of Cnx ΔTMC (data not shown). Hence, the access of glucosidase II to the Glcα1→3Man linkage is likely masked by the binding of Cnx ΔTMC.

The presence of Cnx ΔTMC also conferred protection against PNGase F (Figure 5A). The shift to greater electrophoretic mobility of the nonglucosylated proteins seen by Coomassie staining indicates that PNGase F was not inhibited by Cnx ΔTMC. Native RNase B was not digested by PNGase F, as reported previously (Chu, 1986), and was not affected by the presence or absence of Cnx ΔTMC.

In contrast, Endo H has been reported to cleave oligosaccharides of proteins bound to calnexin (Arunachalam

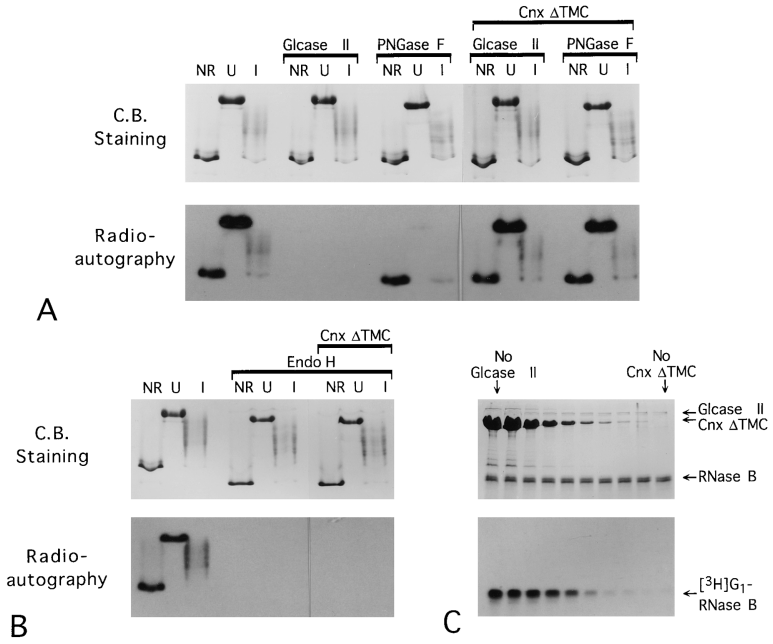

Figure 5. Protection from Glycanases by Cnx ΔTMC

(A) The three forms of G_1RNase B prepared as shown in Figure 2B were incubated with or without glucosidase II (Glcase II) or PNGase F, in the presence or absence of Cnx ΔTMC. Analysis was performed as described in Figure 2B.

(B) Same as (A) but with Endo H.

(C) U-G_1RNase B was incubated with glucosidase II in the absence or the presence of various amounts of Cnx ΔTMC. Samples were analyzed by reducing SDS-PAGE. Similar results were obtained with PNGase F.

C.B., Coomassie blue.

and Cresswell, 1995; Ware et al., 1995; Zhang et al., 1995). This was confirmed by the results shown in Figure 5B, in which the presence of Cnx ΔTMC did not prevent the action of Endo H. This differential protection against PNGase F and Endo H was unexpected because both enzymes cleave the oligosaccharide far from the glucose that confers specificity for binding to calnexin. Endo H cleaves between the 2 GlcNAc residues, whereas PNGase F cleaves the β-aspartylglucosylamine bond. However, protection against digestion by glucosidase II and PNGase F required high concentrations of Cnx ΔTMC. Cnx ΔTMC at low concentrations, but still in excess over G_1RNase B, did not provide complete protection over 4 hr (Figure 5C). This finding is consistent with the dynamic nature of the association between G_1RNase B and Cnx ΔTMC. Unbound molecules can be digested by the glycanases, and protection is effective only if the rate of binding to Cnx ΔTMC (which is proportional to its concentration) is greater than the rate of glycan removal. The sensitivity to digestion by Endo H even in the presence of Cnx ΔTMC indicates that digestion by this enzyme was faster than digestion by PNGase F or glucosidase II.

Because Endo H was active on G_1RNase B in the presence of high concentrations of Cnx ΔTMC, it was used to test whether the polypeptide chain of RNase B remained associated with Cnx ΔTMC after cleavage of the oligosaccharide. To monitor the polypeptide chain and not the oligosaccharide, the thiol groups of fully reduced RNase B were alkylated with iodo[1-^{14}C]acetamide. This radiolabeled unfolded protein was glucosylated using nonradioactive UDP-glucose. After incubation with Cnx ΔTMC followed by digestion with Endo H, the association of the polypeptide chain was assayed by gel filtration. Lane 1 in Figure 6 shows that when RNase B was not glucosylated, none of it coeluted with calnexin. However, when RNase B was monoglucosylated, a fraction of the total RNase B was isolated with Cnx ΔTMC (Figure 6, lane 2). After treatment with Endo H, no RNase B coeluted with Cnx ΔTMC (lane 3). Hence, the polypeptide of RNase B did not remain bound to Cnx ΔTMC after cleavage of the oligosaccharide.

PDI-Catalyzed Refolding of RNase B in the Presence of Calnexin

The kinetics of refolding of RNase B coupled to disulfide bond formation were examined by terminating the reaction after various time intervals and analyzing the conformation of the species by nondenaturing PAGE. RNase B that had been monoglucosylated in its reduced form

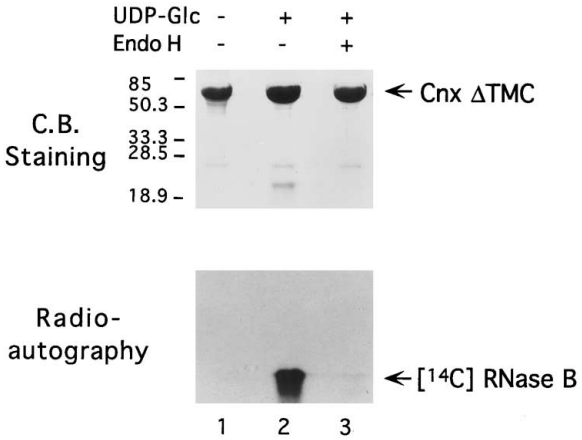

Figure 6. Release of the Polypeptide Chain of RNase B Bound to Cnx ΔTMC, by Treatment with Endo H

Reduced denatured RNase B was radiolabeled by alkylation of the free thiol groups with [^{14}C]iodoacetamide and subsequently incubated with UGGT in the absence (lane 1) or the presence (lanes 2 and 3) of nonradioactive UDP-glucose. After incubation with Cnx ΔTMC, without (lanes 1 and 2) or with (lane 3) subsequent treatment with Endo H, the mixture was separated by gel filtration chromatography and the fraction containing calnexin was analyzed as described in Figure 3A.

C.B., Coomassie blue.

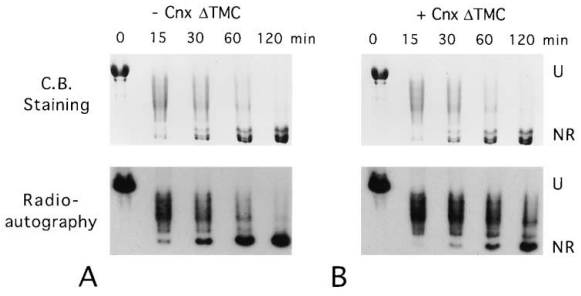

Figure 7. Effect of Cnx ΔTMC on the Oxidative Refolding of G_1RNase B in the Presence of PDI

Refolding of reduced denatured monoglucosylated RNase B was initiated by the addition of an oxidative glutathione mixture and PDI in the absence (A) or presence (B) of Cnx ΔTMC. The reaction was stopped, in aliquots withdrawn after various time intervals, by reacting all thiol groups with iodoacetamide. The conformation of RNase B was then analyzed as described in Figure 2.
C.B., Coomassie blue.

was incubated with a glutathione redox buffer and PDI in the presence or absence of Cnx ΔTMC (Figure 7). The nonglucosylated species of RNase B, visualized by Coomassie staining, were refolded at the same rate in the presence or absence of Cnx ΔTMC. Although the formation of the intermediate species of G_1RNase B was unaffected by the presence of Cnx ΔTMC (Figures 7A and 7B, bottom, at 15 min), the monoglucosylated species adopted the native conformation more slowly in the presence of Cnx ΔTMC. The possibility that this was due to the inaccessibility of PDI to bound G_1RNase B was tested in experiments in which PDI was omitted. No difference was then observed in the rate of progression to the native conformation in the presence or absence of Cnx ΔTMC (data not shown).

Discussion

Calnexin Acts Solely as a Lectin

Calnexin has been shown to increase the yield of folding and assembly of MHC class I molecules in vivo and of influenza hemagglutinin in microsomes (Hebert et al., 1996; Vassilakos et al., 1996). Calnexin can therefore be considered a genuine molecular chaperone, according to the functional part of the definition of this class of proteins, in that it "facilitates the correct fate" of its substrates (Hendrick and Hartl, 1993). The present work shows that the luminal domain of calnexin binds native as well as denatured RNase B if monoglucosylated (Figure 4). Therefore Cnx ΔTMC does not match the physical part of the definition because it is not "binding to and stabilizing an otherwise unstable conformer of another protein" (Hendrick and Hartl, 1993). The model proposed in Figure 1A is not valid. The luminal domain of calnexin appears to act in vitro solely as a lectin, at least with RNase B as a substrate.

The small proportion of G_1RNase B generated with the purified UGGT was not unexpected, given the characteristics of this enzyme (Sousa et al., 1992; Sousa and Parodi, 1995). However, this provided a useful internal control. Only the radiolabeled G_1RNase B was found to bind Cnx ΔTMC, despite the large excess of nonglucosylated RNase B, indicating the remarkable specificity of Cnx ΔTMC for monoglucosylated substrate. Since monopyranosides did not bind to calnexin, the terminal glucose residue alone is not sufficient for binding, but the recognition site involves at least the terminal disaccharide Glcα1→3Man. Observations in vivo have suggested that oligosaccharides with 2 terminal glucose residues do not bind to calnexin (Ora and Helenius, 1995). It is noteworthy that the only difference between Glcα1→3Glc and Glcα1→3Man is the orientation of the hydroxyl group at position C2 (S. M. P. et al., unpublished data).

Glucosidase II Acts on Unbound Molecules

The dynamic nature of the binding equilibrium renders unlikely a model in which a monoglucosylated substrate would be tightly bound to calnexin and only the trimming of the glucose while the substrate is associated with calnexin would trigger the release (Figure 1C). Moreover, the observation that Cnx ΔTMC protects the oligosaccharide of RNase B from digestion by purified glucosidase II indicates that this glycosidase does not act in a ternary complex, ruling out the model presented in Figure 1C.

More likely, free molecules of monoglucosylated substrates, in equilibrium with calnexin-bound molecules, would be available for glucose removal by glucosidase II. In vivo, optimal conditions would allow glucosidase II to compete with calnexin for monoglucosylated substrates, as predicted by the results shown in Figure 5C. The model depicted in Figure 1B, in which calnexin acts only as a lectin, is the simplest with which to describe the actions of UGGT, glucosidase II, and calnexin.

However, when taken together, the system consisting in vivo of UGGT, glucosidase II, and calnexin may be considered a chaperone apparatus in which binding and release are driven by deglucosylation and reglucosylation. There is remarkable similarity in the sugar specificity of the three components of this system. All interact with a higher affinity toward $GlcNAc_2Man_9Glc_1$ than toward oligosaccharides containing fewer mannose residues (Grinna and Robbins, 1980; Sousa et al., 1992; Ware et al., 1995). The soluble luminal homolog of calnexin, calreticulin, was found to be a lectin with a similar specificity (Spiro et al., 1996). This may indicate tight control in the ER of the substrates for these proteins acting in concert as a chaperone. The apparent specificities of this chaperone apparatus for various newly synthesized glycoproteins in vivo is probably dependent on the relative abundance and rate of folding of each particular substrate as well as on the availability and specificity of the other abundant chaperones and folding enzymes of the ER.

Folding of Monoglycosylated Proteins Can Occur on Calnexin

Binding of G_1RNase B to Cnx ΔTMC does not prevent but only slows its refolding catalyzed by PDI (Figure 7). No topological hinderance of the folding reaction should result from the binding of calnexin to the single oligosaccharide of RNase B. Indeed, the rate of disulfide bond formation to yield the mixture of intermediate species was not affected by Cnx ΔTMC. The formation of the

intermediates is rapid and only marginally increased by PDI, whereas the spontaneous disulfide rearrangement is extremely slow and the generation of the native protein is almost entirely due to PDI (Lyles and Gilbert, 1991). Only the catalyzed disulfide bond rearrangements were slowed by the presence of Cnx ΔTMC. However, this was not due to competition for PDI by calnexin, although calnexin contains two disulfide bonds. In effect, the catalysis of disulfide rearrangements of unbound RNase B was not affected. As for glucosidase II and PNGase F, PDI had a restricted access to bound G_1RNase B. This effect may be relevant in vivo.

In contrast, the folding of influenza hemagglutinin is blocked while it is bound to calnexin in microsomes (Hebert et al., 1996). However, influenza hemagglutinin has seven N-linked glycans distributed throughout the protein. Therefore it might associate at different points along its chain with a multivalent calnexin or with several calnexin molecules, thus preventing the formation of a compact native structure (Hebert et al., 1996). In this way calnexin may indeed discriminate between unfolded and folded molecules, without interacting with polypeptide chains.

Calnexin Does Not Bind to Polypeptides

The binding function of Cnx ΔTMC had been shown to be intact in vivo, as revealed by its association with coexpressed human immunodeficiency virus gp120. Furthermore, the conformational changes induced by Ca^{2+} and Mg-ATP were similar for Cnx ΔTMC and the full-length protein (Ou et al., 1995). Although it cannot be ruled out that Cnx ΔTMC has lost part of the binding functionality of the full-length protein, it appears unlikely that this extends to the substrate-recognition feature of calnexin. Indeed, a shorter proteolytic fragment also was found to bind G_1RNase B.

The studies with Cnx ΔTMC failed to substantiate a direct interaction with the polypeptide chain of RNase B. Although it has been shown that MHC class I and II molecules, invariant chains, and α_1-antitrypsin remain bound to calnexin after cleavage of the N-linked glycans (Arunachalam and Cresswell, 1995; Ware et al., 1995; Zhang et al., 1995), the insolubility of these substrates, released from immunocomplexes after Endo H treatment, is an alternative explanation.

Further indications that calnexin binds polypeptides has come from reports of calnexin association with nonglycosylated proteins. When TCRϵ, a nonglycosylated subunit of the T-cell receptor, was coexpressed with a truncated calnexin that lacks its ER retention motif, both proteins were found to be colocalized in an ER distal compartment of the secretory pathway (Rajagopalan et al., 1994). Coimmunoprecipitation with calnexin was observed with thyroglobulin, even when glycosylation was prevented by tunicamycin (Kim and Arvan, 1995), and with P-glycoprotein and MHC class I and II chains, which had their glycosylation site removed (Arunachalam and Cresswell, 1995; Carreno et al., 1995; Loo and Clarke, 1995). The same observation was made in microsomes with a mutant nonglycosylated form of the vesicular stomatitis virus G protein. However, in this case, coimmunoprecipitation of the nonglycosylated protein with calnexin occurred only when the mutant protein was forming large aggregates, whereas the glycosylated protein associated with calnexin as a monomer (Cannon et al., 1996). This aggregation phenomenon may account for all of the observations of the binding of nonglycosylated protein to calnexin.

The action of calnexin as a lectin and its participation in a chaperone system are now well established. The same is probably true for the soluble luminal homolog, calreticulin (Nauseef et al., 1995; Peterson et al., 1995; Hebert et al., 1996; Spiro et al., 1996). The reasons underlying the evolution of a soluble and a membrane-bound lectin as well as a testis-specific calnexin homolog, calmegin (Watanabe et al., 1994), remain to be addressed. Studies should focus on the role of anchoring into the ER membrane and the exact function of the cytoplasmic tails of calnexin and calmegin.

Experimental Procedures

Materials

Affinity chromatography–purified RNase B was from Sigma. Purified human PDI expressed in Escherichia coli was a generous gift from N. Darby and T. Creighton (Darby and Creighton, 1995). PNGase F and Endo H were from Boehringer Mannheim. 1-Deoxynojirimycin was from Calbiochem. GSH, GSSG, iodoacetamide, and sodium iodoacetate were from Sigma; GdHCl was from Gibco–BRL; and solutions were prepared fresh. The 2-aminobenzamide (2-AB) signal labeling kit was from Oxford GlycoSystems. UDP-D-[6-^{3}H]glucose (2.2–7.1 Ci/mmol) and iodo[1-^{14}C]acetamide (60 mCi/mmol) were from Amersham.

Purification of UGGT

UGGT was purified essentially as described by Trombetta and Parodi (1992). Rat livers (60 g) were homogenized in 10 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 1 mM PMSF, and 10 μg/ml each of aprotinin and leupeptin. After centrifugation (120,000 × g, 90 min), the pellet was solubilized in 10 mM Tris–HCl, 0.3 M NaCl, 5 mM $CaCl_2$, 1 mM PMSF, and 0.2% CHAPS. After further centrifugation as described above, the diluted supernatants were loaded onto a DEAE-Sepharose column equilibrated with 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, and 5 mM $CaCl_2$. Proteins were eluted with a gradient of 50–500 mM NaCl in the same buffer. UGGT-containing fractions were loaded onto a Con A Sepharose. After washing with 10 mM Tris–HCl (pH 7.5), 0.5 M NaCl, 5 mM $CaCl_2$, and 1 mM $MgCl_2$, proteins were eluted at 37°C with 0.25 M methyl α-D-mannopyranoside and 0.1 M methyl α-D-glucopyranoside in the washing buffer. The eluate was loaded onto a Mono Q column equilibrated with 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, and 5 mM $CaCl_2$. Proteins were eluted with a gradient of 50–600 mM NaCl in the same buffer. UGGT activity was monitored by the incoporation of [^{3}H]glucose from UDP-[^{3}H]glucose into urea-denatured thyroglobulin (Trombetta and Parodi, 1992).

Expression and Purification of Calnexin ΔTMC

Calnexin ΔTMC was expressed in Sf9 insect cells with a baculovirus vector and purified to apparent homogeneity as described previously (Ou et al., 1995).

Purification of Glucosidase II

α-Glucosidase II was purified from rat liver as described previously (Brada and Dubach, 1984). The pellet obtained as described above was resuspended in 80 mM triethylamine– acetic acid (pH 7.0), 0.5% NP-40. After centrifugation (100,000 × g, 1 hr), the supernatant was applied onto a Con A column equilibrated with 80 mM triethylamine–acetic acid (pH 7.0),1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 0.1% NP-40. Proteins were eluted with 0.2 M of methyl α-D-glucopyranoside in the same buffer containing 25 mM octyl glucoside rather than NP-40. After dialysis against 80 mM triethylamine–acetic acid (pH 7.0), the sample was loaded onto a Mono Q column and eluted with a gradient of 0–500 mM NaCl in the same

buffer. The active fractions were dialyzed against 0.1 M sodium phosphate (pH 6.8) and 0.1 M NaCl. Finally, proteins were separated by gel filtration on a Superose 12 column equilibrated with the same buffer supplemented with 10% glycerol. The purified enzyme was shown to be free of contaminating α-glucosidase I and α-mannosidases. The specific enzymatic activity of the final preparation was 10,000 U/mg, where 1 U is the amount of enzyme necessary to digest 1 pmol of 2-AB–labeled $GlcNAc_2Man_7Glc_2$ in 10 μl at 37°C in 1 hr.

Denaturation of RNase B

RNase B was reduced and denatured by incubation for 20 min at room temperature with 0.1 M Tris–HCl (pH 8), 6 M GdmCl, and 20 mM dithiothreitol. To isolate the reduced form, the protein was then desalted in 0.1% trifluoroacetic acid on a NAP-5 column (Pharmacia) and freeze-dried. Alternatively, the free thiols were blocked by the addition of one-quarter volume of 0.5 M iodoacetamide or sodium iodoacetate, 1.5 M Tris–HCl (pH 8.7) 5 min prior to desalting.

To prepare radiolabeled unfolded RNase B, reduced protein prepared as described above was dissolved in a solution of 2 mM iodo[1–^{14}C]acetamide (60 mCi/mmol) and 0.3 M Tris–HCl (pH 8.7). After incubation at room temperature for 20 min, the protein was desalted and dried as described above.

The conformation of RNase B was analyzed by nondenaturing PAGE with the low pH gel system (15% acrylamide) used previously with RNase A (Creighton, 1979). The incorporation of radiolabel was revealed by fluorography (Amplify, Amersham).

Glucosylation Reaction

RNase B was incubated at 37°C for 2.5 hr with 14–45 μM UDP-[^{3}H]glucose and ~5 μg/ml UGGT in 5 mM Tris–HCl (pH 8), 5 mM $CaCl_2$ containing 100 μM 1-deoxynojirimycin. In experiments with ^{14}C-labeled unfolded RNaseB, 0.1 mM unlabeled UDP-glucose was used.

Oligosaccharide Release and Labeling

Oligosaccharides were released from RNase B with anhydrous hydrazine using the Oxford GlycoSystems GlycoPrep 1000. The glycan pools were fluorescently labeled with 2-AB. Normal-phase HPLC was carried out using a GlycoSep-N column (4.6 × 250 mm; Oxford GlycoSystems). Gradient conditions were as follows: solvent A was 50 mM ammonium formate (pH 4.4), and solvent B was acetonitrile. Initial conditions for gradient 1 were 20% A at 0.4 ml/min, followed by a linear gradient of 35%–53% A over 132 min followed by 53%–100% over the next 3 min. The flow rate was then increased to 1 ml/min over the next 2 min and the column washed in 100% A for 5 min, before being reequilibrated in 35% A before injection of the next sample. The total run time was 180 min. Column temperature was maintained at 30°C. The sample was dissolved in acetonitrile–water (80:20). The column was calibrated in glucose units with a standard mixture of glucose oligomers.

Refolding of RNase B

After monoglucosylation, reduced RNase B was incubated with 0.5 mM GSSG and 2 mM GSH in 20 mM HEPES (pH 7.5), 150 mM NaCl, and 2 mM $CaCl_2$ either for 4 hr in the presence of ~1.8 mM PDI to regenerate the native conformation or for 30 min in the absence of PDI to generate a mixture of folding intermediates. One quarter volume of 0.5 M iodoacetamide in 1.5 M Tris–HCl (pH 8.7) was added to end the reaction. After 5 min incubation, samples were desalted and dried as described above.

To monitor the kinetics of refolding, aliquots of the reaction mixture were withdrawn after various time intervals and reacted with iodoacetamide as described above. After 5 min incubation at room temperature, the samples were stored on ice prior to analysis by nondenaturing PAGE.

Binding Assay

About 5 μM glucosylated RNase B was incubated for 15 min at room temperature with ~1.5 μM Cnx ΔTMC in 550 μl of 20 mM HEPES (pH 7.5), 150 mM NaCl, and 2 mM $CaCl_2$. Five hundred microliters of the mixture was loaded onto a refrigerated Superose 12 column (1 × 30 cm; Pharmacia) equilibrated with the same buffer. The flow rate was 0.5 ml/min, and the elution was monitored at 280 nm. Fractions of 0.9 ml were collected, and proteins were precipitated with 10% (w/v) trichloroacetic acid prior to analysis by reducing SDS-PAGE on 15% acrylamide gels. Gels were analyzed by Coomassie staining and radioautography.

In experiments in which glucosylated RNase B was treated with a glycanase prior to incubation with Cnx ΔTMC, each collected fraction was divided in two. One half was analyzed as described above; in the other half the presence of radiolabeled oligosaccharide was determined by liquid scintillation counting.

To reveal possible differences of affinity for various conformations of G_1RNase B, ~0.75 μM of Cnx ΔTMC was incubated with an ~15 μM mixture of the three conformational states of glucosylated RNase B. Fractions of 0.5 ml were analyzed by nondenaturing PAGE after desalting and freeze-drying. To show where Cnx ΔTMC eluted, fractions of a run without RNase B were analyzed by SDS-PAGE.

In competition experiments, the concentration of Cnx ΔTMC was ~0.5 μM, and about 5 μM nonradioactive glucosylated RNase B was added either 5 min or immediately before the gel filtration. Fractions of 0.9 ml were analyzed by liquid scintillation counting. Alternatively, the nonradioactive glucosylated RNase B had been previously digested for 3 hr at 37°C with a mixture of trypsin and proteinase K (each at a 1:100 ratio) and the reaction stopped by the addition of 3 mM PMSF.

In experiments using ^{14}C-labeled unfolded RNaseB, the whole peak of Cnx ΔTMC was collected in one fraction, and the proteins were precipitated with trichloroacetic acid.

Glycanase Digestions

About 20 μM glucosylated RNase B was incubated in 20 mM HEPES (pH 7.5), 150 mM NaCl, and 2 mM $CaCl_2$ at room temperature (22°C) for 4 hr with either 1 U/ml of PNGase F, 0.1 U/ml of Endo H, or 120 μg/ml of purified glucosidase II in the presence or absence of about ~8 μM Cnx ΔTMC or 2-fold serial dilutions thereof. Samples were analyzed by nondenaturing or reducing SDS-PAGE with Coomassie staining and fluorography. The activity of glucosidase II under the same conditions was tested colorimetrically by using 1 mg/ml of p-nitrophenyl α-D-glucopyranoside and monitoring the absorbance at 405 nm.

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