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Isothermal titration calorimetric study defines the substrate binding residues of calreticulin

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Abstract

Earlier we established using modeling studies the residues in calreticulin (CRT) important for sugar-binding (M. Kapoor, H. Srinivas, K. Eaazhisai, E. Gemma, L. Ellgaard, S. Oscarson, A. Helenius, A. Surolia, Interactions of substrate with calreticulin, an endoplasmic reticulum chaperone, J. Biol. Chem. 278 (8) (2003) 6194–6200). Here, we discuss the relative roles of Trp-319, Asp-317, and Asp-160 for sugar-binding by using site-directed mutagenesis and isothermal titration calorimetry (ITC). Residues corresponding to Asp-160 and Asp-317 in CNX play important role towards sugar-binding. From the present study we demonstrate that the residue Asp-160 is not involved in sugar-binding, while Asp-317 plays a crucial role. Further, it is also validated that cation–π interactions of the sugar with Trp-319 dictate sugar-binding in CRT. This study not only defines further the binding site of CRT but also highlights its subtle differences with that of calnexin.

Keywords: Calreticulin; Calnexin; Isothermal titration calorimetry; Carbohydrate-binding site

Protein–carbohydrate recognition plays a crucial role in several cellular functions such as cell signaling, growth, differentiation, host–pathogen interactions, etc. It is also involved in folding, assembly, and trafficking of polypeptides in which the carbohydrate, binding chaperones, calreticulin (CRT) and calnexin (CNX), residing in endoplasmic reticulum (ER) aid these processes [1–5]. These proteins regulate protein folding by binding to the core glycan of glycoproteins and by interacting with other ER folding catalysts such as protein disulfide isomerase and ERp57 [6,7]. Although, they share the same carbohydrate specificity, they bind different polypeptides. An insight into the sugar-binding specificity of CNX can be obtained from its crystal structure [8]; however the structure of CRT is not available. CRT has a molecular weight of 46 kDa and comprises of three domains: N-terminal, C-terminal, and central P-domain. N-terminal domain harbors the lectin fold [4–7]. C-terminal domain is rich in acidic residues and helps in storage of calcium [9–11]. P-domain interacts with ERp57 and is similar to the P-domain of CNX [12–14]. The lack of a crystal structure of CRT and its importance in regulating correct folding of proteins in the ER emphasize the importance of further studies on the residues important for its sugar-binding activity.

Molecular modeling of CRT lectin domain based on CNX structure, together with modeling of the interaction of CRT with the glucosylated mannose oligosaccharide, suggested several potential hydrogen bonds between sugar and residues of CRT, which were validated using isothermal titration calorimetry (ITC) [15,16]. In this study, we analyze the role of two aspartates, Asp-160 and Asp-317, of CRT in its sugar-binding activity by site-directed

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mutagenesis. Mutants of Asp-160 and Asp-317 were made and the effect of these mutations on calreticulin's sugar-binding capacity was determined by ITC. Further we elucidate the importance of Trp-319 in CRT, which is speculated to have OH–π interactions with the sugar. The experimental observations are corroborated by modeling studies.

Materials and methods

Materials. Media components were obtained from Hi-media (Delhi, India). GST–agarose, reduced glutathione, thrombin, IPTG, MOPS, and SDS–PAGE reagents were obtained from Sigma Chemicals Co. (St. Louis, MO).

Synthesis of sugars. The trisaccharide (Glcα1–3Manα1–2ManαMe) was synthesized as described in [17].

Construction of mutants. All mutants were generated by PCR overlap extension [18] using pGEX–KG/CRTwt as the template [19]. The two PCR products with overlapping ends were generated by using "CRTFor" primer together with the reverse primer of each mutant and "pGex3"' primer together with forward primer of the same mutant [15]. The PCR products so obtained were used in a second round of amplification and the products obtained digested using *Bgl*II and *Eco*RI, and cloned into *Bam*HI–*Eco*RI site of pGEX–KG/CRTwt. The double mutants were created by PCR overlap extension using one of the mutants as template and proceeding in a similar way as described above.

Expression and purification of recombinant calreticulin. GST–CRT full-length fusion protein and the mutants were purified as described earlier [15].

Protein estimation. A_{280} for GST–CRT solution (1 mg/ml) was determined as 1.06 according to the method of Gill and von Hippel [20].

Circular dichroism. Far UV–CD was performed with both the wild type protein and mutants. All the CD experiments were done in a JASCO-J715 polarimeter (JASCO Corporation, Tokyo, Japan) in a 0.1 cm path length cell, with a slit width of 1 nm, response time of 4 s, and scan speed of 50 nm/s. Each data point was an average of three accumulations. The protein concentration used is 10 μM (in 10 mM MOPS, 5 mM $CaCl_2$, and 150 mM NaCl, pH 7.4).

ANS binding. ANS (1-anilino-8-naphthalene sulfonate) binding experiments were executed with both the wild type and mutants. All the experiments were done in a JOBIN YVON HORIBA fluorometer (JOBIN YVON SPEX division, Cedex, France) in a 1 cm water-jacketed cell using a protein concentration of 10 μM and ANS concentration of 50 μM.

Isothermal titration calorimetry. ITC was performed by using VP–ITC calorimeter from Microcal Inc. (Northampton, MA) as described previously [15,16]. Protein, 90–100 μM in 1.5 ml buffer (10 mM MOPS, 5 mM $CaCl_2$, and 150 mM NaCl, pH 7.4), was titrated with 10–12 μl of sugar solution (10 times the protein concentration) at an interval of 3 min using a syringe rotating at 400 rpm. The data so obtained were fitted via non-linear least squares minimization method to determine binding stoichiometry (n), binding constant (K_b), and change in enthalpy of binding (ΔH^0_b) using Origin software (Microcal). The change in free energy of binding (ΔG^0_b) was calculated using Eq. (1):

$$\Delta G^0_b = -RT \ln K_b \quad (1)$$

where R is the gas constant and T is the temperature in kelvin. The thermodynamic quantities so obtained were used to determine the change in entropy (ΔS) using Eq. (2):

$$\Delta G^0_b = \Delta H^0_b - T\Delta S \quad (2)$$

The experimental conditions ensured that c-value ranged from 2 to 130 for all the titrations, where $c = K_b M_t$ (0) and $M_t(0)$ is the initial macromolecular concentration. The value of the binding constant (K_b) was used to compare the relative binding of CRT mutants with the trisaccharide.

Modeling studies. The structure of CRT was modeled using MODELLER suite of programs as described earlier [14,21,22]. The site-specific mutants were modeled using the program O [23] and optimized using INSIGHTII. Specifically, optimization of the protein structure after single-residue replacement was performed by minimizing the conformation of mutated residue, constraining the movement of the rest of the molecule. The minimization procedure included an initial 100 cycles of steepest descent followed by 1000 cycles of conjugate gradient methods. The protein–ligand interactions were analyzed using CONTACT from CCP4 package. Visual inspection of the interactions was done using programs O and INSIGHTII. All the oligosaccharide structures were obtained from SWEETdB [24]. Molecular docked structures of oligosaccharides to CRT obtained earlier provided the template for modeling of various sugars into the binding site of CRT [14]. The atomic coordinates of these sugars were rotated and translated such that they replaced those of the disaccharide Glcα1–3ManαMe. Superposition of atomic molecules was performed using the program ALIGN. The binding site was optimized by subjecting the sugar molecule and the interacting residues to minimization protocol as described in [14].

Results

Purification of CRT and its mutants

Wild type CRT (wtCRT) and its CRT mutants were purified as fusion proteins with GST on glutathione–agarose column. As the fusion protein (GST–CRT) and the cleaved protein (CRT) behave in a similar manner [15], we have used the fusion proteins in the present study.

Circular dichroism (CD) and ANS (1-anilino-8-naphthalene sulfonate) binding experiments

CD spectra of wild type CRT and its mutants shown in (Supplementary Fig. 1) suggest that mutant CRTs retain fold of their native CRT (wtCRT). Binding of ANS exhibits similar fluorescence spectra for wild type and its mutants suggesting that there are no significant differences in their surface hydrophobicities (Supplementary Fig. 2). Taken together these data indicate that CRT mutants being studied here retain their structure similar to wtCRT.

Isothermal titration calorimetry

ITC experiments were carried out to determine the binding of mutant CRTs to the trisaccharide (Glcα1–3Manα1–2ManαMe) and thermodynamic parameters associated with it (Table 1). The interaction of Trp-319 mutants and two Asp-160 mutants (D160A and D160E) with trisaccharide is entropically driven while that of D317E and the double mutants (D317E/D160E and D317E/D160A) is enthalpically driven. The single mutants of Asp-160 showed same binding as wild type CRT, while the D317A did not bind the trisaccharide. This shows that Asp-317 has a crucial role towards sugar-binding and the role of Asp-160 is nominal if at all (Fig. 1a and c). The double mutants D317A/D160A and D317A/D160E rendered the protein inactive. The enhanced substrate-binding of the mutant D317E and the double mutants D317E/D160E and D317E/D160A as compared to wild type CRT is dealt in detail in the modeling section (Fig. 1b). The conservative mutants of Trp-319 (Trp to Tyr and

Table 1
Thermodynamic quantities for the binding of wild type and mutant CRTs to the trisaccharide (Glcα1–3 Manα1–2ManMe) at 293 K

Construct	N	$K_b \times 10^{-4}$ M^{-1}	$-\Delta H_b$ (kcal/mol)	$-\Delta G_b$ (kcal/mol)	ΔS_b (cal/mol/K)
WtCRT	0.93(±0.12)[a]	58.41(±0.33)	7.31(±0.05)	7.81(±0.17)	1.41(±0.03)
W319F	1.01(±0.11)	54.90(±0.12)	7.12(±0.15)	7.70(±0.21)	2.20(±0.26)
W319Y	1.20(±0.11)	52.21(±0.35)	7.40(±0.07)	7.73(±0.32)	1.07(±0.24)
W319I	0.81(±0.09)	23.01(±0.23)	5.61(±0.18)	7.21(±0.16)	5.61(±0.32)
W319A	0.90(±0.08)	16.62(±0.03)	4.23(±0.06)	7.02(±0.26)	9.71(±0.16)
D317E	0.92(±0.14)	125.61(±0.41)	9.93(±0.14)	8.20(±0.18)	−5.72(±0.08)
D160E	0.81(±0.08)	57.43(±0.33)	7.31(±0.03)	7.71(±0.11)	1.53(±0.06)
D317E/D160E	0.82(±0.09)	130.12(±0.75)	10.23(±0.10)	8.35(±0.45)	−6.70(±0.05)
D317E/D160A	0.91(±0.11)	126.31(±0.76)	10.51(±0.09)	8.22(±0.03)	−7.72(±0.17)
D317A	No binding				
D160A	0.93(±0.0.14)	45.71(±0.09)	5.82(±0.07)	7.61(±0.09)	6.20(±0.13)
D317A/D160A	No binding				
D317A/D160E	No binding				

[a] Each value is an average of three determinations. The values in parentheses are errors for each fitted parameter for a given experiment.

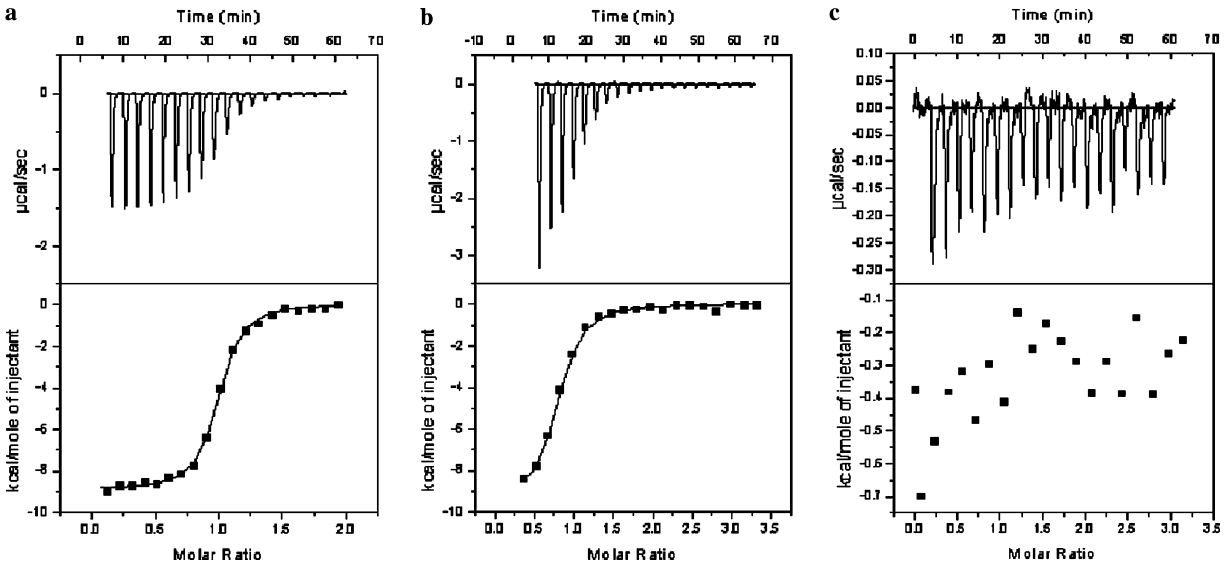

Fig. 1. Isothermal titration calorimetry profiles obtained for CRT mutants D160E (a), D317E (b), and D317A (c) with trisaccharide. Raw data fitted to single site model, obtained after injecting 10–15 µl of 1–2 mM trisaccharide solution into 90–150 µM protein in 10 mM MOPS buffer containing 5 mM $CaCl_2$ and 150 NaCl at 293 K are given on top, with a non-linear least-squares fit (−) of the heat released as a function of the ligand added (solid square), at the bottom. Thermodynamic parameters obtained for different mutants are given in Table 1.

Phe) showed similar binding as the wild type, however, the non-conservative mutants (Trp to Ile and Ala) showed a decrease in affinity towards the trisaccharide.

Modeling studies

A ligplot representation of the glucose interactions in CNX–glucose and CRT–glucose complex is shown in Fig. 2a and b, respectively. An overlap of the residues of calreticulin and calnexin in close vicinity of glucose is shown in Fig. 3 whereas Fig. 4 depicts the interaction of the tetrasaccharide (Glcα1–3Manα1–2Manα1–2ManαMe) with residues in the combining site in CRT. Asp-160 (CRT) is at a distance of greater than 4 and 10 Å, respectively, from the glucose moiety and the second mannose sugar. Hence, mutations at this site are less likely to affect the binding. Asp-317 which is within a distance of less than 4 Å was proposed to interact with the sugar by hydrogen bonding and hence mutation at this site affects binding affinity (Table 2) [14].

The phenyl ring of Trp-319 side chain might have OH–π interactions with the sugar residue within a distance of 2.9–3.6 Å (Table 3). Mutants W319F and W319Y showed no change in affinity but mutants W319I and W319A showed a decrease in affinity when compared to the wild type CRT. This can be explained by the loss of OH–π interactions in case of W319I and W319A mutants.

Discussion

In the present study, we have mutated residues in the binding site of CRT corresponding to those in CNX to understand the significance of the differences in their

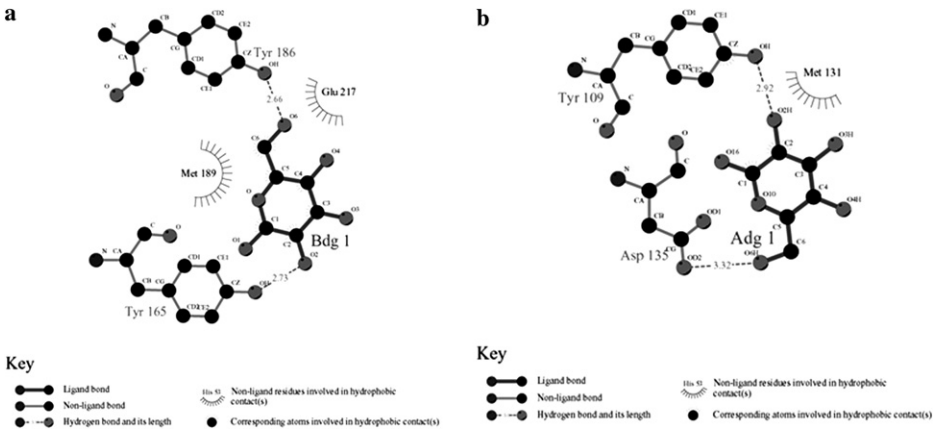

Fig. 2. Ligplot representation of the glucose interactions in (a) CNX–glucose and (b) CRT–glucose complex. The hydrogen-bonded atoms upto a distance of 4 Å are shown as dotted lines.

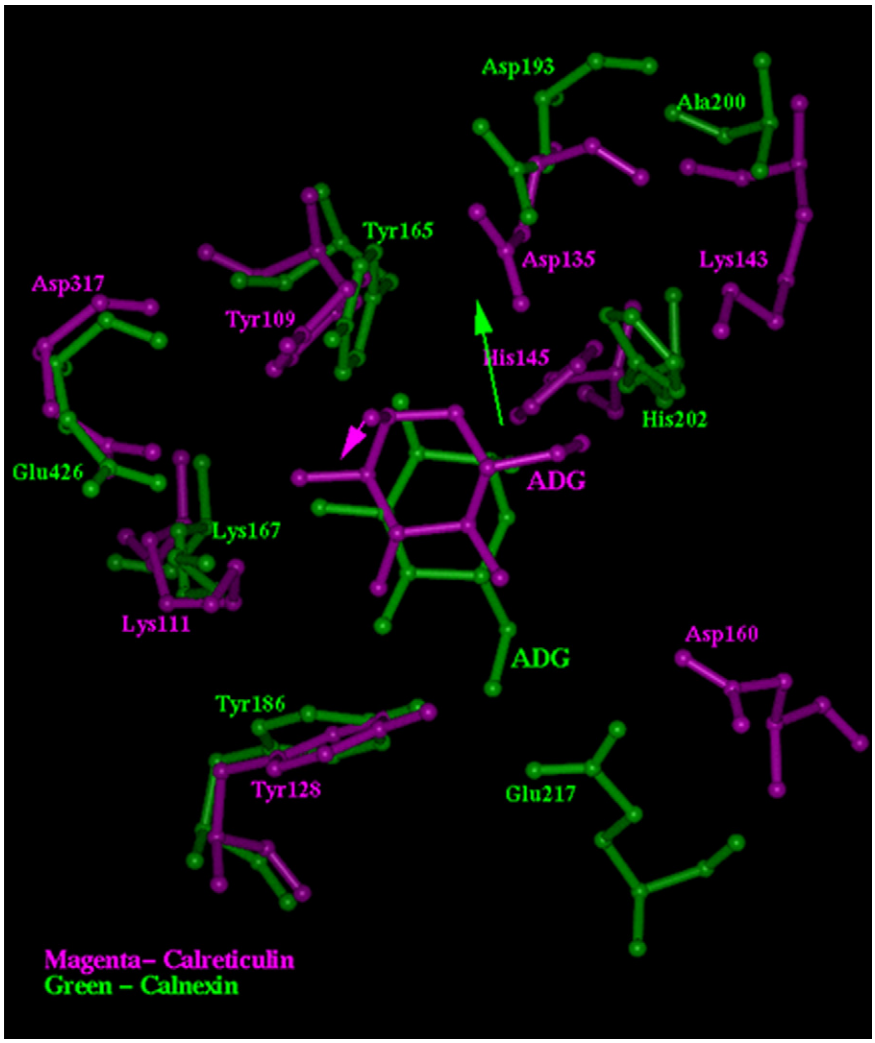

Fig. 3. Schematic representation of the interaction of glucose with the active site residues of calreticulin (magenta) and calnexin (green). (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

carbohydrate-binding regions. The sequence alignment in Fig. 3 of supplementary information highlights the differences in the residues involved in their substrate-binding sites.

The mutation of Asp-317 to Glu increased the binding constant and the mutant D317A rendered the protein completely inactive. The mutation of Asp-160 to Glu or Ala did

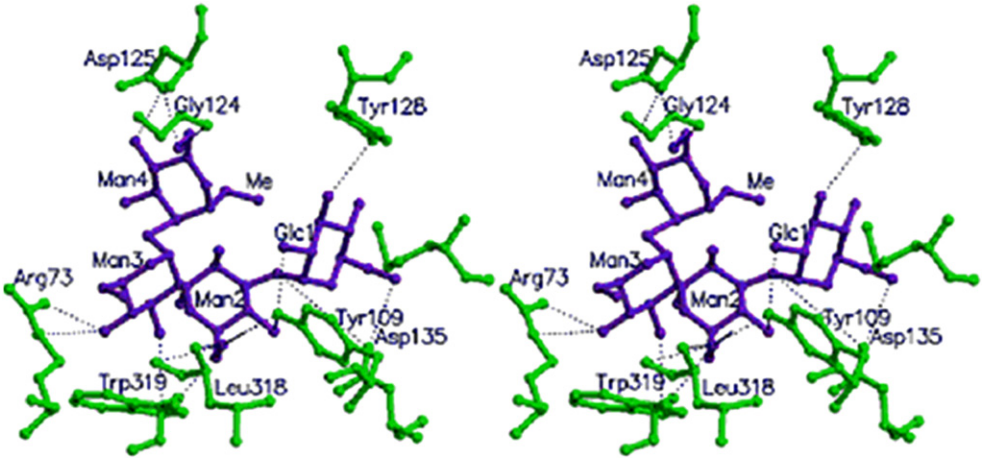

Fig. 4. Schematic representation of the interactions of tetrasaccharide (Glcα1–3Manα1–2Manα1–2Man) with CRT carbohydrate binding site residues. The residues of calreticulin are shown in green and the tetrasaccharide is in blue. Hydrogen bonds are depicted as dotted lines. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Sugar–polypeptide interactions observed in wild type (WT) and mutant CRTs (D317E and D317A)

Interaction	WT (D317)	D317E	D317A
Glc1 O2...OD2 D317	3.4	—	—
Glc1 O2...OE2 E317	—	3.8	—
Man2 O6...O D317/E317	3.3	2.9	—
Man2 O2...OE2 E317	—	3.2	—

A distance criterion of 4.0 Å was used.

Table 3
Sugar–polypeptide interactions observed in wild type (WT) and mutant CRTs, (W319F, W319Y, W319I, and W319A)

Interaction	WT	W319F	W319Y	W319I	W319A
Man 2 O6...Trp319 N	3.2	3.3	3.3	3.2	3.3
Man 3 O3...Trp319 NE1	3.4	—	—	—	—

A distance criterion of 3.5 Å was used.

not change the binding constant and as expected, the double mutants D317E/D160E and D317E/D160A showed higher binding affinity. On the other hand, double mutants D317A/D160A and D317A/D160E did not show any binding.

Asp-160 is not conserved across different CRT sequences. In *Caenorhabditis elegans* and mouse, the residue corresponding to Asp-160 is Glu and in human CRT it is Leu, indicating that it might not be important for sugar binding (Supplementary Fig. 3). This supports our experimental data which show that mutants D160A/D160E retained their activities (Table 1). Modeling Asp-160 to Ala in CRT did not have any effect on the interactions observed (hydrogen bonding and van der Waals). During the course of writing this manuscript, Thomson et al. reported similar results wherein mutation of Asp-160 to Gly had no effect on oligosaccharide binding [25].

Asp-317 is conserved across CRTs but is replaced by Glu in calnexins (Glu-426 in CNX) (Fig. 2b). Modeling studies have predicted that OD2 of Asp-317 forms a hydrogen bond with O2H of glucose [14]. D317A mutant is incapable of binding the trisaccharide (Table 1).This might be due to the loss of the hydrogen bond mentioned above (Table 2). D317E mutant, however, showed greater binding as compared to wild type CRT. A possible explanation for this increased affinity could be that upon mutation, the interactions of glucose moiety and Asp-317 weaken, but the interactions with the second mannose increase and hence cause an overall increase in affinity (Table 2 and Fig. 4). The presence of conserved Glu in human, mouse, and *Oryza sativa* CRTs in place of Asp as in rat CRT (Fig. 2b) emphasizes the point that the presence of Glu or Asp might play a subtle role in differentiating sugar-binding specificities between the two chaperones.

Asp-317 of CRT is located in the concave β-sheet region. The residue composition of this stretch is GLE/ALW, which is highly conserved. The conformation of this stretch is also similar in both CNX and CRT. In native CRT, Asp-317 does not interact with any of the protein residues, while the terminal carboxylate is hydrogen bonded to the first glucose moiety (O2; 3.44 Å). The sequentially and structurally equivalent residue in CNX, Glu-426, makes hydrogen bond with Lys-167 and a very weak hydrogen bond with the glucose moiety (O3; 3.5 Å). Modeling studies appear to support the suggestions that while the D317E mutation could potentially weaken the interactions between the polypeptide and the first sugar residue, it has led to increase in both the number and the strength of interactions with the second mannose residue (Table 2). Thus, the modeling studies provide a rationale for observed differences in binding affinities of the various mutants and support the role of Asp-317 in the sugar-binding activity of CRT.

The importance of Asp-317 is highlighted further by characterization of four double mutants (D317E/D160E, D317A/D160A, D317A/D160E, and D317E/

D160A). Among these the mutants D317E/D160A and D317E/D160E showed high affinity to the sugar whereas D317A/D160A and D317A/D160E showed no binding. The mutant D317A might have lost the activity due to the loss of hydrogen bonding interactions between O2H of glucose and the OD2 of Ala as observed for the single mutant. All these studies suggest that Asp-317 plays a critical role towards binding of oligosaccharides to CRT.

Based on modeling studies Trp-319 was predicted to make hydrophobic interactions with the oligosaccharide (Glcα1–3Manα1–2Manα1–2Man) along with Arg-73, Tyr-109, Asp-125, Met-131, and Leu-318 [14]. The first mannose of Glcα1–3Manα1–2Manα1–2Man was suggested to be involved in OH–π interactions with Trp-319 [14,26–29]. The O-4 hydroxyl group of mannose is predicted to make contacts with all the six carbon atoms of the phenyl ring of Trp-319 (within a distance ranging from 2.9 to 3.6 Å). It was also predicted that Trp-319 could make two hydrogen bonds involving its main chain nitrogen atom with O6H of first mannose and the nitrogen atom of the indole with O3 of second mannose residue of the trisaccharide. Mutations of Trp-319 were made in such a way as to retain the size and hydrophobicity. The mutants include W319F, W319Y, W319I, and W319A. The mutation of Trp319 to either Tyr or Phe did not cause any change in the binding constant. However, the mutation to Ile and Ala decreased the binding constant to less than half in the former and close to 1/4th in the latter case.

Trp-319 is conserved in calreticulin and calnexin from different sources. Upon mutating Trp-319 to Tyr, Phe, Ile, or Ala, the interaction between main chain nitrogen with O2 of the second moiety will be retained, but the interaction of the nitrogen atom of the indole to O3 of third mannose will be lost (Table 3). However, the OH–π interactions are conserved in the aromatic substitutions and replaced by other hydrophobic interactions in case of non-aromatic mutations. Therefore, the lack of OH–π interactions and weakened compensatory hydrophobic interactions explain the poor affinity of W319A for the trisachharide (Table 1).

In conclusion, Asp-317 of CRT functions similarly to Glu-426 of CNX but Asp-160 does not function equivalently to Glu217 of CNX. Comparative analysis of the interaction of glucose with the active site residues of CRT and CNX is shown in Fig. 2. Based on this we can rationalize the observed experimental results. Asp-317, Tyr-109, Lys-111, and Tyr-128 of CRT occupy similar three-dimensional space, as residues Glu-426, Tyr-165, Lys-167, and Tyr-186 of CNX. The position of Asp-160 of CRT does not overlap with Glu-217 of CNX explaining its non-involvement in glucose recognition. Moreover, the model in Fig. 3 highlights a subtle yet significant difference present between the glucose binding sites of CRT and CNX, which may be of some relevance to their substrate recognition.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.09.164.

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