Calreticulin is a lectin-like molecular chaperone of the endoplasmic reticulum in eukaryotes. Its interaction with N-glycosylated polypeptides is mediated by the glycan, Glc\textsubscript{1}Man\textsubscript{9}GlcNAc\textsubscript{2}, present on the target glycoproteins. In this work, binding of monoglucosyl IgG (chicken) substrate to calreticulin has been studied using real time association kinetics of the interaction with the biosensor based on surface plasmon resonance (SPR). By SPR, accurate association and dissociation rate constants were determined, and these yielded a micromolar association constant. The nature of reaction was unaffected by immobilization of either of the reactants. The Scatchard analysis values for $K_a$ agreed well with the one obtained by the ratio $k_+/k_{-1}$. The interaction was completely inhibited by free oligosaccharide, Glc\textsubscript{1}Man\textsubscript{9}GlcNAc\textsubscript{2}, whereas Man\textsubscript{9}GlcNAc\textsubscript{2} did not bind to the calreticulin-substrate complex, attesting to the exquisite specificity of this interaction. The binding of calreticulin to IgG was used for the development of immunoassay and the relative affinity of the lectin-substrate association was indirectly measured. The values are in agreement with those obtained with SPR. Although the reactions are several orders of magnitude slower than the diffusion controlled processes, the data are qualitatively and quantitatively consistent with single-step bimolecular association and dissociation reaction. Analyses of the activation parameters indicate that reaction is enthalpically driven and does not involve a highly ordered transition state. Based on these data, the mechanism of its chaperone activity is briefly discussed.

The endoplasmic reticulum (ER)\textsuperscript{1} of most eukaryotic cells contains two homologous calcium and carbohydrate binding chaperones: calnexin and calreticulin (1–3). Although calnexin is a luminaly oriented type I membrane protein (4), calreticulin is a soluble luminal protein (5). The most striking and conserved region between them is located in the center of these molecules, the proline rich P-domain, which consists of motifs repeated four and three times each in tandem in calnexin and calreticulin respectively (6). Glycans bind to the P-domain. Binding is exquisitely specific for the monoglucosylated N-linked oligosaccharides which appear as transient intermediates, consequent to the sequential action of glucosidase I and II that remove two glucose residues from Glc\textsubscript{1}Man\textsubscript{5–9}GlcNAc\textsubscript{2} from N-linked glycoproteins in the ER (7–9). Display of monoglucosylated (Glc\textsubscript{1}Man\textsubscript{5–9}GlcNAc\textsubscript{2}) structure allows recognition of newly translocated glycoproteins in ER by calreticulin and calnexin, which is essential for their proper folding, assembly, and exit out of ER (6, 10, 11). Both calreticulin and calnexin, like other chaperones, interact transiently with the newly synthesized glycoproteins and crucially assist in their folding process (12–16). However, they differ strikingly from other chaperones as the cycles of binding and release of substrates to them are regulated by enzymes of contrasting catalytic activities, UDP-glucose:glycoprotein glucosyltransferase (UGGT) and glucosidase II, respectively, that bring about addition and release, respectively, of glucose on target glycoproteins. UGGT transfers glucose on the non-reducing end mannose of $\alpha$1–3 arm of Man\textsubscript{5–9}GlcNAc\textsubscript{2} chains of unfolded glycoproteins, only thereby acting as a folding sensor (17, 18). Once folded or assembled glycoproteins escape calnexin/calreticulin as they are refractory to reglucosylation by UGGT and are ready to leave ER.

Although lectin-like activities of calnexin and calreticulin are indispensable for their chaperone function, additional interactions with the polypeptide segments of the substrate have also been proposed. Thus, two extreme views about their chaperone function invoke lectin only (19–21) and a lectin/polypeptide (10, 22) dual mode of binding.

Since the consequences of calreticulin-nascent glycoprotein interactions have immense implications in physiological, developmental, and pathological processes, study of calreticulin-substrate interactions at a molecular level such as the delineation of the nature and mechanism of this reaction will provide a basic framework for understanding of this recognition process. As the elementary steps for the binding of a macromolecule to its ligand and the activation parameters involved therein shed valuable insights on the mechanism of the reaction, we report here the study of the recognition of the monoglucosylated chicken IgG and the monoglucosylated oligo-
Saccharide, GlcMαnGlcNAc2 with calreticulin by surface plasmon resonance (SPR). These studies show that the reactions are several orders of magnitude slower than the diffusion controlled processes largely due to a considerable enthalpic barrier for calreticulin-monoglucosyl glycoprotein/glycan (GlcMαnGlcNAc2) interactions.

EXPERIMENTAL PROCEDURES

Materials—DEAE-Sephalac and Sephacryl S-300 were obtained from Amersham Pharmacia Biotech. Stains-All, ribonuclease A, anti-chicken IgG antibody raised in rabbit, and 5,5,5′-tetramethylbenzidine dihydrazide were obtained from Sigma. Standard molecular weight markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bangalore Genei Ltd., India. Bovine liver was purchased from a local slaughterhouse. All other chemicals were of highest purity available from Sigma. Centrprep and Centricron microconcentrators were products of Amicon Corp. Chicken IgG was a kind gift from Dr T. Shantha Raju (Genentech Inc., CA). Heavy chain was prepared by denaturing gel electrophoresis of chicken IgG, followed by electroelution of its heavy chain.

Calreticulin Purification—Bovine liver calreticulin (CRT) was purified from microsomes prepared by subcellular fractionation of liver homogenate. The microsomes were repeatedly freeze-thawed to extract the luminal proteins and the extract was separated by centrifugation. Further, the extract was subjected to ammonium sulfate precipitation at 65% saturation, followed by 85% saturation of the supernatant and finally adjusted to pH 4.65 as reported earlier (23). The precipitate was diazylized against 50 mM Tris-Cl, 10 mM EDTA and purified by ion-exchange chromatography on DEAE-Sephalac equilibrated with the same buffer. The protein was eluted with increasing NaCl (0–0.5 mM NaCl) gradient. The protein was detected in the fractions by aqueous staining and SDS-PAGE gel staining with the dye Stains-All (24). Under these conditions, the protein eluted between 0.25 and 0.35 mM NaCl. The partially purified protein was rechromatographed on DEAE-Sephalac after desalting in a Centrprep (10-kDa cut-off) concentrator. The purified protein showed the presence of nucleic acid and was subjected to ribonuclease A digestion (2 mg of enzyme/mg of protein). The protein was separated from RNase by Centricron (30-kDa cut-off) centrifugation until no more RNase eluted in the filtrate. The protein was found pure by SDS-PAGE, fast protein liquid chromatography, and mass spectrometry (electrospray ionization) using a Hewlett Packard series 1100 MSD mass spectrometer.

Protein Estimation—The B290 of calreticulin was re-estimated using the method outlined in Gill and von Hippel (25). For this purpose, a 1 mg ml−1 calreticulin solution, A290 was determined to be 2.1, close to the value determined by Waisman et al. (26). Chicken IgG was quantitated by B280 = 14 (27).

Gel Filtration—Calreticulin (0.92 mg) was added to chicken IgG (1.5 mg), and the resulting mixture was subjected to gel filtration chromatography on Sephacryl S-300 column (dimensions: 1.15 × 62 cm) equilibrated with 10 mM HEPES, pH 7.5, 5 mM CaCl2 at a flow rate of 1.9 ml h−1. The eluted fractions (315 μl volume) were checked for protein by UV absorbance at 280 nm and further by SDS-PAGE.

Electrophoretic Procedures—SDS-PAGE under reducing conditions was carried out as described by Laemmli (28).

Carbohydrate Content—Neutral sugars were determined by phenol-sulfuric acid method of Dubois et al. (29) using mannose as a standard. The carbohydrate composition of the oligosaccharides prepared from chicken IgG fraction with monoglucosylated oligosaccharides (peaks 1 and 2) was determined as described earlier (30).

Preparation of Oligosaccharide GlcMαnGlcNAc2 and ManGα GlcNAc2—Chicken IgG fractions with two monoglucosylated oligosaccharide moieties (GlcMαnGlcNAc2α and GlcMαnGlcNAc2β) per IgG molecule, i.e. [monoglucosyl]2 IgG (peak 1) and with one monoglucosylated oligosaccharide (GlcMαnGlcNAc2α), moiety per IgG, respectively, molecule [monoglucosyl] (peak 2) were purified using a larger scale gel filtration experiment on the above Sephacryl S-300 as described above. Peaks 1 and 2 were incubated in 50 mM acetic acid for 5 min and subjected to gel filtration on a Sephacryl S-300 column equilibrated with 50 mM acetic acid to obtain [monoglucosyl]2 IgG in acetic acid. The IgG fractions obtained were treated with anhydrous hydrazine as described in Ref. 29. After the reacetylation of free amino groups with acetic anhydride, oligosaccharide were freed of protein degradation material and salts by gel filtration chromatography on Sephadex G-25 column (1.15 × 45 cm). A portion of the oligosaccharide was then derivatized with anithranilic acid and analyzed by normal phase high performance anion exchange chromatography (HPAEC) as described in Ref. 31. Anthranilic acid-labeled oligosaccharides were separated on an amine (-NH2)-bonded polymeric column (polymer -NH2, 5 μm, 0.46 × 25 cm; Astec). Separations were carried out at 50 °C using a flow rate of 1.0 ml/min. Solvent system A consisted of 2% acetic acid and 1% tetrahydrofuran (inhibited) in acetonitrile, and solvent system B consisted of 5% acetic acid, 3% triethylamine, and 1% tetrahydrofuran (inhibited) in water. The gradient consisted of 30% B increased linearly to 50% B over 60 min. Column was washed with 95% B for 15 min and equilibrated with initial conditions for 15 min before the next injection. A Waters HPLC with Waters 420AC fluorescence detector was used for the separation and analysis. The oligosaccharide, ManGlcNAc2α was prepared from soybean agglutinin and was characterized using the above HPAEC system.

SPR Analysis—Binding kinetics were determined by SPR using a BIAcore 2000TM (Amersham Pharmacia Biotech) biosensor system. Calreticulin or chicken IgG, [monoglucosyl], or [monoglucosyl] were covalently immobilized on the research CMS sensor chips at concentrations of 40 μg/ml in 10 mM sodium acetate, pH 4.8, using the amine coupling kit (1-ethyl-3-(dimethylaminopropyl)carbodiimide, (N-hydroxysuccinimide) supplied by the manufacturer. Nearly 1500 resonance units (RU) of the protein were immobilized under these conditions, where 1 RU corresponds to immobilized protein concentration of ~1 pg/mm2. The unreacted moieties on the surface were blocked with ethanolamine. To study the binding of the monoglucosylated oligosaccharide (GlcMαnGlcNAc2α) calreticulin was immobilized on certified grade CMS sensor chip, using the above procedure, wherein 75 RU were immobilized which are equivalent to 20 pg of CRT. All measurements were carried out in 10 mM HEPES, pH 7.5, containing 5 mM CaCl2. For the determination of association rate constants (k1), calreticulin or IgG (25–125 nm) or the oligosaccharide (5–40 μM) flowing at the rate of 10 μl/min, in the same buffer were used. Dissociation rate constant (k−1), was evaluated by passing the same buffer at a flow rate of 10 μl/min. Inhibition of IgG binding by the oligosaccharide was done by preincubating IgG with varying concentrations of competing sugar (1–15 μM) for 30 min. The experiments were repeated three times for each concentration. The surface was regenerated by a 10-s pulse of 10 mM glycine-HCl (pH 4.2) flowing at 50 μl/min.

Data Analysis—Rate constants k1 and k−1 were obtained by fitting the primary sensorgram data using the BIA evaluation 3.0 software. The dissociation rate constant is derived using Equation 1.

\[
R_d = R_e \left( e^{-k_{d}[1-e^{-k_{d}t}]} \right)
\]

Rd is the response at time t, Re is the amplitude of the initial response, and k1 is the dissociation rate constant. The association rate constant k1 can be derived using Equation 2, from the measured k−1 values.

\[
R_d = R_{max} \left( 1 - e^{-k_1 t} \right)
\]

Rd is the response at time t, Rmax is the maximum response, C is the concentration of the analyte in the solution, and k1 and k−1 are the association and dissociation rate constants, respectively. The ratio of k1 and k−1 yields the value of association constant Kd (k1/k−1).

The ligand binding parameters, namely concentration and RU, from the sensogram data were plotted by the Scatchard method (32). The Arrhenius plot for the temperature dependence of rate constants were plotted and the activation parameters obtained according to Equations 3–5 (33).

\[
\Delta H = E_b - RT
\]

\[
\ln(k(T)) = \Delta H/RT + \Delta S/RT + \ln(k(0)/k(T))
\]

\[
\Delta G = \Delta H - T \Delta S
\]

k is the appropriate rate constant, k′ is Boltzman’s constant, and h is Planck’s constant.

Enzyme-linked Immunosorbent Assay (ELISA)—Calreticulin was dissolved in 10 mM PBS buffer, pH 7.5. Fifty μl of calreticulin solution were dispensed into each well of polystyrene microtiter plate (flat-bottomed; Corning) and incubated overnight at 37 °C in an incubator in a moist chamber. After dissolving the sensitizing CRT solutions, 200 μl of 5% skim milk was added to each well of the microtiter plate. Plates were washed twice with 200 μl/PBS well. Chicken IgG was added to varying concentrations (0.1–120 μg) in PBS in 6.25 μl of 5% skim milk powder solution to avoid nonspecific binding. Appropriate controls without calreticulin and IgG were kept. Plates were incubated for 4 h at 25 °C in a moist chamber.
After discarding the supernatants and washing three times with PBS, 50 μl/well of the appropriate dilution of rabbit anti-chicken IgG conjugated to horseradish peroxidase in PBS were incubated at 25 °C for 30 min in a moist chamber. After discarding supernatants, plates were washed four times with PBS before adding the substrate (200 μl/well). The substrate 3,3',5,5'-tetramethylbenzidine dihydrochloride was freshly prepared in citrate phosphate buffer (100 mM to 10 ml of 10 mM, pH 6.2 with 10 μl H₂O₂). The plates were then kept in dark for 10 min. After terminating the enzymatic reaction with 50 μl of 1 N sulfuric acid, the absorbance at 450 nm was recorded on an ELISA reader.

**Oligosaccharide Inhibition Assay**—Inhibition assay was performed by preincubating the adsorbed calreticulin with various concentrations of oligosaccharide Glc₃Man₉GlcNAc₂ in 0.5% bovine serum albumin for 4 h. The plates were then incubated with 10 μg of IgG for 4 h and the assay carried out as mentioned above.

**Ouchterlony Immunodiffusion Test**—The calreticulin-IgG reaction was performed in agar wells with 10 μg of CRT in the center and 0.625, 1.25, 2.5, 5, 10, and 20 μg of IgG in the circumference wells on agarose.
A representative sensogram for the interaction of varying amounts of [monoglucosyl]$_2$ IgG immobilized on a CM-5 chip is depicted in Fig. 2a. A rapid enhancement and diminution in RU's when calreticulin is followed by buffer containing [monoglucosyl]$_2$ IgG is flown over the immobilized IgG suggests that the accompanying changes in the mass correspond, respectively to the association and dissociation phases of the reaction. Mass transport limited analyses of the sensogram yields the $k_1$ and $k_2$ of $3.9 \times 10^4$ M$^{-1}$ s$^{-1}$ and $0.08$ s$^{-1}$, respectively, at 25 °C (Table I). A satisfactory distribution of residuals for both the phases of reaction confirms further the monoequponential nature of the reaction (data not shown). The ratio of $k_1/k_2$ yields a value of $4.87 \times 10^5$ M$^{-1}$ for $K_0$ at 25 °C. Calreticulin does not exhibit any binding to quail ovalbumin, which predominantly contains Man$_6$GlcNAc$_2$, Man$_6$GlcNAc$_2$, and Man$_5$GlcNAc$_2$. Additionally, the non-glucosylated IgG counterpart (Man$_6$GlcNAc$_2$) does not bind to calreticulin even at high concentrations (data not shown). Similar values of rate constants were obtained when [monoglucosyl]$_2$ IgG, I.e., IgG molecules containing a single monoglucosyl oligosaccharide chain, was immobilized and calreticulin was flown over it. This suggests that the density of the oligosaccharide chains in the substrate does not alter the kinetic parameters of the interaction (Fig. 2b and Table I). The association constants for these interactions were also evaluated by Scatchard analysis of the above SPR data. The similarities of $K_0$ obtained by Scatchard analysis and those determined by the ratio of $k_1/k_2$ is noted (Table I). Moreover, the interaction between calreticulin and [monoglucosyl]$_2$ IgG were inhibited completely by the free oligosaccharide which not only attest to the specificity of these interactions but also provide an estimate of $K_0$ for the latter (Fig. 3b). Additionally, the binding to [monoglucosyl]$_2$ IgG was totally abrogated in presence of chelator, EGTA, thereby showing the requirement of Ca$^{2+}$ ions in the chaperone-substrate interaction (data not shown). In a previous study on calreticulin interaction with glycosylated laminin of undefined overall carbohydrate chain composition, a binding constant of $2.1 \times 10^8$ M$^{-1}$ and an association rate constant of $2 \times 10^5$ M$^{-1}$ s$^{-1}$ were determined indirectly with an assumed value of $K_0$ for the latter (Fig. 3b). Similarly, various values of kinetic constants are obtained when calreticulin is immobilized and [monoglucosyl]$_1$ IgG is flown over it (Fig. 5a); thus, the nature of the reaction is unaffected by immobilization of either of the reactants. Studies on interaction between oligosaccharide and plant lectins and C-type animal lectins by SPR showed that the binding affinity values were in good agreement with those previously determined by affinity chromatography and Scatchard analysis (41, 42). Moreover, the rate constants for the association of Ricus communis agglutinin as determined by temperature jump spectroscopy and the SPR method are also found in perfect agreement (42, 43). Likewise, good agreement has been observed between the SPR data and the isothermal titration calorimetry data for other systems (44–46). Thus, our SPR data are reporting parameters intrinsic to the system. Similarity of binding parameters for the free oligosaccharide, intact IgG molecule, and its heavy chain underscore the lectin-only mode of interaction between calreticulin and its substrates.

In order to obtain an independent estimate for the association constants for the [monoglucosyl]$_2$ IgG and the free monoglucosylated oligosaccharide (Glc$_6$Man$_6$GlcNAc$_2$), an enzyme-linked lectin adsorbent assay, which has provided a wealth of information on lectin sugar interaction in the past, was used (47–50). The assay is analogous to the system wherein antigen adsorbed onto the polylysine plates are used to capture the antibody. Binding of calreticulin to the ELISA plate wells was followed by binding chicken IgG. Subsequently,
FIG. 2. a, overlay plot of sensograms depicting the interaction of calreticulin to the immobilized [monoglucosyl] IgG at 25 °C. Inset shows Scatchard plot analysis of the sensogram data. Calreticulin ranging in concentration from 25 to 125 nM was injected for 600 s at a flow rate of 10 μl/min and the concentration from bottom to top: 25, 50, 75, 100, and 125 nM. Sensogram depicted with crosses (+ + +) exhibits that no reaction
secondary antibody to chicken IgG conjugated with horseradish peroxidase was used to monitor the amount of chicken IgG-[monoglucosyl]1 or 2 captured by calreticulin as per the scheme depicted in Fig. 4a. Conditions for calreticulin concentration, blocking reagent, buffer, time, and secondary antibody dilution were optimized. These parameters of the assay with respect to calreticulin concentration, IgG concentration, and inhibition of this interaction by the oligosaccharide, Glc1Man$_9$GlcNAc$_2$, binding at 25 °C are shown in Fig. 4 (b, c, and d, respectively). When 125 ng of calreticulin was coated on the plates, maximum binding of IgG was observed at 4 μM showing a sigmoidal binding curve. The $K_d$ of the association from this curve is derived to be $4.9 \times 10^6$ M$^{-1}$. The binding of oligosaccharide to calreticulin was also studied by the ELISA method. By using various concentrations of the oligosaccharide, IgG binding could be inhibited at 50% of the maximum at 1.45 μM of the oligosaccharide Glc1Man$_9$GlcNAc$_2$, thereby indicating the affinity of the interaction to be $6.9 \times 10^5$ M$^{-1}$. The $K_a$ values thus determined are in the range obtained with the SPR method. In another study, similar inhibitory concentrations for the Glc$_1$Man$_3$Man$_1$-2Man fragment of the oligosaccharide as well as for the entire oligosaccharide chain were noted when incubated with labeled $[^3]$H$[$Gluc$_1$Man$_9$GlcNAc$_2$]* for binding to glutathione S-transferase-CRT immobilized on agarose (6).

No precipitin line was observed in the immunodiffusion test slides for the reaction between CRT and [monoglucosyl] 2 IgG, even after Coomassie Blue staining, thereby indicating that the chaperone has only one oligosaccharide binding site per monomer and behaves as a monovalent carbohydrate binding
protein. Consequently, it is not surprising to find similar $k_1$, $k_2$, and $K_a$ values for the binding of calreticulin to [monoglucosyl]$_1$ IgG, [monoglucosyl]$_2$ IgG as well as the free oligosaccharide, Glc$_1$Man$_9$GlcNAc$_2$. This is perhaps a key feature, which distinguishes it from lectins, which both by design and by definition are multivalent (51). In the latter, multivalency increases not only their binding affinities but also their specificities and physiological responses such as cell agglutination, mitogenicity, etc. (51–54). A wealth of information on the affinities of plant lectin-saccharide/glycopeptide interaction is available, which in turn has been exploited for the isolation and the structural characterization of the latter by affinity chromatography (55–57). These studies have clearly shown that glycans with affinities lower than $10^6$ M$^{-1}$ are merely retarded on lectin columns. Our studies thus provide a rationale for the mere retardation of glucosylated oligosaccharide chains (Glc$_1$Man$_9$GlcNAc$_2$) on calreticulin-Sepharose column as being due to its moderate affinity ($10^5$ M$^{-1}$) for its substrate (11). Nonetheless, this moderate affinity appears to be a favorable property for its chaperone function as it would allow multiple rounds of association and dissociation with the unfolded/misfolded glycoprotein substrate. The values of $k_1$ and $k_{-1}$ obtained for calreticulin-substrate interactions are in the range of those obtained for HSP 47-collagen and GroEL-substrate interactions (58, 59). Thus, in analogy with these chaperones, moderately rapid binding and relatively slower release of substrate by calreticulin are sufficient for its chaperone function. However, the cytosolic chaperone SecB of Escherichia coli has been reported to interact with its substrate within diffusion limits ($K_1 = 5 \times 10^9$ M$^{-1}$ s$^{-1}$) (60).

Fig. 5a shows the overlay plot of sensograms depicting the binding of [monoglucosyl]$_1$ IgG to immobilized CRT as a function of temperature. The values of activation parameters obtained by the temperature dependence of $k_1$ and $k_{-1}$ using Arrhenius plots (Fig. 5b) are also listed in Table II. CRT-substrate interaction shown in these studies is several orders of magnitude slower than the diffusion controlled reaction. Formation of an intermediate for reactions that are several orders of magnitude slower than the diffusion controlled processes is generally invoked for such reactions (61–63). However, the agreement between the kinetically determined values of $K_a$ and $\Delta H$ with those obtained by Scatchard plots indicate that these parameters reflect truly the total binding process and that there does not exist any unobservable faster process that contributes appreciably to either the association constant or enthalpy change. Linearity of Arrhenius plot also rules out such an event, as well as the occurrence of dramatic conformational changes during these reactions. Thus, the kinetics of calreticulin-substrate interaction is both quantitatively and qualitatively consistent with a single-step bimolecular reaction. An
unfavorable activation energy term indicates that a large amount of energy has to be expended for the calreticulin-substrate interaction, which perhaps accounts for the slow second order rate constants. The observed entropy of activation for the association process is small, implying that the association process does not involve a highly ordered transition state. Hence, the monoglucosyl oligosaccharide chain of the substrate can approach the binding pocket of this chaperone in a several ways, which is in stark contrast to many legume lectin-sugar interactions (64, 65). This is consistent with a fairly rigid conformation of the oligosaccharide epitope (66). Thus, much of the energetic barrier is related to an unfavorable activation enthalpic term, which could be utilized in overcoming some steric constraints, such as some localized conformational changes in calreticulin and or more so in breaking hydrogen bonds between the solvent and the chaperone as well as the carbohydrate chain and the solvent before the new ones can be established between calreticulin and the oligosaccharide chain in the complex. Occurrence of a considerable reorientation of water molecules during the reaction could also be responsible for the unfavorable activation enthalpic term.

Several mechanisms have been proposed for the chaperone function of calreticulin. The most widely accepted mechanism proposes a cycle of binding and release of the unfolded protein through its monoglucosylated oligosaccharide chain (3, 10). In this cycle, deglucosylation by glucosidase II prevents its binding to the chaperone and reglucosylation by UGGT of only the unfolded protein promotes its binding. Should the protein fold in the meantime, the consequence of glucosidase II action predominates over that of UGGT as the latter cannot reglucosylate the folded protein. In other words, the lectin-only function of calreticulin is sufficient to account for its chaperone function.

Although it has also been proposed that subsequent to the recognition of the substrate through its carbohydrate moiety, calreticulin also recognizes regions of the unfolded molecules. However, the agreement between the kinetic constants and $K_a$ values for the interaction of calreticulin with intact IgG, its heavy chain and the free oligosaccharide underscore that the chaperone recognizes exclusively the oligosaccharide chain of its substrate, ruling out any contribution of protein-protein interactions to either the kinetics or energetics of the reaction.

Most chaperones such as GroEL, crystallin, etc., bind with unfolded protein in a deep cavity through hydrophobic interactions, which perhaps imposes constraints on their folding in the bound state (67). Hence, much of the unfolded substrate folds subsequent to the dissociation from the chaperone. In other words, these chaperones facilitate the protein folding by thermodynamic partitioning of the unfolded intermediates during protein folding reactions. Calreticulin apparently should function differently from these chaperones, as it does not seem to possess a deep pocket for binding to the misfolded proteins.

In conclusion, these studies clearly show that calreticulin binds to its substrate through its lectin function only and that protein-protein interactions do not stabilize these reactions. Since protein-protein interaction between the chaperone and the glycoprotein substrate do not seem to contribute to the binding reaction, calreticulin, if at all, has a very shallow and highly solvent exposed region for binding to the protein part of the substrate. Consequently, calreticulin acts as a chaperone by sequestering and sterically excluding interactions between the unfolded polypeptide chains of the nascent glycoproteins. These data also lead us to speculate that the initiation of folding of the unfolded glycoprotein substrate could begin in the complex with calreticulin, unlike the case of other chaperones wherein there are direct protein-protein interactions precluding such an event to a great extent. Should this be the case, calreticulin could facilitate folding both by kinetic as well as thermodynamic partitioning of the substrate.

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**TABLE II**

| Temperature | $k_1$ $M^{-1} s^{-1} \times 10^2$ | $k_2$ s$^{-1}$ | $K_a$ $M^{-2} \times 10^{-5}$ |
|-------------|---------------------------------|---------------|-------------------------------|
| 10          | 1.5                             | 0.02          | 7.5 (7.1)                     |
| 15          | 2.1                             | 0.04          | 5.2 (4.9)                     |
| 20          | 2.6                             | 0.06          | 4.3 (4.0)                     |
| 25          | 3.1                             | 0.09          | 3.4 (3.1)                     |

The value of $k_{on}$ is obtained for plotting (dRU/[concentration]) vs. RU where RU is the maximum response obtained in the sensogram for respective concentrations.
