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Structural features of the combining site region of *Erythrina corallodendron* lectin: Role of tryptophan 135

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Abstract

The role of Trp 135 and Tyr 108 in the combining site of *Erythrina corallodendron* lectin (ECorL) was investigated by physicochemical characterization of mutants obtained by site-directed mutagenesis, hemagglutination-inhibition studies, and molecular modeling, including dynamics simulations. The findings demonstrate that Trp 135 in ECorL: (1) is required for the tight binding of Ca^{2+} and Mn^{2+} to the lectin because mutation of this residue into alanine results in loss of these ions upon dialysis and concomitant reversible inactivation of the mutant; (2) contributes to the high affinity of methyl α -N-dansylgalactosaminide (Me α GalNDns) to the lectin; and (3) is solely responsible for the fluorescence energy transfer between the aromatic residues of the lectin and the dansyl group in the ECorL-Me α GalNDns complex. Docking of Me α GalNDns into the combining site of the lectin reveals that the dansyl moiety is parallel with the indole of Trp 135, as required for efficient fluorescence energy transfer, in one of the two possible conformations that this ligand assumes in the bound state. In the W135A mutant, which still binds Me α GalNDns strongly, the dansyl group may partially insert itself into the place formerly occupied by Trp 135, a process that from dynamics simulations does not appear to be energetically favored unless the loop containing this residue assumes an open conformation. However, a small fraction of the W135A molecules must be able to bind Me α GalNDns in order to explain the relatively high affinity, as compared to galactose, still remaining for this ligand. A model for the molecular events leading to inactivation of the W135A mutant upon demetallization is also presented in which the *cis-trans* isomerization of the Ala 88–Asp 89 peptide bond, observed in high-temperature dynamics simulations, appears not to be a required step.

Keywords: *Erythrina corallodendron* lectin; metal ion binding; molecular dynamics simulations; N-dansylgalactosaminide binding; site-directed mutagenesis

Lectins, nonenzymic proteins that bind mono-, di-, and oligosaccharides reversibly and with high specificity, are ubiquitous in nature (Sharon & Lis, 1989). They act as mediators of cellular recognition in a variety of biological systems and are widely employed in biomedical research as carbohydrate-specific reagents. Understanding the atomic basis of lectin–carbohydrate interactions is of interest for theoretical reasons, as well as for the design of

novel drugs for treatment of microbial infections and inflammatory diseases.

The best-characterized lectins are those of the legumes (Sharon & Lis, 1990; Jordan & Goldstein, 1994; Konami et al., 1995). They represent a family of close to 100 metalloproteins. The nearly 40 sequences of these lectins that have been determined exhibit identities or similarities in close to 40% of the amino acid positions. In addition, the three-dimensional structures of 10 legume lectins, all in complex with sugar ligands, have been elucidated by high-resolution X-ray crystallography (Dessen et al., 1995; Rini, 1995; Hamelryck et al., 1996; Srinivasan et al., 1996; Weis & Drickamer, 1996). Although their quaternary structures are sometimes different, the tertiary structures of these proteins are practically superimposable. They are in the form of elaborated jelly-rolls derived from antiparallel β -strands arranged as two β -sheets. Such a tertiary structure, referred to as “the lectin fold,” has been found by X-ray analysis during the last four years in a number of other lectin

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Abbreviations: ECorL, *Erythrina corallodendron* lectin; rECorL, recombinant *Erythrina corallodendron* lectin; Con A, concanavalin A; GalNDns, dansylgalactosamine; LacNAc, N-acetylglucosamine; LnNT, lacto-N-neotetraose; Me α GalNDns, methyl α -N-dansylgalactosaminide; MD, molecular dynamics; RMSD, RMS deviation; all monosaccharides are of the D-configuration, except L-fucose.

families, among them the galectins and pentraxins, despite the absence of detectable similarity in their primary structures with those of the legume lectins (Srinivasan et al., 1996). More recently, sequence similarities to the legume lectins have been observed in intracellular animal lectins, such as MR60/ERGIC53 and VIP36, that are involved in the control of glycoprotein traffic in cells (Fiedler & Simons, 1995; Roche & Monsigny, 1996).

ECorL, the lectin from the seeds of the leguminous tree *Erythrina corallodendron* (also known as coral tree), is specific for galactose and *N*-acetylgalactosamine ($K_a = 1-2 \times 10^3 \text{ M}^{-1}$) and, among the oligosaccharides tested, it exhibits the highest affinity for $\text{Fuc}\alpha 2\text{Gal}\beta 4\text{GlcNAc}$ (Teneberg et al., 1994; Moreno et al., 1997). Recent site-directed mutagenesis studies (Adar & Sharon, 1996) based on the three-dimensional structure of the ECORL-lactose complex (Shaanan et al., 1991) have shown that three combining-site residues (Asp 89, Asn 133, and Phe 131) are essential for ligand binding by the lectin, and have led to the proposal that a similar constellation of amino acids has the same function in other legume lectins, irrespective of their specificity (except that Phe can be replaced by other hydrophobic residues, preponderantly aromatic ones) (Adar & Sharon, 1996).

ECorL binds *N*-dansylgalactosamine and its methyl glycosides more than two orders of magnitude stronger than it binds galactose. Thus, for Me α GalNDns, $K_a = 3.5 \times 10^5 \text{ M}^{-1}$ as measured both by spectrofluorimetry (Arango et al., 1993) and microcalorimetry (Surolia et al., 1996). Interaction of the dansyl derivatives with the lectin is accompanied by a marked increase in the emission spectrum of the dansyl fluorescence, as well as a blue shift (Kinzy et al., 1992), demonstrating that the high affinity of these compounds to the lectin is due largely to hydrophobic bonding, as concluded in the case of the closely related *Erythrina cristagalli* lectin (De Boeck et al., 1984).

Preliminary manual modeling indicated that the dansyl group of these ligands could fit into a cavity surrounded by Tyr 108, Pro 134, and Trp 135 in the combining site of the lectin and be in close contact with the indole group of the latter residue (Arango et al., 1993). Earlier site-directed mutagenesis experiments (Arango et al., 1993) have shown that substitution of Tyr 108 by threonine decreased the affinity of the α -glycoside to the lectin by about one half, whereas it did not affect the affinity of the β -glycoside. This result suggested that Tyr 108 plays a marginal role in the binding of the dansyl group by the lectin. Pro 134 appears not to be involved in ligand binding either, because, in the recombinant lectin, expressed in *Escherichia coli*, this residue is replaced by glutamine (Young et al., 1995), yielding approximately the same activity and affinity for different sugars as the native lectin (Arango et al., 1992, 1993; Adar & Sharon, 1996). To examine the role of Trp 135 and Tyr 108 in ligand binding, the former residue was replaced by tyrosine or alanine and the latter by threonine or alanine using site-directed mutagenesis. Characterization of these mutants by different techniques, including binding studies and molecular dynamics simulations, were performed, and the results are reported below.

Results and discussion

For the purpose of this study, three new single-point mutants of ECORL, in which combining-region amino acids were replaced by other residues, namely Y108A, W135A, and W135T, were constructed employing the PCR technique (Higuchi et al., 1988). Because this technique is known to be subject to some degree of error,

all the recombinant cDNAs were fully sequenced to confirm the planned mutations. Upon expression of the new mutant DNAs in *E. coli*, the proteins accumulated in the bacteria in the form of inclusion bodies in large quantities of up to 250 mg/L bacterial culture, as found previously for rECorL (Arango et al., 1992) and its other mutants (Arango et al., 1993; Adar & Sharon, 1996). The mutant lectins were isolated from the inclusion bodies by denaturation and refolding followed by purification by gel filtration on Sephadex G150 as described (Arango et al., 1992, 1993; Adar & Sharon, 1996). The final protein yield was usually 10–20 mg/L bacterial culture, similar to what has been found in the earlier studies.

Physicochemical characterization

The purified mutant proteins possessed the same physicochemical properties as the native lectin, although they were devoid of carbohydrate. They all assembled into the expected dimers of 53 kDa, as found by the gel filtration used for their purification (Fig. 1). When examined by affinity electrophoresis in their native state, they bound fully and specifically to immobilized galactose, *N*-acetylgalactosamine, and lactose (data not shown). Upon SDS-PAGE, the mutant proteins migrated as single or double bands, having an apparent molecular weight of 26 kDa, as revealed by Coomassie blue and by immunostaining with rabbit anti-ECorL antiserum (Fig. 2). The appearance of double bands is most likely due to the C-terminal processing that legume lectins undergo both in the seeds and in the bacteria, as demonstrated by molecular weight measurements by electrospray mass spectrometry (Young et al., 1995).

The CD spectra of the mutants were similar to those of the native and recombinant lectin (Adar & Sharon, 1996), with λ_{\min} of

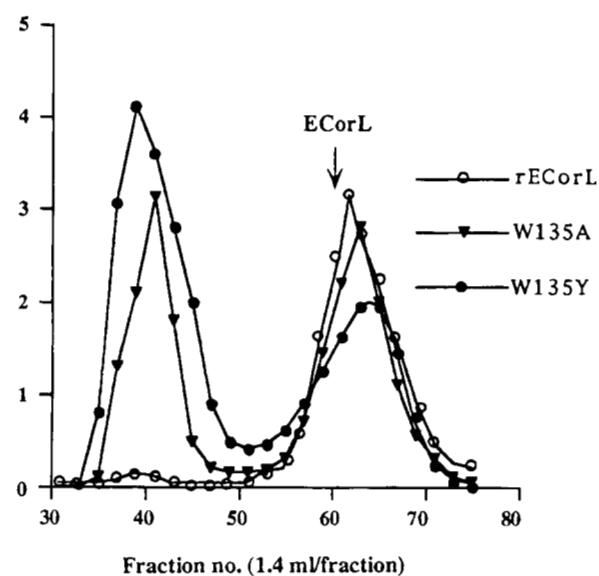


Fig. 1. Gel filtration of refolded recombinant ECORL and mutants W135A and W135Y on Sephadex G-150 (1.5 × 90 cm). Fractions (1.4 mL) were eluted at a flow rate of 10 mL/h and those emerging at the same position as rECorL were collected, pooled, and concentrated by Centriprep-10. ECORL marks the elution position of the native lectin. The large flow-through peak most likely consists of incorrectly folded and aggregated protein, the amount of which is highly variable, both for the recombinant and mutant lectin (Adar & Sharon, 1996).

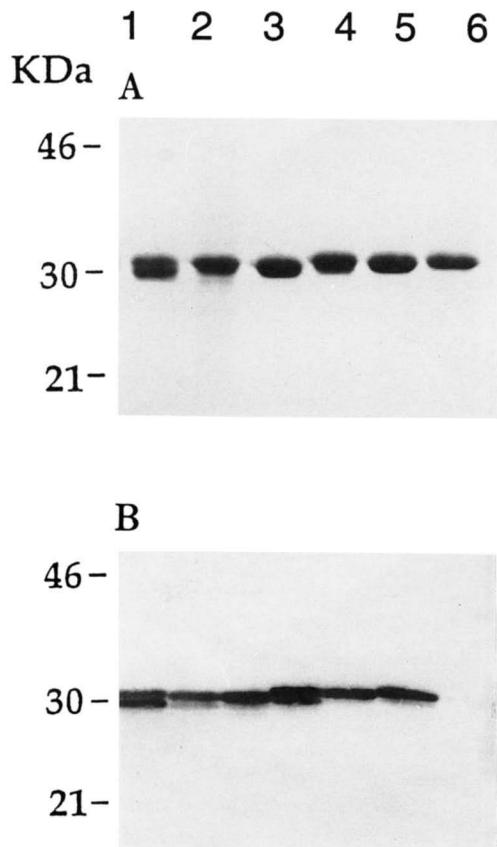


Fig. 2. SDS-PAGE and western blot of ECORL mutants. Proteins obtained from inclusion bodies and purified on Sephadex G-150 were analyzed by electrophoresis on 10% gels and stained either with Coomassie blue (**A**) or transferred to a nitrocellulose membrane and probed with polyclonal rabbit anti-ECORL IgG as described in Materials and methods (**B**). Lane 1, ECORL; lane 2, rECORL; lane 3, Y108A; lane 4, Y108T; lane 5, W135A; lane 6, W135Y. Double bands are most likely due to C-terminal processing of the lectin (Young et al., 1995), which is variable (Adar & Sharon, 1996; Arango et al., 1993).

224 ± 1 nm and mean residue ellipticities Θ nearly identical and the same as those reported for Con A (Yang et al., 1986). The CD data therefore suggest that the gross conformation of the different proteins is essentially the same, consisting largely of β -sheets, as shown also by X-ray crystallography of ECORL as well as other legume lectins. Tyr 108 and Trp 135 are located in loop regions of ECORL, so that their replacement by other residues might be expected to cause only minor local changes in the structure of the lectin, and not in their CD spectra.

Spectrofluorimetry

In Figure 3, the fluorescence excitation spectra of rECORL and W135A, both with bound Me α GalNDns, are shown. In the W135A spectrum, the peak at an irradiation wavelength of 280 nm, which represents the indole absorption responsible for the dansyl emission at 530 nm, is completely abolished. The excitation spectrum resembles the superposition of the spectra of tryptophan and *N*-dansylalanine, showing that the energy transfer is virtually complete. One cannot exclude, however, the contribution of energy

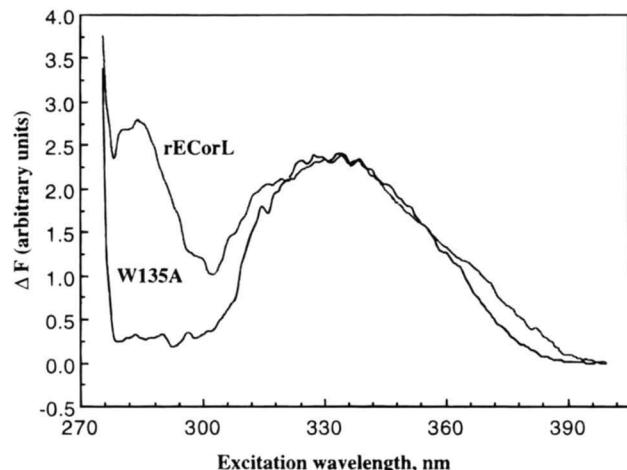


Fig. 3. Excitation spectra of ECORL (3.5 nM) and mutant W135A (normalized to the same intensity at 330 nm) in complex with Me α GalNDns (2.9 nM). Detection of emission was at 530 nm.

transfer between the various tryptophan residues of the protein. If this is so, Trp 135 will act as a fluorescence energy sink. In W135Y, there was negligible energy transfer (data not shown). Enhancement of the dansyl fluorescence of Me α - and Me β -GalNDns when bound to the lectin has been described (Kinzy et al., 1992) and, as shown below, the indole of Trp 135 and the dansyl group are parallel, as required for efficient energy transfer. These findings thus demonstrate that, among the five tryptophan residues present in ECORL (Arango et al., 1990), Trp 135 solely is responsible for energy transfer to the ligand.

Metal ion analyses

Analysis of the Ca²⁺ and Mn²⁺ content of Y108T, Y108A, and W135Y gave, as expected, values close to one mole of each metal per mole of lectin subunit (Fig. 4). The Mn²⁺ ESR spectra of these mutants did not differ from that of the native or recombinant lectin (Fig. 4) and were essentially the same as those reported for the previously described ECORL mutants (Adar & Sharon, 1996) as well as for other legume lectins, e.g., Con A (Nicolau et al., 1969; Reed & Cohn, 1970), demonstrating that, in the above three mutants, Mn²⁺ is in the usual octahedral environment. The importance of the metal ions for both activity and stabilization of the legume lectins has been well established (Sharon & Lis, 1990; Emmerich et al., 1994; Bouckaert et al., 1995). The fact that these mutants have a normal metal content and normal Mn²⁺ ESR spectra may thus be taken as evidence that the overall topology of the metal sites is unchanged.

Quite surprisingly, however, no Mn²⁺ or Ca²⁺ could be detected by atomic absorption spectrometry in W135A dialyzed similarly to the other protein preparations, as required for metal analysis (Fig. 4). This mutant also was completely devoid of ions such as Cd²⁺, Co²⁺, or Ni²⁺, which can substitute for Mn²⁺ and Ca²⁺ in legume lectins to give active proteins (Jaffe et al., 1977; Emmerich et al., 1994). No Mn²⁺ could be detected in the ESR spectrum of W135A either (Fig. 4). When tested for hemagglutinating activity, the mutant was inactive even at concentrations as high as 1 mg/mL (300 times the minimal hemagglutinating concentration of ECORL

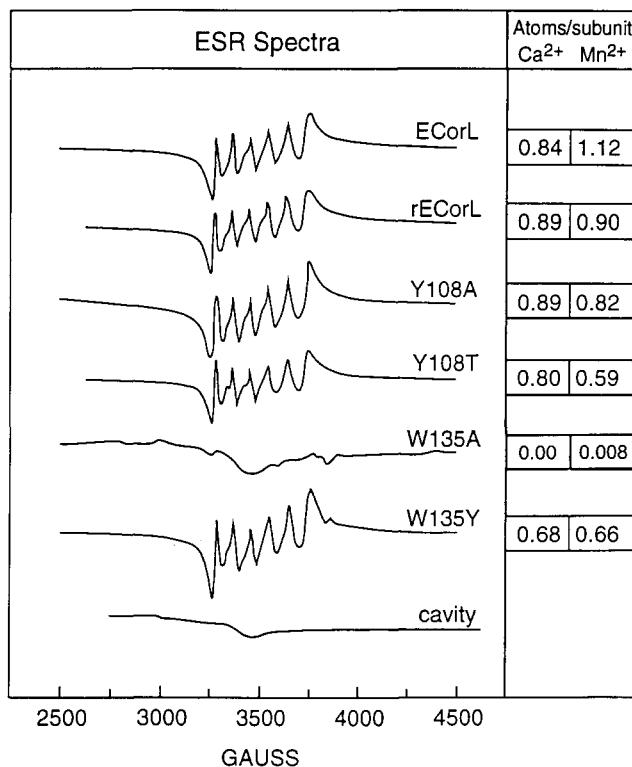


Fig. 4. ESR spectra of Mn²⁺ and metal content (determined by atomic absorption spectrometry) of ECORL, rECORL, and the mutants, extensively dialyzed against double-distilled water and lyophilized prior to analysis.

or rECORL). This is in agreement with findings with other legume lectins, showing that demetallization leads to loss of activity. However, in the few other legume lectins examined (e.g., Con A and soybean agglutinin), both Mn²⁺ and Ca²⁺ were found to be tightly held and could not be removed by prolonged dialysis against water, nor against chelating agents such as EDTA (Jaffe et al., 1977). Demetallization could only be achieved at acidic pH (5 or below) and usually in the presence of chelating agents. It should be noted that W135A was fully active before dialysis (Table 1), as well as after 12 h of dialysis against deionized water, when its metal ion content, measured by atomic absorption spectrometry, was 0.44 gram atom Mn²⁺ and 3.71 gram atom Ca²⁺ per subunit.

To obtain an idea of the metal ion binding strength to W135A, it was dialyzed against 1 mM EDTA. As can be seen in Figure 5A, a 50% loss of activity was obtained after 30 min of dialysis, whereas almost complete loss occurred within 2 h. The loss of activity is due to removal of the metal ions, because it could be fully restored by dialysis against Mn²⁺ and Ca²⁺ (Fig. 5B) or against other appropriate metal ions (Fig. 5C).

Biological activity and carbohydrate specificity

The biological activity of the mutants and their specificity for different monosaccharides and disaccharides was established by hemagglutination assays. This is a simple, rapid, and convenient technique, although accurate only within a factor of two, as dictated by the twofold dilution of the assay. Some variation in the results may also be due to differences in the sensitivity of human

Table 1. Properties of ECORL, rECORL, Tyr 108, and Trp 135 mutants

Lectin	MHC ^a ($\mu\text{g/mL}$)	Gal (mM) ^b	R _{Gal} ^c		
			GalNAc	MeαGalNDns	2'FL ^d
ECORL	3.5	18	3	500	15
rECORL	3.0	24	3	750	NT ^e
Y108A	12	12.5	1.8	260	7.5
Y108T	9	12.5	1.5	430	7.5
W135A	6	10	0.12	70	3.7
W135Y	9.5	12.5	1.0	100	10

^aMinimal hemagglutinating concentration.

^bConcentration of galactose required for complete inhibition of hemagglutination by four units of the lectin.

^cAffinity relative to galactose. The lectins listed had essentially the same affinities for the other sugars tested: L-fucose ($R_{Gal} = 0.3$), lactose (2–4), LacNAc (20), MeαGal (0.75–3), MeβGal (0.5–1.5), 6-fluorogalactose (0.5), L-arabinose (0.025), LnNT (16–32), and *p*-nitrophenyl α-galactoside (2–4).

^d2'-Fucosyllactose, L-Fuc α 2Gal β 4GlcNAc.

^eNot tested.

erythrocytes to sialidase treatment and in their reactivity with the lectin. Still, the relative affinities for different ligands measured by such assays compare well with those obtained by more accurate techniques, such as ELISA or spectrofluorimetric titration (Arango et al., 1993) or microcalorimetry (Surolia et al., 1996). The results obtained for the mutants are summarized in Table 1. For comparison, the data for the native and recombinant lectins (both used routinely as controls) are also included. Each value is an average of at least three different experiments, often done with different preparations of the same protein and with different blood preparations.

As can be seen, the hemagglutinating activities of the mutants are close to those of the native or recombinant lectins, as are their affinities for galactose, based on the minimal concentration of the monosaccharide required for complete inhibition of hemagglutination (Table 1). Therefore, neither Tyr 108 nor Trp 135 interacts with the galactose in the combining site, as is also apparent in the high-resolution structure (2.0 Å) of the ECORL-lactose complex (Shaanan et al., 1991). The relative affinities of mutants Y108A and Y108T for all the other mono- and disaccharides tested, of which the complexes with ECORL have not been examined by X-ray crystallography, are nearly identical to those found for the parent lectin. This demonstrates that Tyr 108, although close to the binding site, is not involved in binding to any of these compounds.

Replacement of Trp 135 by alanine or tyrosine did not affect the activity of the lectin, nor its affinity for most of the saccharides tested (Table 1). However, a decrease in the affinity for *N*-acetylgalactosamine, MeαGalNDns, and Fuc α 2Gal β 4Glc of mutant W135A, and to some extent also of W135Y, was observed.

Ligand docking and MD simulations

The global minimum energy conformation of MeαGalNDns was found through grid searches to be centered around $-70^\circ/120^\circ$ for the C2-N-S-C1 and N-S-C1-C2 dihedral angles (starting from the galactose end) with the H2-C2-N-H dihedral angle in the *trans* conformation (Fig. 6). Docking of the *trans* conformer into ECORL resulted in the dansyl moiety making contact with Ala 218 instead

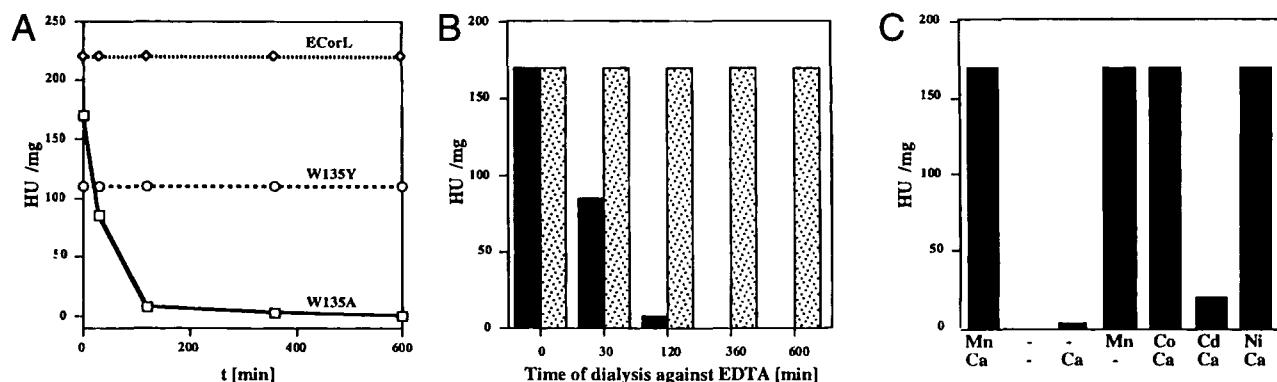


Fig. 5. Dialysis and remetallization experiments. **A:** Effect of dialysis against EDTA (1 mM, pH 8, 4 °C) on the activity of ECORL (1.0 mg/mL), W135A (0.55 mg/mL), and W135Y (1.23 mg/mL). **B:** Activity of mutant W135A, dialyzed against EDTA (1 mM, pH 8, 4 °C) (black bars), and reactivated by dialysis against Mn²⁺ and Ca²⁺ (10 mM, H₂O, 4 °C, 12 h) (dotted bars). **C:** Impact of other divalent metal ions on the activity of W135A, obtained by dialysis of demetallized W135A against solutions of various metals (10 mM, 4 °C, 12 h). HU, hemagglutinating units.

of Trp 135. However, rotation around the C2-N-S-C1 dihedral angle to -160° , whereby a local minimum energy region (centered around $170^\circ/120^\circ$ and 1.4–2.0 kcal/mol higher in energy) is reached, results in a parallel arrangement of the Trp 135 indole and the dansyl moiety which, furthermore, come into van der Waals contact. Conformers of MeαGalNDns corresponding to other energy minima caused either serious interference between the dansyl moiety and the protein or no interactions at all.

Moreover, the conformation of MeαGalNDns in the ECORL complex, in which the dansyl moiety is parallel with the indole of Trp 135, was found to be very stable in a 100-ps MD simulation made in the presence of a water shell having a 14-Å radius centered around the ligand. Only residues in the vicinity of the ligand and in contact with the solvent were allowed to move. Figure 7A shows a representative time frame 22 ps into the simulation with the dansyl group in van der Waals contact with the side chains of Tyr 108 and Trp 135. The Asn 133 and Tyr 106 side chains also make contact with the dansyl moiety, whereas the Pro 134 ring is slightly away from this group. The galactosyl unit displays the same interactions as found for the ECORL-lactose complex (Shaanan et al., 1991) except for the side chain of Asn 133, which interacts less frequently with the Gal 3-OH. Thus, the energetically somewhat unfavorable conformation of the isolated MeαGalNDns mol-

ecule must be more than compensated for by the protein interactions just described. The rECORL-MeαGalNDns complex is very similar to that of ECORL, except for the C_βH₂-C_γH₂ segment of Gln 134 (replacing Pro) making van der Waals contacts with the dansyl group and a reorientation of the methoxy group of MeαGalNDns away from the dansyl moiety toward the Gln 219 side chain, but this does not significantly affect the lectin affinity for MeαGalNDns (Table 1).

The slightly reduced affinity of the Y108A mutant for MeαGalNDns (Table 1) may be explained by recent molecular modeling studies by Moreno et al. (1997), suggesting that this substitution affects the adjacent Tyr 106 such that, in a fraction of the lectin molecules, the side chain of this residue is oriented into the galactose binding pocket instead. For the fraction retaining the native conformation, loss of van der Waals contacts with the Tyr 108 side chain may be a contributing factor to the lowered affinity. In the Y108T mutant, the somewhat larger threonine side chain appears to disallow the transition described above for Tyr 106.

Previous investigations of the effect of other mutations around the combining site surprisingly revealed that A218G exhibited a reduced affinity for MeαGalNDns by about a factor of 10 (Adar & Sharon, 1996). This may be because the only other MeαGalNDns conformation accepted by the lectin resulted in the dansyl moiety making hydrophobic contact with the A218 methyl group (Fig. 7B), suggesting that the dansyl moiety significantly populates this site as well.

The structural role of the Trp 135 side chain in the native lectin appears mainly to be the closing of the metal-binding cavity by firmly anchoring the 132–136 loop, largely through hydrophobic interactions, to the rest of the protein. This strategically located residue, not too far away from the sugar binding site (5–6 Å), may be important also for the affinity of the lectin for various ligands, as will be described below. Significantly, this residue is conserved among the legume lectins, except that, in some instances, it is conservatively exchanged for other bulky hydrophobic residues and interacts with the likewise conserved Ile 150 (Table 2). Mutation of Trp 135 into Tyr results in a substantial drop of the lectin affinity for MeαGalNDns (Table 1). Because the ability of this mutant to retain the metal ions is unimpaired (Fig. 5A), the only

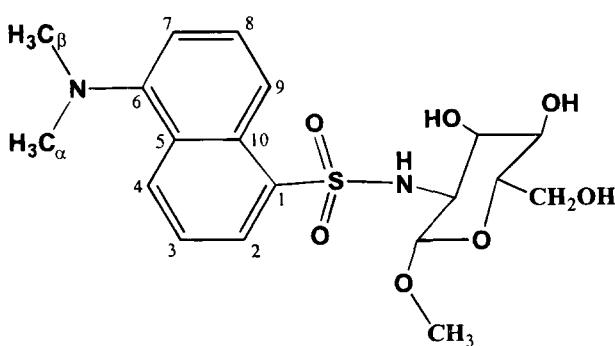


Fig. 6. Structure of methyl α -N-dansylgalactosaminide (MeαGalNDns).

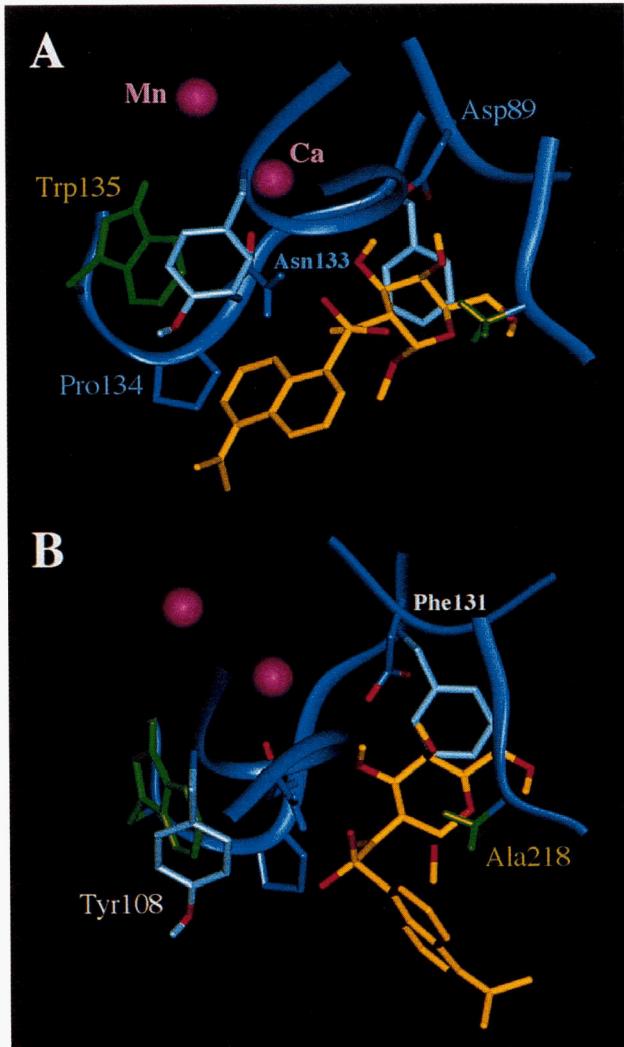


Fig. 7. Models of the complex between ECORL and Me α GalNDns in two different conformations. **A:** Conformation in which the dansyl moiety is parallel and in van der Waals contact with the indole of Trp 135. **B:** Global minimum energy conformation of Me α GalNDns in which the dansyl moiety interacts with the methyl group of Ala 218. In both panels, parts of the protein backbone have been drawn as blue ribbons; the side chains of Tyr 108 and Phe 131 are shown in light blue and those of Trp 135 and Ala 218 are colored green; the metal ions are shown in magenta. Oxygen atoms are marked in red. Only polar hydrogens of protein side chains and Me α GalNDns, apart from the methyl hydrogens of Ala 218, are included. Both panels show the same amino acid residues, their labels being complementary.

reasonable explanation is that the tyrosine side chain occupies the same space as that taken up by tryptophan indole moiety in the native protein. The decreased affinity in this case is thus most likely due to the more polar environment afforded by the phenol hydroxyl group.

However, for the decreased affinity of W135A for Me α GalNDns, an entirely different mechanism must be operative because the small alanine side chain is unable to provide enough hydrophobic interactions with the surrounding residues necessary in order for the 132–136 loop to firmly adhere to adjacent loops, and thus close the metal-binding cavity, unless major conformational changes occur. The flexibility of the 132–136 loop in W135A was therefore

explored during a 200-ps MD simulation in vacuum in which this loop and neighboring loops were allowed to move. The simulation reveals that only the 132–136 loop experiences significant changes as monitored, e.g., by the distance between the C β atom of Gln 134 and O_{para} atom of Tyr 108, the position of the latter residue remaining roughly the same throughout (Fig. 8, top panel). When these two residues are at their closest, they come into van der Waals contact with each other, closing the metal-binding cavity efficiently and thus also denying the dansyl moiety of Me α GalNDns access to the space occupied by the indole of Trp 135 in the native lectin. Simultaneously, Ala 135 moves toward the C γ H₃ of Ile 150 and C α H-C β H₂ of Arg 151 and remains mainly within van der Waals distance of these groups, whereas Asn 133 moves toward the Asp 89 carboxylate, frequently within hydrogen bonding distance (Fig. 8, middle and bottom panels). However, at around 125 ps, the 132–136 loop starts to veer away substantially from its previous close association with the rest of the protein, reaching a maximal opening around 150 ps (Fig. 8, top panel), corresponding to the van der Waals thickness of the dansyl moiety (6.7 Å), suggesting that the dynamic behavior of W135A infrequently results in conformations that would allow Me α GalNDns to bind with the dansyl moiety, filling up the space occupied by the Trp 135 indole in the native lectin.

Using the same starting conformation for W135A–Me α GalNDns as for the rECORL complex described above in an MD run in vacuum (100 ps) reveals that the dansyl moiety, already during the equilibration period, partially inserts itself into the space occupied by the Trp 135 indole in ECORL and remains there during the whole simulation period (Fig. 9). The dansyl group makes numerous van der Waals contacts of hydrophobic nature with surrounding residues: N-C α H₃ interacts with the Ala 135 C β H₃, Asn 133 C β H₂, and Asp 136 C β H₂; N-C β H₃ with Ser 115 C β H₂; the innermost dansyl ring with Ile 150 C γ H₃ and C α H of Gly 107. The galactose is displaced ~1.5 Å toward Ala 135, but retains its normal interactions, whereas the C2-N-S-C1 and N-S-C1-C2 dihedral angles change from -160° to 160° and from 132° to 102°, respectively. These interactions indicate that the ligand is more strongly bound by this mutant than by rECORL, whereas the binding data reveal an approximately ten-fold reduction in binding strength. However, these seemingly contradictory observations may be rationalized by assuming that a kinetic barrier in the form of a conformational equilibrium involving open and closed states of the 132–136 loop exists in which the closed state is energetically favored in accordance with the results from the 200-ps MD run described above. This reasoning is also consistent with the slow release of the metal ions from W135A (Fig. 5A). The second Me α GalNDns conformation accepted by the lectin (see Fig. 7B) is probably also represented in the W135A–Me α GalNDns complex and would most likely have to entail ligand binding to one of the more open conformations of the 132–136 loop, as discussed below for other ligands.

Binding of N-acetylgalactosamine and Fuc α 2Gal β 4Glc were also affected for W135A, the former more severely than the latter (Table 1). Inspection of the ECORL–GalNAc complex shows that the acetamido methyl group makes hydrophobic contact with the rim of the Trp 135 indole, which most likely explains the higher affinity of this ligand relative to galactose. In the case of W135A, this interaction is lost and, coupled to the fact that the Asn 133 side chain forms a hydrogen bond with the Asp 89 carboxylate in the closed conformation of the 132–136 loop, suggests that N-acetylgalactosamine would be denied access to the galactose binding

Table 2. Alignment of legume lectin sequences for a segment containing residues involved in metal binding

	127	133	135		146	150
*	E CorL	... E FDT----FS	N Q-	W -DP PQVP-----HIGI	D VNS	I ...
*	SBA	... E FDT----FR	N S-	W -DP PN-P-----HIGI	N VNS	I ...
DBL	... E FDT----LS	N SG	W -DP SM-K-----HIGI	D VNS	I ...	
DB58	... E FDT----FS	N TD	W -DP TS--R-----HIGI	D VNS	I ...	
PHA-L	... E FDT----LY	N KD	W -DP TE--R-----HIGI	D VNS	I ...	
PHA-E	... E FDT----LY	N VH	W -DP KP--R-----HIGI	D VNS	I ...	
LBL	... E FDT----CH	N LD	W -D- KN-S-----IAV	N LG-	I ...	
DLL	... E FDT----DYL	N PD	Y GDP -NYI-----HIGI	D VNS	I ...	
*	LOL-I	... E FDT----FY	N TA	W -DP SNGDR-----HIGI	D VNS	I ...
LSL	... E FDT----FH	N QP	W -DP -DYI-----HIGV	D INS	I ...	
*	LCL	... E FDT----FY	N AA	W -DP SNKER-----HIGI	D VNS	I ...
*	PSA	... E FDT----FY	N AA	W -DP SNRDR-----HIGI	D VNS	I ...
VFL	... E FDT----FY	N AA	W -DP SNGKR-----HIGI	D VNT	I ...	
*	CONA	... E LDT----YP	N TD	I GDP -SYP-----HIGI	D IKS	V ...
DGL	... E LDS----YP	N TD	I GDP -NYP-----HIGI	D IKS	I ...	
BMA	... E FDT----YP	N TD	I GDP -NY-R-----HIGI	D VNS	I ...	
*	PNA	... E FDT---YS	N SE	Y NDP -PTD-----HVGI	D VNS	V ...
SL	... E FDT----FS	N R-	W -DP -ANS-----HIGI	N VNS	V ...	
UEA-I	... E FD T I-GSPV	N --	F DDP -GFP-----HIGI	D VNR	V ...	
UEA-II	... E FD S YFGKTY	N P-	W -DP -DF-K-----HIGI	D VNS	I ...	
CSII	... E FDT---YY	N SA	W -DP QTNP-----HIGI	D VNT	I ...	
LAA-I	... E FD T YFGKAY	N P-	W -DP -DF-K-----HIGV	D VNS	I ...	
LTA	... E FDS----YH	N I-	W -DP KSLRSS-----HVGI	D VNS	I ...	
MTL-I	... E IDT----FH	N T-	W -DP KINR-----HIGI	N VNC	I ...	
MTL-II	... E IDT----FY	N AQ	W -DP NPGNISSTGRHIGI	D VNS	I ...	
BPA	... E FDT----WP	N TE	W S D L RY-P-----HIGI	N VNS	T ...	
* GS4	... E FDT----WI	N KD	W NDP PY-P-----HIGI	D VNS	I ...	

^aComparison of amino acid sequences of 27 legume lectins for segment 127–150 (ECorL numbering), which contains residues important for metal binding. Positions 127, 129, 133, 135, 136, 146, and 150 are marked in bold (see text). Gaps (-) were introduced for optimal alignment. Asterisks (*) indicate the availability of crystal structure(s). Sequences are grouped by suborders and tribes. Abbreviations used for the lectins are: ECorL, *Erythrina corallodendron*; SBA, *Glycine max* (soybean); DBL and DB58, *Dolichos biflorus*; PHA-L, *Phaseolus vulgaris* leucoagglutinin; PHA-E, *Phaseolus vulgaris* erythrocytes agglutinin; LBL, *Phaseolus lunatus*; DLL, *Dolichos lab lab*; LOL-I, *Lathyrus ochrus* isolectin I; LSL, *Lathyrus sphaericus*; LCL, *Lens culinaris* (lentil); PSA, *Pisum sativum* (pea); VFL, *Vicia faba*; ConA, *Canavalia ensiformis*; DGL, *Dioclea grandiflora*; BMA, *Bowringia mildbraedii*; PNA, *Arachis hypogaea* (peanut); SL, *Onobrychis viciifolia*; UEA-I and UEA-II, *Ulex europeus* isolectins I and II, respectively; CSII, *Cytisus scoparius* lectin II; LAA-I, *Laburnum alpinum* lectin I; LTA, *Lotus tetragonolobus*; MTL-I and MTL-II, *Medicago truncatula* isolectins I and II, respectively; BPA, *Bauhinia purpurea*; GS4, *Griffonia simplicifolia* isolectin IV.

pocket mainly due to steric interference between the acetamido and 3-OH groups and Asn 133, but, once bound, the more hydrophilic environment experienced by the acetamido group would be the contributing factor to the lowered affinity. The very weak binding that is observed can thus, in analogy with the MeαGalNDns case, be ascribed to complexation with the open conformation of W135A. Concerning Fuca2Galβ4Glc, the same mechanism must be operative even though the affinity is reduced only by a factor of 3. However, once bound, the fucosyl residue provides more interactions with the lectin of both hydrophobic and hydrogen bonding nature (Moreno et al., 1997).

The basis for the loss of metal ions and inactivation of W135A was next investigated by an MD simulation (100 ps) where the metal ions had been removed from the lectin while letting the internal water molecules remain. The metal-binding cavity contains four negatively charged residues, three of which, namely Glu 127, Asp 129, and Asp 136, participate directly in the metal-binding network and are strictly conserved in the known legume lectin sequences (Table 2), whereas Asp 146, which is replaced by Asn in several legume lectins, interacts with Mn²⁺ through a water molecule. Furthermore, both Mn²⁺ and Ca²⁺ are coordinated to other electronegative atoms belonging to neutral amino acid resi-

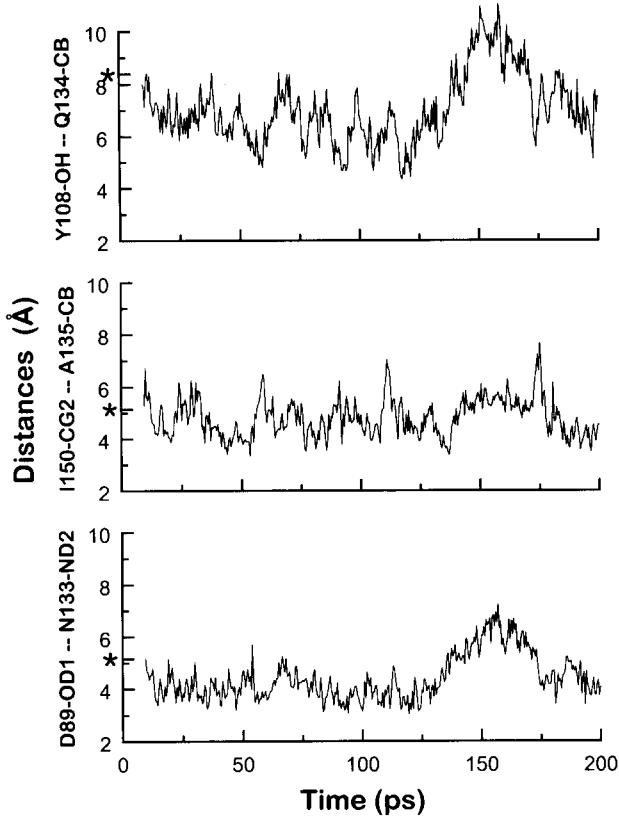


Fig. 8. Time versus distance traces extracted from a 200-ps MD simulation in vacuum of the W135A mutant of ECORL showing distances between Tyr 108 O_{para} and Gln 134 C_β (upper panel), Ala 135 C_β and Ile 150 C_{γ2} (middle panel), and Asp 89 O_{δ1} and Asn 133 N_{δ2} (bottom panel). Asterisks indicate distances in the starting conformation of the lectin.

dues or water molecules, which envelop the positive charges carried by these metal ions. When the metals are removed, the charge balance is disrupted, which must lead to a reorientation of some amino acid side chains.

During the MD run, a complex set of events occurred where initially two of the four water molecules in the metal cavity were expelled during the equilibration period, leading to a reorientation of the Asp 136 side chain upward and to the left of the cavity (by ~3.3 Å compared to the starting conformation), where it may form a single or double hydrogen bond with the backbone NH of Ser 152 (not included in Fig. 10). This results in Ala 135 being able to move further into the cavity by 3.6 Å (2.5 Å relative to its position in the metal-containing mutant in its closed conformation) with its methyl becoming lodged between Ile 150 C_αH₂ and C_γH₃, Ser 115 C_βH₂, and Gly 107 C_αH₂ groups (Fig. 10). The Ile 150 and Ser 115 side chains also move in the same direction (~1.7 Å), whereas the Gly 107 moves toward the Ala 135 methyl (~2.1 Å). These concerted movements, together with slighter adjustments of other residues—the Leu 109 and Asp 129 side chains move inward by 1.2 Å and 1.4 Å, respectively, whereas the Glu 127 carboxylate moves away from the cavity by 1.3 Å—reduce the size of the cavity, thus allowing only two of the internal water molecules to remain, and also account for the rebalancing of the charge distribution within the cavity.

The movement of Gly 107 is propagated to the right-hand side of the galactose binding pocket by a concomitant lowering of the

Tyr 106 residue (~2.7 Å) toward the binding site, pushing away the Ala 218 methyl group by approximately the same distance but to the right, which in turn results in the Trp 45 side chain flipping almost 180° to avoid interference with Ala 218 (Fig. 10). As Ala 218 moves, the 218–219 peptide bond rotates approximately 90° away from the galactose binding site, leaving room for the Gln 219 side chain to swing beside Phe 131 and come within hydrogen bonding distance of the Asp 89 side chain, which moves slightly downward. Together with a movement of Asn 133 toward Asp 89 on the left-hand side, these changes appear to destroy the binding capability of the galactose site. This chain of events was completed within 45 ps, whereafter no significant changes were observed.

As mentioned, W135A is able to release its metal ions (Fig. 5A). The mode by which this process occurs was therefore investigated by high-temperature dynamics simulations by heating the native lectin and W135A, with and without metals, up to 1,500 K. In these simulations, the whole protein, including internal water molecules, was allowed freedom of movement without any constraints whatsoever. The tertiary structure of native ECORL remains remarkably stable over almost the whole temperature range, with the only significant change occurring around 1,000 K, in which the *cis* conformation of the Ala 88–Asp 89 peptide bond, a conserved feature among leguminous lectins (see e.g., Bouckaert et al., 1995), isomerized to the *trans* conformation (not shown). The *cis* configuration is stabilized by interstrand hydrogen bonds and is structurally linked to the nearby Ca²⁺ via the Asp 89 side chain, which forms a hydrogen bond with one of the internal water molecules coordinated to Ca²⁺ (Shanaan et al., 1991). The triggering event of the isomerization is the migration of the above-mentioned internal water molecule toward the saccharide binding site, which allows the Asp 89 side chain to swing down and directly coordinate to the calcium ion. However, other structural features remain essentially unchanged as reflected by the 1.5-Å and 2.0-Å RMSDs for the C_α atom positions and for the whole protein, respectively, as compared to the starting conformation, and the metals essentially remain in their original position. The *cis-trans* isomerization of the Ala 88–Asp 89 bond in ECORL at the above-mentioned temperature is not due to restrictions imposed by the peptide bond parameterization because the energy barrier for such a transition was measured to be approximately 14 kcal/mol, considerably lower than the activation energy (21.7 kcal/mol) observed for the corresponding process in Con A (Koenig et al., 1978). Changing the dielectric constant from 6r to 4 caused the transition point to be lowered by approximately 50°. Otherwise, events took place as described above.

Performing the same type of simulation for metal-containing W135A reveals a different behavior in that the Ca²⁺ ion starts to migrate away from the metal cavity toward the 132–136 loop at approximately 800 K. This loop subsequently assumes a more open conformation around 1,000 K, which allows the Ca²⁺ ion to completely escape from the protein at ~1,150 K, whereas the Mn²⁺ ion starts to migrate only slightly in the same direction around 1,400 K. The migration of the metal ions should be interpreted with caution, however, because force field parameterizations are incomplete for systems containing metals (Halgren, 1995). Even so, these events serve as an indicator of the lowered stability of W135A. At 1,150 K, the RMSD of the C_α atom positions, as compared to the starting conformation, is 1.7 Å and, for the whole protein, 2.4 Å, only slightly higher than for the native lectin. Surprisingly, the *cis-trans* isomerization is seen to occur at a significantly higher temperature (~1,500 K) than is the case for ECORL, suggesting that the disappearance of the metals relieves the protein

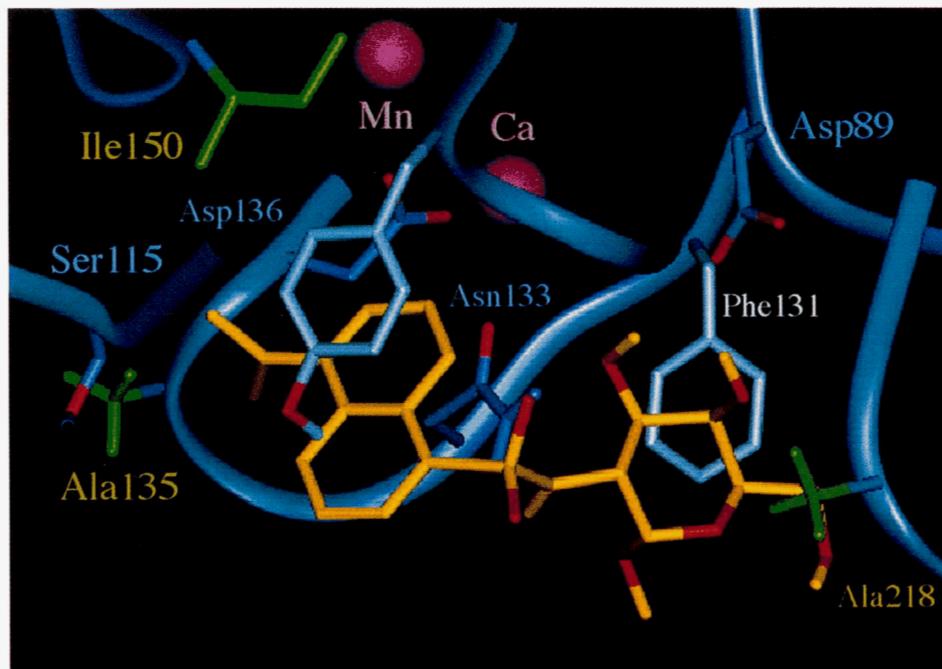


Fig. 9. Model of the complex between MeaGalDns and the W135A mutant, as obtained from MD simulations. Compared with the conformation shown in Figure 7A, the dansyl moiety in this case has moved toward the space occupied in part by the Trp 135 indole in ECORL. Only polar hydrogens are displayed, with the exception of the methyl hydrogens of Ala 135 and Ala 218, which, together with Ile 150, are shown in green. The remainder of the picture follows the same color code as in Figure 7.

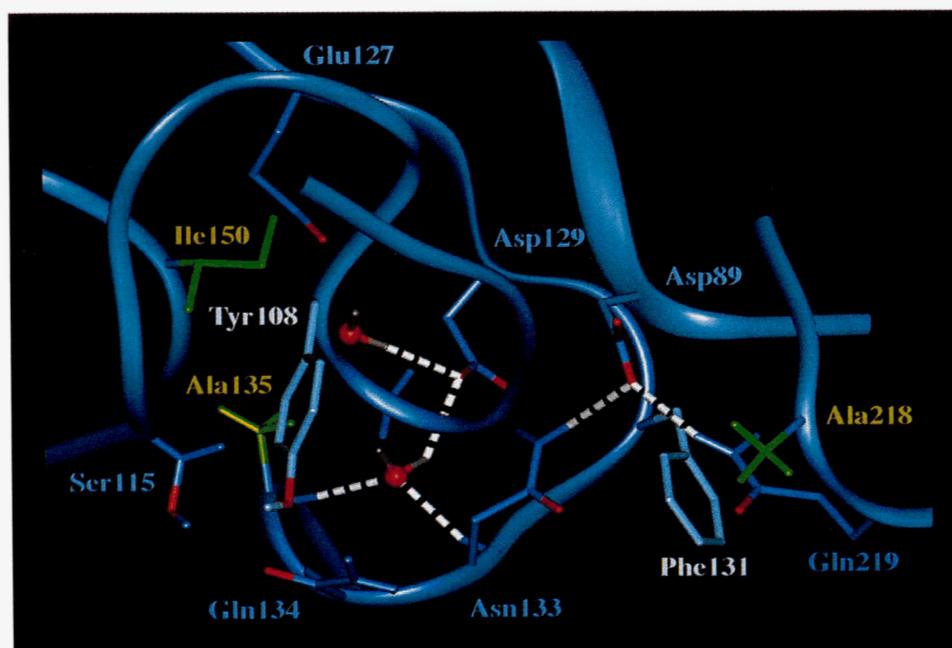


Fig. 10. Model of the metal and saccharide combining sites of the W135A mutant of ECORL, obtained from a 100-ps MD simulation in vacuum, showing the stable configuration arrived at after approximately 45 ps. The color code is the same as in Figures 7 and 9, except as detailed below. Of the four water molecules conserved in the metal binding site of all legume lectins (Loris et al., 1994), the two remaining here shown in red and gray, are held by hydrogen bonds to the Asp 129 side chain and the backbone NH protons of Asn 133 and Asp 136, which are shown as blue sticks. The 132–136 loop assumes a closed conformation, allowing the side chains of Tyr 108, Ser 115, and Gln 134 to interact frequently. The methyl group of Ala 135 is lodged between Ile 150 C_γH₂ and C_γH₃, Ser 115 C_βH₂, and Gly 107 C_αH₂ groups, the two latter protons being shown as blue sticks (not labeled). The Asp 136 side chain (not included) is pointing toward the Ser 115 backbone NH as described in the text (cf. Fig. 8 where it is pointing into the cavity). Access to the saccharide binding site is blocked by the side chains of Asn 133 and Gln 219, which form hydrogen bonds to the Asp 89 side chain.

of some steric strain around the Ala 88–Asp 89 peptide bond, resulting in the *cis* isomer being energetically more favored. Metal-free W135A confirms this picture.

Taken together, these results suggest that metal release from W135A is due to the increased mobility of the 132–136 loop and not directly coupled to the isomerization process. In Con A, the remetallization process has been investigated thoroughly: the metal-free protein was thus found to exist in either of two conformational states termed “locked” and “unlocked,” respectively (Brown et al., 1977). The predominant unlocked form binds metals and ligands weakly and has the Ala 207–Asp 208 peptide bond in the *trans* conformation, whereas the minor locked form has full metal-binding capability and the Ala 207–Asp 208 peptide bond in the *cis* conformation. Once the metals are bound to the locked form, the saccharide affinity of the lectin is restored. The unlocked state of the lectin has also been examined by X-ray crystallography, most recently by Bouckaert et al. (1995), who found extensive conformational changes around the Ca^{2+} and saccharide sites, whereas the Mn^{2+} site was relatively unperturbed. However, this structure was obtained at low pH (5), which also is a requirement for demetallization, suggesting that protonation of residues involved primarily in Ca^{2+} binding is responsible for the disruption of this site as well as the neighboring saccharide site. Because the isomerization in W135A does not appear to be dependent on the loss of metals, and coupled to the fact that the latter process may occur around neutral pH, this indicates that the locked form of metal-free Con A, rather than the unlocked form, would provide a more relevant comparison in terms of the structural changes.

Materials and methods

Materials

Native *E. corallodendron* lectin from mature dry seeds was prepared by affinity chromatography on lactose-Sepharose (Lis et al., 1985). Sialidase (Test Neuraminidase) was from Behring (Behringwerke AG, Marburg). Sugars were from commercial sources of the highest purity available. MeαGalNDns (Kinzy et al., 1992) was a gift of Dr. Willy Kinzy; *N*-acetyllactosamine and lacto-*N*-neotetraose ($\text{Gal}\beta 4\text{GlcNAc}\beta 3\text{Gal}\beta 4\text{Glc}$) were gifts of Dr. David Zopf and Fuca2Galβ4Glc was a gift of Dr. P. Prieto and Dr. A. Surolia. Construction of mutant cDNAs, expression of mutant lectins, and their isolation from inclusion bodies were performed as described by Adar and Sharon (1996).

Analytical techniques

The following techniques were performed as described by Adar and Sharon (1996): protein determination and analysis by SDS-PAGE; electrophoretic transfer of proteins from the gel to a nitrocellulose membrane and their immunodetection; affinity electrophoresis and CD spectroscopy.

Affinity electrophoresis

The mutants were analyzed by affinity electrophoresis on polyacrylamide gels substituted by the α -anomers of galactose, *N*-acetylgalactosamine, or lactose under nondenaturing conditions, both in the absence and presence of inhibitory sugars (Horejsi et al., 1977).

Metal ion analysis

The content of calcium and manganese in exhaustively dialyzed and lyophilized protein samples dissolved in boiling nitrous acid (13%) was assayed by atomic absorption spectrometry in an ICP apparatus at the Faculty of Agriculture, Hebrew University, Rehovot.

ESR measurements

These were performed on a Varian E-12 spectrometer, operating at X-band (~9 GHz). Spectra were recorded at room temperature.

Dialysis experiments

To ensure the absence of divalent cations, water treated with Chelex-100 (BIORAD) was used for buffer preparation and dialysis. For dialysis, only plastic dishes, exhaustively rinsed with dilute HCl (0.01 M), EDTA (0.01 M, pH 8), and Chelex-100-treated double-distilled water, were used. Dialysis bags (MW cutoff 15 kDa) were treated with boiling EDTA solution (1 mM) prior to use.

Demetallization and remetallization experiments

Five aliquots (1 mL each) of ECorL (1.00 mg/mL), W135Y (0.55 mg/mL), and W135A (1.23 mg/mL) in buffer (10 mM Tris-HCl, 150 mM NaCl, 0.02% NaN₃, pH 7.5) were prepared. The first aliquot was taken as a control and the others were dialyzed against 5 L of 1 mM EDTA, pH 8 (4 °C). After 30 min, 2 h, 6 h, and 10 h, respectively, one aliquot of each protein was removed and frozen at –20 °C. When dialysis was completed, the aliquots were thawed and centrifuged to remove any precipitate formed. The protein content was determined by measuring the absorbance at 280 nm, and subsequently the hemagglutinating activity of the samples was assayed.

Solutions of demetallized W135A, obtained as described above, were dialyzed against 10 mM solutions of different metal ions, alone or in combination of two, prepared in double-distilled water (4 °C, 12 h). As above, the protein content of the remetallized samples was checked by reading the OD at 280 nm and the hemagglutinating activity was assayed.

Spectrofluorimetry

Fluorescence excitation spectra were recorded at room temperature in a Perkin-Elmer LS-50B spectrofluorimeter. Measurements were performed in a 1-cm pathlength quartz (QS) glass cuvette and a final reaction volume of approximately 2 mL. The slit width for excitation and detection was 8 nm. To minimize the contribution of unbound MeαGalNDns to the overall fluorescence emission, an excess of lectin over ligand was always employed. Fluorescence excitation was scanned from 250 to 400 nm and detection of dansyl emission was at 530 nm. To facilitate comparisons of the energy transfer, fluorescence excitation spectra were normalized to the same intensity at 330 nm, the absorption maximum of the dansyl group.

Hemagglutinating activity and inhibition of hemagglutination

These were assayed by the serial dilution method in microtiter plates in a total volume of 50 mL, of which 25 mL was a suspension of sialidase-treated (Lotan et al., 1975; Arango et al., 1992)

human erythrocytes obtained from the blood bank of Sheba Hospital, Tel Hashomer. Variations in the minimal hemagglutinating concentration of native ECorL, used as standard in all assays, were observed (2–5 mg/mL) irrespective of the blood type of the erythrocytes employed. The minimal inhibitory concentration of different sugars was measured by mixing serial dilutions of the sugars in phosphate-buffered saline (10 mM KH₂PO₄, 40 mM Na₂HPO₄, 0.9% NaCl, pH 7.4) with four hemagglutinating units of the lectin (final volume of 25 mL) 20 min before adding the erythrocytes. One hemagglutinating unit is the lowest concentration of lectin giving visible erythrocyte agglutination.

Molecular modeling

Molecular modeling was conducted on a Silicon Graphics Indigo² Extreme workstation using the Quanta4.1/CHARMM23 software package (Molecular Simulations, Inc.). Protein models were constructed essentially according to Moreno et al. (1997), based on the crystal structure of the complex between ECorL and lactose (Shaanan et al., 1991) deposited in the Brookhaven Protein Data Bank (entry 1LTE). Solvent molecules were removed, except those that were completely surrounded by the protein environment. The N-linked heptasaccharide of ECorL (at Asn 17) was also removed because it is far from the binding site. Subsequently, both polar and non-polar hydrogens were added to the structure. To refine the stereochemistry and molecular contacts, constrained energy minimizations of the protein were performed. Harmonic constraints of 20 kcal/(mol Å²) were applied to all α carbons and also to the two metal ions present in the lectin (Ca²⁺ and Mn²⁺), and the oxygen atoms of the internal water molecules, during 100 steps of a steepest-descent geometry relaxation. Afterward, harmonic constraints were reduced to 5 kcal/(mol Å²) in order to perform 300 steps of conjugate gradient minimization. A distance-dependent dielectric constant ($\epsilon = 6r$) or a 15-Å cutoff distance were used for nonbonded interactions. At the end of this procedure, the RMSD from the crystal structure was 0.10 Å when calculated for main-chain atoms and 0.41 Å for all atoms in the protein. The Ca²⁺ and Mn²⁺ ions were displaced from their crystal positions 0.05 Å and 0.15 Å, respectively. This structure was used subsequently as starting geometry for generation of mutants and in subsequent docking procedures.

Mutants were constructed by placing each new side chain in a conformation as similar as possible to the old one. After such automatic replacements, the side-chain conformations were adjusted, if necessary, taking into account rotamer populations and steric contacts with the rest of the protein. Each mutant listed in Table 1 carries an additional mutation because rECorL differs from ECorL at position 134 by having a Gln residue instead of Pro (Young et al., 1995). Finally, rECorL and the respective mutants were subjected to local energy minimization around the residue(s) in question.

Docking of ligands into the binding pocket of ECorL, rECorL, or mutants was done as described previously (Moreno et al., 1997) by superimposing the galactosyl part of either MeαGalNDns, N-acetylgalactosamine, or Fucα2Galβ4Glc onto the galactose of lactose in the ECorL-lactose complex (Shaanan et al., 1991). Local energy minima for MeαGalNDns were located by performing grid searches every 10° around the N-S-C bonds connecting the galactosyl and dansyl moieties. Each generated conformer was subjected to 200 steps of conjugate gradient energy minimization without any dihedral constraints. Conformers corresponding to the

found local energy minima were subsequently minimized further. Starting values for the glycosidic dihedral angles of the Fucα2Galβ4Glc trisaccharide were taken from the literature (Bock et al., 1985; Imberty et al., 1995).

MD simulations were run in vacuum and/or in a water environment. Vacuum simulations were performed mainly to study local conformational properties of the proteins in the absence of a ligand. A distance-dependent ($\epsilon = 6r$) or distance-independent ($\epsilon = 1$) dielectric constant was used for vacuum and water simulations, respectively. The SHAKE algorithm was used to constrain bonds containing hydrogen atoms, allowing a time step of 0.001 ps in the presence of water and 0.002 ps in vacuum. Simulations were started by heating the system from 0 K up to the final temperature (300 K unless otherwise specified) using a 5 K increment every 100 time steps, followed by an equilibration period of 10 ps.

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