Structural basis of redox-dependent modulation of galectin-1 dynamics and function

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Galectin-1 (Gal-1), a member of a family of multifunctional lectins, plays key roles in diverse biological processes including cell signaling, immunomodulation, neuroprotection and angiogenesis. The presence of an unusual number of six cysteine residues within Gal-1 sequence prompted a detailed analysis of the impact of the redox environment on the functional activity of this lectin. We examined the role of each cysteine residue in the structure and function of Gal-1 using both experimental and computational approaches. Our results show that: (i) only three cysteine residues present in each carbohydrate recognition domain (CRD) (Cys², Cys₁₆ and Cys₈₈) were important in protein oxidation, (ii) oxidation promoted the formation of the Cys₁₆–Cys₈₈ disulfide bond, as well as multimers through Cys₂, (iii) the oxidized protein did not bind to lactose, probably due to poor interactions with Arg₄₈ and Glu₇₁, (iv) in vitro oxidation by air was completely reversible and (v) oxidation due to poor interactions with Arg₄₈ and Glu₇₁, (iv) in vitro oxidation by air was completely reversible and (v) oxidation by hydrogen peroxide was relatively slow (1.7 ± 0.2 M⁻¹ s⁻¹ at pH 7.4 and 25°C). Finally, an analysis of key cysteines in other galectins is also provided in order to predict their behavior in response to redox variations. Collectively, our data provide new insights into the structural basis of Gal-1 redox regulation with critical implications in physiology and pathology.

Keywords: circular dichroism / cysteine / galectin-1 / molecular dynamics / oxidation

Introduction

Galectins are members of a family of multifunctional lectins widely distributed in the animal kingdom (Cooper 2002). They are defined by their specificity for β-galactoside-containing glycans and carbohydrate recognition domain (CRD) (Cooper 2002). Galectins participate in diverse functions, including cell signaling and death, immunomodulation, host–pathogen interactions, neuroprotection and angiogenesis (Ilarregui et al. 2009; Dam and Brewer 2010; St-Pierre et al. 2011; Rabinovich and Croci 2012; Starosom et al. 2012; Thijssen et al. 2013). In humans, about 16 different galectins’ CRDs have been discovered and identified (Guardia et al. 2011), being galectin-1 (Gal-1) the first and most studied so far. However, there are still members of the family that are not completely characterized, such as Gal-12 and galectin-related protein folds such as hGRPC (C-terminal of human galectin-related protein, previously known as HSPC159 for hematopoietic stem cell precursor), PP13 (placental protein 13, also known as Gal-13) and PPL13 (placental protein 13-like, or Gal-14). These proteins display a high degree of sequence identity with members of the galectin family, although their lectin activity is uncertain. Since their discovery, it was established that most galectins require a reducing microenvironment in order to fulfill their function (Vasta and Ahmed 2009). The relevance of protein oxidation in galectin structure and function has been demonstrated by biochemical characterization (Pande et al. 2003; Shahwan et al. 2004; Ashraf et al. 2011) and the contribution of cysteine residues to lectin inactivation has been demonstrated by site-directed mutagenesis (Abbott and Feizi 1991; Hirabayashi and Kasai 1991) and chemical modification (Oda and Kasai 1983; Whitney et al. 1986; Hirabayashi et al. 1987). However, in spite of considerable evidence showing the importance of oxidation in Gal-1 function, a clear consensus on the importance of each cysteine residue and the molecular basis of this oxidative mechanism has not been reached.

Human Gal-1 is a small lectin composed of 135 amino acids which folds into a three-dimensional structure in the form of a β-sandwich, consisting of two slightly bent sheets with variable long connecting loops. Gal-1 has been widely used as a model of ligand binding and multimerization, but it has also emerged as an interesting model to explore other molecular hallmarks of the galectin family such as the presence of a high number of...
cysteine residues in its sequence (six cysteines per monomer). This biochemical property makes this glycan-binding protein highly sensitive to oxidation leading to loss of lectin activity (Tracey et al. 1992). Interestingly, the first reported X-ray structure of human Gal-1 (Lopez-Lucendo et al. 2004) revealed not only the spatial distribution of the cysteines but also modifications such as sulfenic acid formation and mixed disulfide formation with 2-mercaptoethanol (ME). In addition, the intramolecular disulfide bonds present in oxidized Gal-1 have been characterized (Tracey et al. 1992; Inagaki et al. 2000). Whereas the reduced form of this lectin appears to be critical for its immunoregulatory and pro-apoptotic activity, oxidized Gal-1 has been postulated to function as a growth factor during axonal regeneration in peripheral nerves (Inagaki et al. 2000; Kadoya and Horie 2005). Thus, fluctuations in the redox status of Gal-1 may control the range and diversity of biological functions displayed by this lectin in physiologic and pathologic settings.

It is known that inactivation of Gal-1 by air is a very slow process that can be catalyzed by traces of heavy metals such as Cu. However, the kinetics of the oxidation process has not been characterized. This is in part because the air-exposed protein is subjected to various oxidants, which operate through still poorly understood mechanisms. In this regard, hydrogen peroxide (H$_2$O$_2$) is one of the most important reactive oxygen species (ROS) (Stone and Yang 2006), not only because of its involvement in microbial activities, aging process and oxidative stress, but also because of its critical function as a signaling molecule (Brigelius-Flohé and Flohé 2011; Veal and Day 2011). To verify whether hydrogen peroxide is a suitable candidate for the oxidation of Gal-1 in vivo, we measured the kinetics of oxidation of this lectin.

Here, using an interdisciplinary approach involving experimental and computational strategies, we investigated the structural and molecular determinants of redox-dependent Gal-1 inactivation.

Results

Selected cysteines are involved in the Gal-1 oxidation process

One of the most striking features of Gal-1 is the high proportion of cysteine residues, being cysteine one of the rarest amino acids employed for biosynthesis (Pe’er et al. 2004). In fact, there are six cysteines in each Gal-1 monomer, namely Cys2, Cys16, Cys42, Cys60, Cys88 and Cys130. Their spatial distribution in the 3D structure of the protein is shown in Figure 1. These cysteine residues exhibit a wide range of solvent accessibility; hence their reactivity toward oxidizing agents is expected to vary according to the solvent environment. To verify this hypothesis, free thiols were measured using Ellman’s reaction (DTNB assay). The experimental analysis of exposed sulfhydryl groups showed a molar ratio of 3.9 ± 0.6, suggesting the presence of about four moles of free thiol per mole of protein. In order to identify the cysteine residues that are exposed, we employed molecular dynamics (MD) simulations to compute the radial distribution functions g(r) of water molecules around the sulfur of the six cysteines and the solvent accessible surface area (SASA) per residue and sulfur atom, showing a differential profile of solvent exposure (Figure 2). The results are in accordance with previous works employing a different type of calculation (Lopez-Lucendo et al. 2004). Cys2 and Cys130 are the most highly exposed (higher SASA) and solvated cysteines in Gal-1 monomer, since both residues are located near the terminal ends of the protein. Cys16 and Cys88 show a similar profile with lower exposure than Cys2 and Cys130, while the absence of a typical profile of solvation (negligible SASA) for Cys42 and Cys60 indicates no solvent accessibility for these internal cysteines. These results suggest that reactivity toward oxidation and/or any kind of functionalization of these cysteines is markedly different, being the Cys42 and Cys60 hardly accessible by water-soluble reagents, as previously suggested (Whitney et al. 1986). Therefore, the Gal-1 redox chemistry associated with oxidation of cysteine residues is directly related with the presence of the four solvent-exposed cysteines: Cys2, Cys16, Cys88 and Cys130.

Using PROPKA and the results of the MD of wild-type Gal-1, we calculated the pK$_a$ values for the six cysteines present in the monomeric structure of the protein (and also for the C2S mutant as a control for single cysteine-to-serine mutants) (Table I). The cysteines that undergo the greatest change in pK$_a$ with respect to the free amino acid are Cys16, Cys42 and Cys60. Since Cys42 and Cys60 are buried in the core of the protein, we may expect a significant disturbance in their side-chain pK$_a$ value with respect to free cysteine in water. The positive value of this difference confirmed that these cysteines are excluded from solvent and surrounded by hydrophobic residues. On the other hand, Cys16 displayed the greatest change (with negative sign), being Cys16 more acidic than free cysteine in water. Since cysteine oxidation often involves the nucleophlic attack of the thiolate to the oxidant, the lower pK$_a$ of Cys16 suggests that among all the solvent-exposed cysteines, this residue might control the onset of the oxidation process as it is the most reactive cysteine due to increased thiolate availability at physiologic pH. However, it is well known that other factors might also affect cysteine reactivity (Ferrer-Sueta et al. 2011). In addition, despite being slightly acidic, other solvent exposed cysteines display pK$_a$ values similar to a free cysteine.

Oxidation and reduction of Gal-1 is a reversible process

Although the redox status appears to be critical for the functional activity of Gal-1 and oxidation has been proposed as a regulatory mechanism to limit the immunoregulatory activity of this lectin (Rabinovich and Ilarregui 2009), it has not been established whether this process is reversible. When we exposed a 5 μM Gal-1 solution in PBS to oxidation, either through exposure to air (5 days) or by treatment with 30 mM H$_2$O$_2$ for 30 min, similar far-UV circular dichroism (CD) spectra were obtained in both cases, which were clearly different from that generated by the reduced protein (Figure 3A). This change occurred in the intensity and position of the minimum of ellipticity, indicating a conformational change toward an alternative folded state. Spectra deconvolution using DichroWeb rendered for reduced Gal-1 a b-strand content of 43%, which diminished to 39% upon air oxidation. In addition, these changes were accompanied by a slight increment of turn components (from 11.5 to 13%). Similar results were obtained for this galectin in particular (Kadoya and Horie 2005) and for other galectins from other species (Pande et al. 2003; Shahwan et al. 2004; Ashraf et al. 2011). Interestingly, when the air-oxidized protein
was treated for 15 min with 2 mM dithiothreitol (DTT), we recovered a spectrum that was similar to that obtained with the reduced protein (Figure 3A), suggesting that oxidation was a reversible process. Furthermore, when an excess of hydrogen peroxide was added again to the samples, it was possible to recover the spectrum of the oxidized protein (data not shown). In addition, CD spectra on the near-UV showed a flattening of the positive band centered at 280 nm upon oxidation, suggesting the loss of the native packing around the aromatic residues (Figure 3B). Since these experiments required higher concentrations of the protein (70 μM), oxidation also induced substantial aggregation of Gal-1. For this reason, upon adding DTT to the oxidized sample only a partial recovery of the initial Gal-1 spectrum was achieved. Finally, when we performed a similar analysis using fluorescence spectroscopy (Figure 3C), we found identical emission spectra when the protein was reduced after being oxidized by air, even in the presence of lactose. Taken together, these results indicate that both the secondary and tertiary structures of the reduced form as well as the lactose-binding capacity were recovered after reducing the oxidized protein.

Cysteines 2, 16 and 88 play key roles in the conformational change experienced by Gal-1 during oxidation

To fully dissect the contribution of each cysteine to the oxidation process, the six single cysteine mutants CXS as well as two

<table>
<thead>
<tr>
<th>Residue</th>
<th>ΔpK_a Wild-type Gal-1</th>
<th>ΔpK_a Gal-1 C2S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys2</td>
<td>-0.34 ± 0.82</td>
<td>-0.26 ± 0.56</td>
</tr>
<tr>
<td>Cys16</td>
<td>-1.02 ± 0.79</td>
<td>-0.80 ± 0.63</td>
</tr>
<tr>
<td>Cys42</td>
<td>0.66 ± 0.43</td>
<td>0.83 ± 0.40</td>
</tr>
<tr>
<td>Cys60</td>
<td>0.84 ± 0.68</td>
<td>0.67 ± 0.49</td>
</tr>
<tr>
<td>Cys88</td>
<td>-0.26 ± 0.27</td>
<td>-0.20 ± 0.56</td>
</tr>
<tr>
<td>Cys130</td>
<td>-0.43 ± 0.48</td>
<td>-0.52 ± 0.26</td>
</tr>
</tbody>
</table>
selected double mutants were expressed and purified. These mutated proteins were exposed to the same reduction and oxidation procedures previously used for wild-type Gal-1. Conformational changes were followed by CD (Figure 4). Similarly to the wild-type protein, mutation of the internal cysteines (Cys42 and Cys60) did not prevent the protein to reach the oxidized conformation. A similar behavior was observed with the C130S mutant, suggesting that this exposed cysteine is not essential for promoting oxidation-driven conformational changes. In contrast, proteins mutated in Cys16, Cys88 and surprisingly Cys2 showed little changes on their CD spectra after the air oxidation protocol. However, C2S mutant underwent a conformational change when exposed to hydrogen peroxide. In addition, no significant changes were observed in the double mutants C2SC16S and C2SC88S, as they lack two of the critical cysteines involved in the conformational change (Figure 4).

Based on these findings it was difficult to understand why the absence of some particular cysteines prevents the conformational transition, as it has been proposed that the final state of oxidation requires the presence of six cysteine residues to form three disulfide bridges (Tracey et al. 1992; Inagaki et al. 2000). Although changes that do not directly influence the CD spectra might also occur, in our experimental conditions only Cys16 and Cys88 (and Cys2 if the oxidation is performed in air) appeared to be essential for inducing conformational changes, as their mutation prevented structural alterations evidenced by CD.

**Oxidation of Gal-1 by H₂O₂**

When reduced Gal-1 (four thiols/protein as measured by the DTNB assay) was exposed to increasing concentrations of hydrogen peroxide, biphase time courses of protein thiol oxidation were observed (Figure 5A). Bi-exponential curves were fitted to the experimental data at each hydrogen peroxide concentration, where the amplitude of each exponential was consistent with the oxidation of approximately two thiyl groups. From the slope of the plot of the observed rate constants of the first exponential vs. hydrogen peroxide concentration, the rate constant for the most reactive thiol in Gal-1 was 1.7 ± 0.2 M⁻¹ s⁻¹ at pH 7.4 and 25°C in PBS (Table II). In the presence of lactose, this value was slightly higher but was still within the error bars of the method. At pH 6.8, the rate constant was approximately 6-fold lower, as expected for a reaction where thiolate is the reactive species. Since the second exponential corresponds to a process that is not fully resolved in time, the value of the constant obtained from these experimental data has larger errors, and can only be estimated as ~10⁻³ M⁻¹ s⁻¹ at pH 7.4 and 25°C. When the three most relevant CXS mutants were subjected to similar experiments, all rate constants were lower than that obtained for Gal-1 (Table II). The value for the rate constant of the C2S mutant provided the real consumption rate for the Cys16–Cys88 pair formation, resulting in a value of 0.4 ± 0.1 M⁻¹ s⁻¹.

Regarding the nature of the first disulfide bond formed, the most reasonable possibility was the formation of a Cys16–Cys88 intramolecular disulfide bond, due to the reasons mentioned above and because any reaction of Cys2 with some cysteine residue from another Gal-1 monomer would generate dimers. Although dimers are indeed formed during the reaction of Gal-1 and hydrogen peroxide, the kinetics of dimer formation did not coincide with the first phase of thiol consumption, which revealed...
a half life of 40 s at 10 mM hydrogen peroxide. On the contrary, dimer formation was not found to be complete after 2 h (Figure 5B). Moreover, the monomer band from the samples subjected to oxidation migrated faster than the same band under reducing conditions, most likely showing a disulfide bond formation that occurs within the monomer (Figure 5B; lanes 9 and 11). This observation was confirmed by testing the mobility of C16S and C88S mutants (Supplementary data, Figure S1). Under nonreducing conditions, both mutants showed the same mobility (slightly lower than that observed for oxidized Gal-1), supporting the hypothesis that Cys16 and Cys88 are responsible for the formation of an intramolecular disulfide bond. When similar oxidation assays were performed using C2S or C130S mutants (Figure 5C), we observed formation of dimers not only in the wild-type (Figure 5B), but also in both mutants. Interestingly, dimers were reduced by ME and they were formed even when iodoacetamide (IAM) was added after hydrogen peroxide treatment (to avoid undesired thiol oxidation during sample manipulation). However, the band corresponding to the dimer was more evident in the C130S mutant, suggesting that the Cys2 is primarily responsible for the formation of these dimers and also for the formation of higher molecular weight aggregates.

To obtain an estimation of the rate of the conformational change experienced by Gal-1 during oxidation by H2O2, kinetics
experiments were performed following the changes in intrinsic fluorescence signal. Fluorescence intensity at 345 nm of a 7 µM Gal-1 solution was registered as function of time at various concentrations of H₂O₂ (Figure 6). As noted above, the fluorescence intensity of samples increased upon oxidation (Figure 3C). However, the most striking trend was the decline in final fluorescence intensity using increasing concentrations of the oxidant. Once reactive cysteines reached the intermediate sulfenic acid, the conformational change has been shown to be independent of the concentration of H₂O₂ (Ferrer-Sueta et al. 2011), so we did not expect this trend in fluorescence spectra. As hydrogen peroxide can induce not only the two-electron oxidation of thiol groups to sulfenic acids, but also the two-electron oxidation of sulfenic acids to sulfenic acids, in a process termed overoxidation (Baker and Poole 2003; Georgiou and Masip 2003; Pascual et al. 2010), we propose a kinetics model that supports an overoxidation process of cysteine, thus providing an explanation for the above-mentioned behavior and allowing the calculation of the rate constant for the conformational change. This reaction competes with the formation of disulfide bonds, thus inhibiting the associated conformational change (Supplementary data). After fitting the model, we obtained a constant for conformational change independent of H₂O₂ of 0.003 s⁻¹ and an overoxidation constant of 0.3 M⁻¹ s⁻¹, comparable with the rates obtained for the kinetics of thiol consumption. At low H₂O₂ concentration, the initial speed of conformational change was greater than that of overoxidation (in 5 mM H₂O₂, the conformational change was about twice faster). On the other hand, at high H₂O₂ concentration, the sulfenic intermediate overoxidation can compete with the reaction that drives the conformational change. Importantly, these oxidant concentrations were beyond any physiologically relevant range.

### Table II. Kinetics of thiol consumption upon oxidation of Gal-1 and selected CXS mutants by H₂O₂

<table>
<thead>
<tr>
<th>Protein</th>
<th>Condition (T = 25°C in PBS)</th>
<th>( k ) [M⁻¹ s⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal-1</td>
<td>pH 7.4</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>pH 7.4 + 100 mM lactose</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>pH 6.8</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>C2S</td>
<td>pH 7.4</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>C16S</td>
<td>pH 7.4</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>C88S</td>
<td>pH 7.4 + 100 mM lactose</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>

![Fig. 6. Kinetics analysis of conformational changes of Gal-1 upon oxidation with H₂O₂. Gal-1 (7 µM) was incubated with hydrogen peroxide at concentrations of 5 mM (squares), 10 mM (circles), 15 mM (triangles) and 20 mM (diamonds) in PBS buffer (100 mM, 0.1 mM DTPA, pH 7.4) at 25°C. The intensity of the emission spectrum at 345 nm was recorded as function of time. A kinetics model taking into account the consumption of reduced Gal-1, the formation of the different oxidized Gal-1 species and the concentration of hydrogen peroxide (Supplementary data) was fitted (line) in order to obtain the rate of conformational changes and the reactions corresponding to overoxidation of cysteines.](https://academic.oup.com/glycob/article-abstract/24/5/428/1988426)

**Oxidized Gal-1 does not bind lactose and formation of Cys16–Cys88 disulfide bond is sufficient to disrupt Gal-1-lactose binding**

We monitored the intrinsic fluorescence intensity of Gal-1 at 363 nm (a wavelength at which the difference in the fluorescence of the free and the ligand-bound protein is maximal) as a function of lactose concentration and found a binding constant \((K_b)\) of \(4.7 \times 10^3\) M⁻¹ (Figure 7A). When the same test was performed with Gal-1 previously incubated in the presence of air or 10 mM H₂O₂, the fluorescence spectra remained constant upon lactose addition (Figure 7A). Therefore, the oxidized protein did not bind lactose at any of the concentrations tested (from 0 to 1.3 mM). To clarify the molecular basis of this effect, we performed a computational thermodynamic analysis of the lactose-binding process by comparing the free energy of lactose binding of the protein with the Cys16-Cys88 disulfide bond (Gal-1 oxidized) vs. the wild-type protein (Gal-1 reduced), i.e. with all the cysteines reduced. Although during the simulation time (80 ns), the lactose molecule remained in the binding site of oxidized Gal-1, the \(\Delta G\) binding calculated with the MM/QM-COSMO method \((\Delta G_{binding} (\text{Gal-1 oxidized}) = -34.21\) kJ/mol\) increased as the simulation time increased, indicating the onset of dissociation, consistent with the fact that the binding process is unfavorable when Gal-1 bears the Cys16-Cys88 disulfide bond \((\Delta G_{binding} (\text{Gal-1 oxidized}) = -17.49\) kJ/mol\). This tendency indicates that lactose binds with higher affinity to the reduced form of Gal-1 as expected from the experimental data. The computational method used, based on a continuum model approach, affords binding \(\Delta G\) values that are useful only on a qualitative basis, since absolute values are typically overestimated (Kongsted et al. 2009; Hou et al. 2011). On the other hand, the simulation of the oxidized protein would probably require longer times to statistically converge than those accessible within the time scale of our simulations. In this context, our results suggest that the \(\Delta G\) for the oxidized protein is less negative than the results computed for the reduced protein, consistent with the experimental data. The amino acids located at the binding site that were more affected by the formation of this disulfide bond were Arg48, Glu71 and Arg73 (Figure 7B) as calculated with MM/GBSA amino acid decomposition approach. Arg48 and Arg73 (and Asn61 and Arg111 to a lesser extent) increased the electrostatic component that weakened lactose binding, while Asn46, Ala51, Lys63, Glu71 and Asp123 promoted...
of the glucose moiety to Glu71(CD) and Arg48(NH2) acids to the ligand. When Gal-1 was oxidized, the distances to
the total energy (sum of all energy inputs) indicated a positive
trend was also re
due bond), the principal amino acids that suffered from that structural
perturbation were mostly located in the ligand-binding groove in contact with
the ligand. These amino acids are: Arg48, Glu71 and Arg73 as they presented the
greatest changes in
fluorescence was observed for Gal-1 oxidized by H2O2
binding.
A
B
Fig. 7. Thermodynamic analysis of Gal-1 binding to lactose. (A) Gal-1 (8 μM)
squares) was titrated by adding aliquots of a 100 mM lactose stock solution.
The intensity of the emission spectrum at 363 nm was recorded and fitted as
function of lactose concentration. Binding constant (Kd) 25°C was calculated
by fitting a single binding site model to the fluorescence data. No significant
change of emission fluorescence was observed for Gal-1 oxidized by H2O2
circles) at any of the lactose concentrations tested. (B) Differences of ΔE_binding
(ΔΔE) between Gal-1_oxidized-lactose and Gal-1_reduced-lactose systems per
residue, using the classical mechanics MM/GBSA approach. Energetic
contributions were computed to the vdW contribution (solid line) and
electrostatic energy (ELE) (dot-dash line) as well as the total free energy
contribution computed by the generalized model (GB_total) (dashed line). Using
this method, we obtained the detailed differences in binding energy per residue
between reduced and oxidized Gal-1. Although the structural modification was
located in the F-sheet of Gal-1 folding (i.e., the formation of the Cys16-Cys88
disulfide bond), the principal amino acids that suffered from that structural
modification were mostly located in the ligand-binding groove in contact with
lactose. These amino acids are: Arg48, Glu71 and Arg73 as they presented the
greatest changes in ΔE_binding.

the opposite effect. Moreover, the His44, Ala51 and Trp68 resid-
ues increased the van der Waals (vdW) energy component by
breaking the dispersive interactions with the ligand. Finally,
Arg48 and Glu71 contributed to the binding in the oxidized
form through a reduction in vdW energy contribution, where
the total energy (sum of all energy inputs) indicated a positive
overall contribution to the binding in the oxidized protein. This
trend was also reflected at the level of distances of these amino
acids to the ligand. When Gal-1 was oxidized, the distances to
O3 of the glucose moiety to Glu71(CD) and Arg48(NH2)
increased 0.06 and 0.3 Å in 80 ns of simulation, respectively.
In addition, Arg48(NH1) approached to O3 lactosamine moiety
by ~0.10 Å, reflecting a significant displacement of the ligand
within the binding site. This approach allowed the discrimina-
tion of the contribution of each amino acid to ligand binding
of Gal-1 upon formation of the disulfide bond between Cys16
and Cys88.

Oxidized Gal-1 does not impair T-cell viability
Gal-1 has been reported to selectively alter the viability of acti-
vated T cells under reducing conditions (Perillo et al. 1995;
Toscano et al. 2007). This effect involves the binding to specific
glycosylated ligands on the surface of T cells and a predomi-
nant presentation of this lectin in a dimeric form. We studied
the impact of oxidation on the structure and function of Gal-1
using cell death assays. We exposed activated Jurkat T cells to
different Gal-1 concentrations under reducing conditions, i.e.,
adding 0.55 mM ME to the medium (Supplementary data,
Figure S2). We used an optimal concentration of wild-type
Gal-1 and mutants corresponding to 3 μM to promote T-cell
apoptosis in culture (Toscano et al. 2007). In contrast to the
effects observed under reducing conditions, we found that oxi-
dation of wild-type Gal-1 and mutants C42S, C60S and C130S
resulted in gradual loss of the pro-apoptotic activity of this
lectin (Figure 8). On the other hand, C2S, C16S, C88S single
mutants and C2SC16S and C2SC88S double mutants did not
change the viability of T cells irrespective of the prevalent
redox condition. These results confirmed the relevance of
Cys2, Cys16 and Cys88 in Gal-1 inactivation and reinforced
the hypothesis that only selected cysteine residues are essential
for regulating Gal-1 function. Interestingly, double mutants suc-
cessfully circumvented oxidative inactivation and were capable
of triggering the same degree of apoptosis as wild-type Gal-1
exposed to reducing conditions. Thus, double mutants of Gal-1
are substantially more resistant to oxidative inactivation than
single mutants of this protein.

Discussion
Although protein oxidation was originally believed to be part
of an inactivating process responsible of attenuating or elimi-
inating galectin activity, recent studies suggested that oxidized
Gal-1 may display alternative functions independently of
glycan ligand recognition, including enhancement of peripheral
nerve regeneration (Kadoya and Horie 2005). During oxidation,
it has been proposed that each subunit of Gal-1 forms three
intramolecular disulfide bridges that result in profound con-
formational changes, thereby preventing Gal-1 dimerization
and ligand recognition (Kadoya and Horie 2005; Stowell et al.
2009). Stability under non-reducing conditions is considerably
improved in cystein-to-serine mutants, while glycan-binding
specificity and affinity are barely affected (Hirabayashi and
Kasai 1991; Nishi et al. 2008). In this regard, it has been
demonstrated that ligand engagement partially protects Gal-1
from oxidation (Stowell et al. 2009). Stowell et al. found that
binding to specific ligands may control Gal-1 sensitivity to oxida-
tion by shifting the monomer-dimer equilibrium toward di-
merization, suggesting that glycan binding protects Gal-1 from
oxidative inactivation. Dimerization may therefore limit the
conformational freedom required to successfully form intramolecular disulfide bonds, thereby protecting Gal-1 from oxidation. Supporting these findings, a mutant form of Gal-1 that exhibits impaired dimerization, showed enhanced sensitivity to oxidation and failed to induce cell surface phosphatidylserine exposure, a biological activity commonly exerted by reduced Gal-1 (Stowell et al. 2009). These results suggested that binding of Gal-1 to specific glycans enhanced dimerization and reduced sensitivity to oxidative inactivation. Accordingly, mutations that impair dimerization and therefore increase monomer formation favor oxidation of the protein.

Here, using a combination of in vitro and in silico experiments, we studied the molecular mechanisms underlying Gal-1 oxidation. We established a hierarchy of reactivity and importance of each cysteine residue and characterized the kinetics of oxidation with hydrogen peroxide, yielding an atomistic vision of this process with the aim of integrating the structural information available. The first surprising result was the high degree of reversibility of the oxidation-reduction process. The secondary structure followed by CD and the intrinsic fluorescence spectra reflected the potential of Gal-1 to reversibly move between two structures, a balance dictated by the redox potential of the environment (Figure 2). Since only four of the six thiols present in Gal-1 are exposed to solvent, we postulated that the cysteine residues responsible for triggering the oxidation-driven conformational change of the protein are among these four residues. This hypothesis was verified by analyzing the CXS single mutants. We found that only Cys2, Cys16 and Cys88 are important for this process, since the C130S mutant displayed a similar behavior to that observed with the wild-type protein (Figure 4). Furthermore, their proximity and the particular acidity of one of these residues (Table 1), Cys16 and Cys88 are good candidates to form a disulfide bridge. This finding is also supported by experimental evidence (Tracey et al. 1992). In this regard, the formation of three disulfide bonds, involving the six cysteine residues of Gal-1, has been reported. However, the conformational change induced by oxidation when Cys42, Cys60 or Cys130 were mutated indicates almost no relevance of these residues in the overall oxidation process (Figure 4).

The critical role of Cys2 in Gal-1 oxidation and stability was first established by the pioneering work of Hirabayashi and Kasai (1991). The results presented here support the idea that the presence of this particular amino acid is essential for the conformational stability against oxidation in vitro. When replacing the Cys2 by serine, a disturbance in the interactions with the surrounding residues was expected. However, MD simulations and pKa calculations (Table 1) showed that this mutation did not significantly affect the structure and energetics of the reactive cysteines. Since the C2S mutant was oxidized in the presence of H2O2, we argue that the difference with air oxidation could be caused by the different concentrations of oxidants in solution. Hence, other possibilities to assign differences in reactivity between C2S and wild-type Gal-1 must be explored. Moreover, our results on the stability of Gal-1 mutants in oxidized microenvironments correlated well with their ability to promote T-cell apoptosis, thus substantiating the relevance of oxidation in the biological activity of this lectin.

Quantification of thiols consumption vs. time was used to follow the kinetics of oxidation of Gal-1 by H2O2 (Figure 5A), Gal-1 reacted with H2O2 with a biphasic kinetics and this phenomenon displayed a specific rate constant of $1.7 \pm 0.2 \text{ M}^{-1} \text{s}^{-1}$, a value very similar to the oxidation rate constant of free cysteine amino acid ($2.9 \pm 0.1 \text{ M}^{-1} \text{s}^{-1}$ at pH 7.4) (Winterbourn and Metodiewa 1999) or cysteine residues in other proteins such as human serum albumin ($2.26 \text{ M}^{-1} \text{s}^{-1}$ at pH 7.4) (Carballal et al. 2003) or thioredoxin ($1.05 \text{ M}^{-1} \text{s}^{-1}$ at pH 7.4) (Goldman et al. 1995). This value is low in comparison with the typical values recorded for thiol-containing proteins specialized on hydroperoxide reduction, i.e. thiol-dependent peroxidases, an effect which

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**Fig. 8.** Effect of Gal-1 and its CXS mutants in T-cell death. (A) Activated Jurkat T cells ($5 \times 10^5$) were incubated with or without 3 mM of Gal-1 or its mutants under different redox conditions. For reducing conditions, 0.55 mM ME (final concentration) was added to complete the medium before adding the cell suspension. For nonreducing conditions, galectins were previously treated with 10 mM H2O2 for 20 min. The oxidation reaction was stopped by using catalase (100 U mL$^{-1}$). After 14 h of exposure to Gal-1 or its variants, cells were washed with PBS. Cell death was determined by annexin V-FITC/PI in staining buffer by flow cytometry using a FACSCanto. (A) Representative data for wild-type Gal-1 presented as the peak fluorescence intensity profile (log scale) of cells stained with annexin V-FITC (control, dashed line; reduced and oxidized Gal-1, solid lines). (B) A Cell death (%) observed for each recombinant Gal-1 tested (wild-type, CXS and two double CXS mutants), reduced (black bars) or oxidized (grey bars). Notably, Cys2, Cys16 and Cys88 appear to be key cysteines that are involved in Gal-1 pro-apoptotic function under different redox conditions. The results shown are representative of three independent experiments (mean ± SD; *P < 0.05).
may be associated with the particular folding of these proteins (10^3–10^7 M^{-1} s^{-1}) (Trujillo et al. 2007; Flohé et al. 2011).

In the presence of lactose, the value of the rate constant of hydrogen peroxide-mediated Gal-1 oxidation was not significantly affected. In this regard, a previous study (Stowell et al. 2009) suggests that sensitivity to oxidation involves changes in dimerization and ligand-binding equilibria. We could not observe these differences probably because of the concentration of Gal-1 used in our experiments, which was higher than the dimerization equilibrium constant (K_d of 7 μM) (Cho and Cummings 1996). Moreover, no changes could be detected in the presence of lactose. In addition, the reversibility of the oxidation process suggests that oxidation may represent a transient factor that limits the biological activity of the protein in inflammatory microenvironments where the risk of oxidation is high but its function could be restored when oxidative stimuli are eliminated or attenuated. Hence, the biological effects observed with the oxidized form of the protein could also be due, at least in part, to a balance between the reduced and the oxidized forms of Gal-1.

The low oxidation rate constant measured with H_2O_2 suggests that Gal-1 oxidation in vivo may be associated with a specific enzymatic catalysis or strictly controlled by the cell and its surroundings. To gain biological relevance in a more complex regulatory circuit, these structural and functional changes should occur faster than those observed here using air or H_2O_2. Indeed, Gal-1 oxidation may be provoked by these or other oxidants indirectly, through thiol–disulfide exchange reactions with more reactive, peroxide-sensing proteins. Such interactions were firstly demonstrated for the yeast transcriptional factor Yap, whose response to hydrogen peroxide was mediated by glutathione peroxidase-3, and then confirmed for other factors (Delaunay et al. 2002; Flohé and Ursini 2008).

The oxidation kinetics and electrophoretic analysis (Figure 5) showed the time-dependent formation of intra- and intermolecular disulfide bridges. The lower molecular weight protein band migrated from the initial position at early stages of the kinetics (Figure 5C), indicating the formation of an intramolecular disulfide bond. Simultaneously, species displaying the molecular weight of a dimer were also apparent but were almost absent under reducing conditions; these species correspond to an intermolecular disulfide bridge between Gal-1 monomers. Formation of this dimer was slower than the first phase of oxidation, which according with the rate constant reported here and a H_2O_2 concentration of 10 mM, must be complete in <10 min (half life of 40 s).

The kinetics of the conformational change observed upon Gal-1 oxidation with H_2O_2 followed by intrinsic fluorescence revealed that the final signal intensity decreased as the concentration of oxidant increased (Figure 6). A plausible model to explain this phenomenon (Supplementary data) required the consideration of a peroxide concentration-dependent reaction which competed with the conformational change: overoxidation of cysteine. Using this model, we were able to obtain a rate constant for the conformational change of 0.003 s^{-1}, which at high H_2O_2 concentration competes with the overoxidation (formation of sulfinic and sulfonic acids) of the particular cysteines that trigger the conformational change. These data are relevant when considering a strict regulation of disulfide bonds formation in Gal-1 in order to generate timely structural modifications according to the requirements of the tissue microenvironment.

Finally, to gain a more integrated picture, we evaluated the relevance of cysteine residues present in other members of the human galectin family. We hypothesized that galectins displaying the key cysteines in the proper environment might exhibit the same profile of oxidation demonstrated for Gal-1 because of the high conserved folding (Guardia et al. 2011). To address this question, we conducted a multiple sequence alignment using Clustal-X program, and incorporated structural information for the gaps in the alignment (Figure 9). The alignment revealed that: (1) the most conserved cysteine is Cys60; (2) the most commonly substituted cysteine is Cys42 (mostly by alanine); (3) the only family members that have the pair of relevant cysteines Cys16 and Cys88 (contained in the Gal-1 sequence) are PP13 and PPL13. Those with Cys88 but without Cys16 are Gal-3 and the N-terminus of Gal-9 (Gal-9N). Thus, the occurrence of Cys60 in this lectin family could play a role in their structural/folding stability, as it is present and conserved in most members of the family. Furthermore, our results suggest that this effect could not be related to a redox function. The same could be concluded for Cys42 but in the opposite way: its role in the oxidation of the protein may not be crucial as it is the most commonly replaced cysteine within the galectin family. It is also possible that both PP13 and PPL13 could exhibit a similar redox behavior to that observed for Gal-1, as they have the key cysteine residues in the same topological location. Interestingly, Gal-1, as well as PP13 and PPL13, has been proposed to play roles in pregnancy maintenance, suggesting a cooperative or redundant role for these lectins in mammalian placentation (Than et al. 2008; Blidner and Rabinovich 2013). On the other hand, Gal-3 and Gal-9N might reflect the opposite situation: these galectins could have become insensitive to oxidation, probably as a consequence of an evolutionary pressure to eliminate Cys16. Alternatively, redox regulation governed by the Cys16–Cys88 disulfide bond would not be required for these particular galectins.

In conclusion, our data provide new insights into the understanding of redox regulation in the structure–function relationship of Gal-1, a key regulatory lectin that plays central roles in the control of immune, neural and vascular signaling circuits.

Materials and methods

All experiments were performed at 25°C in 100 mM phosphate-buffered saline (PBS) containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA), pH 7.4, unless otherwise stated.

Expression and purification of recombinant Gal-1 and CXS mutants

Recombinant human Gal-1 was produced as outlined previously (Pace et al. 2003). A similar protocol was adopted for the production of the six single cysteine mutants. Briefly, Escherichia coli BL21 (DE3) cells were transformed with each plasmid containing different genes inserted into the expression vector pET22b (Novagen), and production of the recombinant galectin was induced at the log phase by addition of 1 mM isopropyl β-D-thiogalactoside. Cells were separated by centrifugation, washed and disrupted by sonication. Debris was eliminated after centrifugation at 15,000 × g, and soluble fractions were obtained.
for subsequent purification by affinity chromatography on a lactosyl-Sepharose column (Sigma-Aldrich), using 0.1 M lactose in PBS supplemented with 4 mM ME. Eluted Gal-1 was further purified using a HiPrep Sephacryl S-100 HR gel filtration column (GE Healthcare). After gel filtration, lectin-containing fractions were subjected to extensive dialysis against PBS containing 4 mM ME at 4°C to remove lactose bound to the protein. To prevent mixed disulfide bridge formation between cysteine residues and ME, prior to any analysis, ME was removed from protein structure by incubating the lyophilized sample in PBS with 10 mM DTT on ice during 30 min and desalted with an NAP-5 column (GE Healthcare). This procedure removes the excess of DDT and ME. The reduced protein samples were immediately purged with argon in a closed vessel and the solution was kept on ice until use.

**Generation of mutants**

Cysteine residues on Gal-1 were mutated to Ser (CXS) using the inverse polymerase chain reaction method (Clackson et al. 1991). The forward sense primer contained a mismatch that changes the appropriate Cys to Ser. These primers were used in combination with antisense primers that start at the beginning of the sense primers. The insert and the vector were amplified on the same step with KOD Hot Start polymerase (Novagen) and the resulting product was ligated with T4 DNA Ligase (Promega). Double cysteine mutants C2SC16S and C2SC88S were generated using the single mutant C2S as starting material and the mutations were introduced using the primers previously employed to generate the single mutants C16S and C88S. Mutations were checked by DNA sequencing of the entire insert. Primers used are listed on Table III.

**Oxidants, protein and thiol quantification**

The concentration of H$_2$O$_2$ (Mallinckrodt Chemicals) stock solutions was measured at 240 nm ($\varepsilon_{240}=43.6$ M$^{-1}$ cm$^{-1}$). Protein concentration after reduction treatment was measured spectrophotometrically using an absorption coefficient at

![Table III. Primers used to generate the Cys to Ser (CXS) mutants. Mutated codons are in bold](https://academic.oup.com/glycob/article-abstract/24/5/428/1988426/1988426)
280 nm of 8480 M\(^{-1}\) cm\(^{-1}\) for Gal-1 and the single cysteine mutants, as assessed from their primary sequences (http://www.expasy.ch/tools/protparam.html). Thiols were determined with 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) after incubating Gal-1 samples with an excess of DTNB in PBS for 30 min in the dark at room temperature. An absorption coefficient at 412 nm of 14,150 M\(^{-1}\) cm\(^{-1}\) (Riddles et al. 1979) was used to quantify the 5-thio-3-nitrobenzoate anion with the absorbance of the DTNB solution and the intrinsic low absorbance of Gal-1 at this wavelength accounted for.

**Spectroscopic measurements**

Far- and near-UV CD spectra were recorded using a Jasco J-815 spectropolarimeter equipped with a Peltier temperature control. Spectra shown are averages of at least eight scans, with background corrected by the subtraction of respective buffer blanks. They were acquired over the wavelength range of 190–360 nm, using a 1 nm path length polarimetrically certified cell (Hellma). Spectra deconvolution was performed using DichroWeb (http://dichroweb.cryst.bbk.ac.uk) with the CONTIN analysis program and the reference set SP175. Intrinsic fluorescence emission spectra were measured at 25°C in a Jasco FP-6500 spectrofluorometer. Excitation wavelength was set to 280 nm (295 nm for the experiments related to the kinetics of conformational change), and spectra were recorded between 300 and 420 nm. Excitation and emission bandpasses were set to 1 and 3 nm, respectively. An average of at least six scans was used for final calculations. Spectra were corrected for dilution effects, and the final dilution of the sample was always <10%.

**Kinetics of the reaction between hydrogen peroxide and Gal-1**

The rate constants of oxidation of Gal-1 or CXS mutants by hydrogen peroxide were determined using pseudo-first-order conditions as described (Carballal et al. 2003). Reactions were conducted at increasing concentrations of H\(_2\)O\(_2\) and the same protein concentration (67 μM) at different time intervals, aliquots of the reactions were mixed with catalase (100 U mL\(^{-1}\)) and the samples thus obtained were measured for the thiol content. Observed rate constants of thiol consumption (\(k^o\)) were determined by fitting time courses of thiol oxidation data at each oxidant concentration to a double exponential function.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 15:1 polyacrylamide gels containing SDS further stained with silver or Coomassie blue.

**Binding of Gal-1 to lactose**

The Gal-1:lactose binding constant under reducing or oxidizing conditions was determined by fitting the fluorescence emission spectrum change in the presence of DTT or H\(_2\)O\(_2\), respectively. Gal-1 (8 μM) was titrated by adding aliquots of a 100 mM lactose stock solution. The intensity of the emission spectrum at 363 nm was recorded and fitted as function of lactose concentration. Binding constant (\(K_b\)) at 25°C was calculated by fitting a single binding site model to the fluorescence data.

**Computational experiments**

Initial structures of Gal-1 CRDs were retrieved from the Protein Data Bank when available: 1GZW for the wild-type protein (X-ray, 1.65 Å resolution) and 1W6N for the C2S Gal-1 mutant. For those CRDs whose structures were not available or required editing of the structure (oxidized form of Gal-1-Cys16-Cys88 disulfide bridged, bound or unbound to lactose) (all initial structures available in Supplementary data), we generated them using Amber9 package of computational simulation programs (Case et al. 2006). In all cases, all crystallographic water molecules were deleted, and a single subunit was then solvated with explicit three-site point charge modeled (TIP3P) water molecules in an octahedral box, localizing the box limits as far as 10 Å from the protein surface. MD simulations were performed at 1 atm and 300 K, maintained with the Berendsen barostat and thermostat (Berendsen et al. 1984; van Gunsteren and Berendsen 1990), using periodic boundary conditions and Ewald sums (grid spacing of 1 Å) for treating long-range electrostatic interactions with a 10 Å cut-off for computing direct interactions. The SHAKE algorithm was applied to all hydrogen-containing bonds, allowing employment of a 2 fs time step for the integration of Newton’s equations. The Amber ff9SB force field parameters (Hornak et al. 2006) were used for all residues. GLYCAM parameters (Kirschner et al. 2008) were employed for lactose. The equilibration protocol involved an optimization of the initial structure, followed by 500 ps constant volume MD run heating the system slowly to 300 K. Finally, 1 ns MD run at constant pressure was performed to achieve proper density. Different production MD runs (60 ns for the structures experimentally determined and 80 ns for the models) were performed. Frames were collected at 1-ps intervals for the last 20 ns of each simulation, and were subsequently saved on disk for further analyses.

Using the MD simulation of the wild-type Gal-1 (one monomer on its reduced form) and C2S Gal-1 mutant, 50 frames were taken at random and for each one, we calculated the value of all cysteines lateral chain pK\(_a\) using PROPKA (Bas et al. 2008), an empirical method for structure-based protein pK\(_a\) prediction which takes into account desolvation effects and intra-protein interactions. The results were expressed as \(\Delta pK_a\), the difference between the average value obtained from all frames and the reference value used by the program (9.00 for cysteine). Data are expressed with the standard deviation. For the same frames, a SASA calculation of the lateral chain and the sulfur SG atom of each cysteine was performed using the vmdICE plug-in implemented in visual molecular dynamics (VMD) (Humphrey et al. 1996; Knapp et al. 2010).

Thermodynamic parameters for MD simulations of CRD: lactose complexes were calculated using two different strategies: The single-trajectory molecular mechanics/generalized Born surface area (MM/GBSA) approach (Jorgensen et al. 1983; Zou et al. 1999; Bashford and Case 2000) implemented in the Amber 9 package (Case et al. 2006) and the linear-scaling quantum mechanical-based end-point method developed by Anisimov and Cavasotto and termed MM/QM-COSMO (Anisimov and Cavasotto 2011). The former method combines molecular mechanical energies, continuum solvent approaches and solvent accessibility in order to elicit free energies from structural information avoiding the
computed molecular intrinsic of free energy simulations. The molecular mechanical energies were determined with the sander program from Amber and represented the internal energy (bond, angle and dihedral) and vdWs and electrostatic interactions. An infinite cut-off for all the interactions was used. The electrostatic contribution to the solvation free energy was calculated with a numerical solver for the generalized Born method (Still et al. 1990). Energetic contributions were computed corresponding to the electrostatic energy (ELE) and vdW contribution. Solvation-free energy was estimated using the generalized Born approximation (GBSolv), which is based on the use of a cavity and electrostatic energy components. The total free energy contribution computed by the generalized model is also presented (GBTot). With this method we obtained the binding energy characterization per residue. In the MM/QM-COSMO approach, MD trajectories are re-evaluated using a semi-empirical Hamiltonian and a continuum solvent model to calculate an enhanced binding-free energy description where translational and rotational entropies are calculated using configurational integrals, and internal entropy is calculated using the harmonic oscillator approximation.

Multiple sequence alignment of 15 publicly available human sequences was performed, using the CLUSTAL-W multiple alignment method and software (Thompson et al. 1994): LGALS-1 to -12, LGALS-13 (or PP13 for placental protein 13), LGALS-14 (or PPL13 for placental protein 13-like) and HSPC159 (hGRPC), all the three last genes correspond to galectin-related proteins. Structural information from the Gal-1 CRD structure deposited in the 1GZW PDB entry was used to set local gap penalties.

**T-cell death assay**

Jurkat T cells ($5 \times 10^5$) were cultured and activated as described (Toscano et al. 2007; Lange et al. 2009) and incubated with or without 3 μM Gal-1 or its variants in Roswell Park Memorial Institute (RPMI) medium supplemented with 5% fetal bovine serum (FBS), penicillin (100 μU/mL) and streptomycin (50 μg/mL) in 24-well culture plates at 37°C in 5% CO2. To generate reducing conditions, 0.55 mM ME (final concentration) was added to complete the medium before adding the cell suspension. To test the functional activity of oxidized galectins, galectins were cultured in RPMI and treated with 10 mM H2O2 for 20 min before assays. The excess of ROS was quenched by using catalase (100 U/mL) and the oxidation reaction was stopped. Then, medium was completed with FBS and antibiotics and cells were added to each well. After 14 h of exposure to Gal-1 or its variants, cells were washed with PBS. Cell death was determined by annexin V-FITC/propidium iodide (PI) staining buffer (100 mM HEPES, 1.4 M NaCl, 25 mM CaCl2) as previously described (Toscano et al. 2007). Fluorescence (FITC and PI) were analyzed on a FACSCanto (BD Biosciences). Cell death was calculated as the percent of annexin V-positive cells in galectin-treated cells minus the percent of annexin V-positive control-treated cells.

**Statistical analysis**

Data are expressed as mean ± SD. Prism software (GraphPad Software) was used for statistical analysis. Two groups were compared with Student’s t-test for unpaired data. P-values of 0.05 or less were considered significant.

**Supplementary data**

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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**Abbreviations**

CD, circular dichroism; CRD, carbohydrate recognition domain; CXS, serine-to-cysteine galectin-1 mutants; DTNB, 5,5’-dithiobis-(2-nitrobenzoic) acid; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; ELE, electrostatic energy; FBS, fetal bovine serum; Gal-1, galectin-1; GBSolv, generalized Born approximation; hGRPC, C-terminal of human galectin-related protein ; IAM, iodoacetamide; MD, molecular dynamics; ME, 2-mercaptopethanol; MM/GBSA, molecular mechanics/generalized Born surface area; PBS, phosphate-buffered saline; PI, propidium iodide; PP13, placental protein 13; PPL13, placental protein 13-like; SASA, solvent accessible surface area; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; vdw, van der Waals; VMD, visual molecular dynamics.

**References**


