Different Architecture of the Combining Site of the Two Chicken Galectins Revealed by Chemical Mapping Studies with Synthetic Ligand Derivatives*

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The detailed comparison of the carbohydrate-binding properties of related galectins from one organism can be facilitated by the application of an array of deliberately tailored methyl β-lactoside derivatives. Focusing on chicken due to its expression of two galectins as a model for this approach, the combining-site architecture of the lectin from adult liver (CL-16) is apparently homologous to that previously observed for bovine galectin-1 (Solís, D., J. Jiménez-Barbero, J., Martín-Lomas, M., and Díaz-Maurinó, T. (1994) Eur. J. Biochem. 223, 107-114). Besides preservation of the key interactions and minor differences, the lectin from adult intestine (CL-14) is able to accommodate an axial HO-3 at the glucose moiety. Homology-based modeling enabled us to tentatively attribute the observed differences to a slightly different orientation of pivotal side chains in the binding pocket due to distinct substitutions of amino acid residues in the variable region within the carbohydrate-recognition domain. Thus, the results suggest overlapping but distinct ranges of potential ligands for the two chicken lectins and provide new information on their relationship to mammalian galectins. The described approach is suggested to be of relevance to design pharmaceuticals with enhanced selectivity to a certain member within a family of related lectins.

Galectins are a growing family of metal ion-independent β-galactoside-binding lectins that are widely distributed in animal tissues, their divergence extending even in one organism to expression of several members of this family (1, 2). Their presumed involvement in cell-cell and cell-matrix interactions has already led to initial attempts to design clinically effective glycoinhibitors for galectins (3, 4). However, their application can be hampered by the fact that the target selectivity is not high enough to reduce undesired cross-reactivity to related agglutinins of this family. Since the molecular features of protein-carbohydrate interaction for the individual lectins of this family are presently not precisely defined, we herein exemplify a systematic approach to address this issue, applicable also to other groups of pharmaceutically interesting lectins such as selectins.

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In the chicken only two different galectins have been identified (5). Although they have been called CL-14 and CL-16 on the basis of their electrophoretic mobilities under denaturing conditions, both have subunits of about 15 kDa, as calculated from their amino acid sequences (6, 7) and confirmed by electrospray mass spectrometry (8). The two lectins are 48% identical in sequence, which is a similar degree of identity to what each lectin shows with the well characterized mammalian galectin-1 and -2 (about 50 and 38%, respectively). Interestingly, the quaternary structure of the two lectins is different since at physiological ionic strength CL-14 is a monomer whereas CL-16 is mostly present as a homodimer (5, 9). Thus, in spite of being isolecins supposedly derived from gene duplication at the time of divergence of birds and mammals (7), CL-14 and CL-16 are not so similar as emphasized, for example, by differential binding to homologous lymphocyte populations and independent tissue-specific regulation throughout development (8, 10). In adult chicken, CL-14 is abundantly present in intestine, whereas CL-16 is found in liver and retina nearly exclusively (5, 9, 10). As similarly acknowledged in the case of the several human galectins, for example, the precise function(s) of this regulation remains to be elucidated. Obviously, histochemically monitoring ligand expression, as initiated recently (11), can provide potentially valuable information. At any rate, the restriction of expression to two galectins in chicken presents a useful model to illustrate the potency of our approach.

There are notable differences in the oligosaccharide-binding specificity of different galectins despite a common recognition of the Galβ(1,4)GlcNAc and Galβ(1,3)GlcNAc backbone structures (12-15). They must be the consequence of a different architecture of the combining sites and/or different atomic features of the recognition process. X-ray crystallography has revealed a similar but not identical carbohydrate-binding geometry for galectin-1 (16) and galectin-2 (17) that involves key interactions of the hydroxyl groups at positions 4 and 6 of galactose and at position 3 of glucose in N-acetyllactosamine. It is essential to emphasize that a similar mode of binding has been observed in solution by chemical mapping studies in which the interaction of different monosaccharide, O-methyl and peracetyldex derivatives of methyl β-lactoside to galectin-1 was investigated (18). Therefore, it is reasonable to suggest that such a deliberate chemical mapping study will prove very informative for the analysis of protein-carbohydrate interactions in solution (19, 20). In the absence of x-ray structures they are a valuable source of information to identify the key interactions stabilizing the complexes and to infer the ligand orientation with respect to donors/acceptors of hydrogen bonding of amino acid side chains in the combining site. In the present work, the molecular recognition of the synthetic methyl β-lactoside de-
Different Modes of Ligand Recognition in Chicken Galectins

EXPERIMENTAL PROCEDURES

Materials—Sources for saccharides were as indicated in detail previously (18). The chicken β-galactoside-specific lectins, CL-14 from adult intestine and CL-16 from adult liver, were isolated by successive steps of affinity and ion exchange chromatography (8). Radiiodination of the lectins using IODO-GEN (Pierce Eurochemie) was carried out under activity-preserving conditions in the presence of 0.1 M lactose as described for bovine galectin-1 (18). CL-14 was also radioiodinated in the presence of lactose using Bolton-Hunter reagent (Amersham Int.) according to the manufacturer’s recommendations. Biotinylation of the lectins was carried out using an N-hydroxysuccinimide-biotin ester (Amersham Int.) also as instructed by the manufacturer. Streptavidin (Sigma) was iodinated using IODO-GEN.

Quantitative Binding Studies—Binding of the 125I-labeled chicken lectins was assayed using asialofibrin films on the surface of plastic microwells essentially as described (18) except that the buffer used was 5 mM sodium phosphate buffer, pH 7.2, containing 0.2 M NaCl, 2 mM 2-mercaptoethanol, and 1% bovine serum albumin. For binding assays with biotinylated lectins, precoated wells were incubated with 50 μl of 3 μg of lectin/ml solution for 2 h at 25 °C, and the extent of bound lectin was monitored by measuring the amount of streptavidin associated to the wells after incubation for 1 h at 25 °C with 50 μl (15000 cpm) of 125I-streptavidin solution in the same buffer and thorough washing steps to remove unbound radioactive material.

The affinity of the lectins for the different methyl β-lactoside analogues was estimated by determining the amount of 125I-lectin bound to 100 μg of asialofibrin films after incubation with 50 μl of the 125I-lectin solution (15000 cpm, approximately 50 nm) containing different concentrations of the sugar (from 0.1 to 8 mM; up to 80 mM for galactose) (18).

RESULTS

Labeling and Storage of the Lectins—The conditions required for the labeling and storage of the lectins were first investigated by testing the binding of the labeled lectins to asialofibrin films on the surface of plastic microwells (18). Tyrosine iodination of CL-14 even in the presence of lactose resulted in an inactive preparation showing negligible binding to asialofibrin. Therefore, a different labeling procedure, targeted to primary amino groups, was assayed. Radioiodination using Bolton-Hunter reagent yielded an active product. It bound to asialofibrin in a concentration-dependent manner, and a similar behavior was observed also after biotinylation of amino groups using N-hydroxysuccinimide-biotin ester (Fig. 1). About 8–10% of the radioiodinated CL-14 preparation bound to 100 μg asialofibrin films, and 80% of the binding was inhibited by 0.1 M lactose. Because the percentage of specifically bound radioactivity was rather low, the possibility that the observed binding could be due to a contaminant, which is not or only weakly reactive in silver staining during the quality control after purification, was ruled out by elution of the radioactivity bound to the films with SDS-polyacrylamide gel electrophoresis sample buffer and subsequent analysis by electrophoresis and autoradiography. The results unequivocally demonstrated that the radioactive material bound to asialofibrin was almost exclusively intact 125I-CL-14.

On the other hand, tyrosine-iodinated CL-16 bound rather readily to the films in proportion to the amount of asialofibrin in the well, and a similar behavior was observed for the biotinylated lectin (Fig. 1). About 20% of 125I-CL-16 bound at 100 μg of asialofibrin/well and, nonspecific binding in the presence of 0.1 M lactose was less than 2%.

Labeled CL-16 could be stored at −20 °C in aliquots for months without significant loss of carbohydrate-binding activity. However, freezing of the NH2-labeled CL-14 resulted in a dramatic decrease of activity, the specific binding to 100 μg of asialofibrin films then being only 1%. Thus, this lectin was pooled after labeling (50 μg of lectin/ml of solution in 5 mM sodium phosphate buffer, pH 7.2, containing 0.2 M NaCl and 2 mM 2-mercaptoethanol) and stored at 4 °C, where it remained stable for at least 2 weeks.

Binding of Methyl β-Lactoside Analogues to the Lectins—Methyl β-lactoside analogues were tested as inhibitors of the binding of the 125I-lectins to 100 μg asialofibrin films. For all the inhibitory glycoligands, the plot of the reciprocal of the CL-14 fraction bound to the film versus the sugar concentration gave a straight line with a mean correlation coefficient of 0.988 ± 0.01, except for the 4-deoxy and 6-fluorodeoxy derivatives which yielded correlation coefficients of 0.9. Concerning CL-16, the plots also gave a straight line with a mean correlation coefficient of 0.986 ± 0.01. A representative plot of the data obtained with methyl β-lactoside is shown in Fig. 2. The apparent dissociation constants were calculated from the inter-
Different Modes of Ligand Recognition in Chicken Galectins

Table I

| Unit derivatized | Compound | \(K_d\) (mM) |
|------------------|----------|-------------|
|                  |          | CL-14       | CL-16       | Galectin-1 |
|                  |          |             |             |
| \(\beta\)-Galactopyranose |            |             |             |
| C-2'             | 2'-Deoxy | 0.92 ± 0.02 | 1.20 ± 0.02 | 0.65 ± 0.005 |
|                  | 2'-O-Methyl | 0.72 ± 0.02 | 0.88 ± 0.01 | 0.22 ± 0.005 |
|                  | 3'-Deoxy | 1.82 ± 0.12 | 3.88 ± 0.21 | 2.42 ± 0.18 |
|                  | 3'-Deoxy-3'-fluoro | 1.31 ± 0.04 | 3.67 ± 0.01 | 1.91 ± 0.10 |
|                  | 3'-O-Methyl | 0.52 ± 0.01 | 0.69 ± 0.01 | 0.86 ± 0.02 |
| C-4'             | 4'-Deoxy | 28.2 ± 4.3 | NM | NM |
|                  | 4'-Deoxy-4'-fluoro | NM | NM | NM |
|                  | 4'-O-Methyl | NM | NM | NM |
| C-6'             | 6'-Deoxy | NM | NM | NM |
|                  | 6'-Deoxy-6'-fluoro | 24.5 ± 3.6 | NM | NM |
|                  | 6'-O-Methyl | NM | NM | NM |
| \(\beta\)-Glucopyranose |            |             |             |
| C-1              | 1-Deoxy | 0.81 ± 0.05 | 1.03 ± 0.01 | 1.43 ± 0.04 |
|                  | Lactose | 0.62 ± 0.02 | 0.69 ± 0.05 | 0.66 ± 0.01 |
|                  | 2-Deoxy | 0.58 ± 0.01 | 1.91 ± 0.04 | 1.72 ± 0.04 |
|                  | Lactal | 0.72 ± 0.02 | 3.59 ± 0.23 | 1.68 ± 0.02 |
|                  | 2-O-Methyl | 0.63 ± 0.02 | 0.70 ± 0.02 | 0.77 ± 0.01 |
|                  | Ladulose | 0.44 ± 0.03 | 0.60 ± 0.02 | 0.37 ± 0.01 |
|                  | N-Acetyllactosamine | 0.27 ± 0.01 | 0.21 ± 0.01 | 0.20 ± 0.005 |
| C-3              | 3-Deoxy | 7.71 ± 0.20 | 15.60 ± 0.67 | 9.75 ± 0.19 |
|                  | 3-O-Methyl | 7.06 ± 0.19 | 16.80 ± 1.49 | 18.15 ± 1.18 |
|                  | 3-Deoxy-3-methyl | NM | NM | NM |
|                  | 3-Deoxy-6-methyl | NM | NM | NM |
|                  | 3-Deoxy | 11.5 ± 0.60 | NM | NM |
| C-6              | 6-Deoxy | 0.61 ± 0.01 | 1.24 ± 0.03 | 0.76 ± 0.01 |
|                  | 6-Nor | 0.75 ± 0.02 | 0.88 ± 0.02 | 0.85 ± 0.17 |
|                  | 6-O-Methyl | 0.50 ± 0.01 | 0.53 ± 0.02 | 0.49 ± 0.01 |

Estimated free energy contributions of the hydroxyl groups of methyl \(\beta\)-lactoside binding to CL-14 and CL-16

| Unit | Hydroxyl group | \(\Delta\Delta G^\circ\) (kJ/mol) |
|------|----------------|-------------------------------|
|      |                | CL-14 | CL-16 |
| \(\beta\)-Galactopyranose | HO-3' | 2.3 ± 0.2 | 3.7 ± 0.4 |
|                  | HO-4' | 9.0 ± 1.5 | >9 |
|                  | HO-6' | >9 | >9 |
| \(\beta\)-Glucopyranose | HO-2 | 5.8 ± 0.4 | 7.1 ± 1.5 |
|                  | HO-3 | 0.5 ± 0.05 | 2.0 ± 0.1 |

In general, the binding of methyl \(\beta\)-lactoside analogues to the lectins resembled that observed for bovine galectin-1 (18), as could be expected due to the remarkable sequence homology. The binding affinities observed for the different derivatives at the \(\beta\)-d-galactopyranose unit indicated that the hydroxyl groups at positions C-4' and C-6' are key groups in the interaction with the chicken lectins; the hydroxyl group at C-3' participates in a much smaller but still noticeable polar interaction whereas the hydroxyl group at C-2' is apparently not involved in the recognition. The key HO-4' and HO-6' act as donors of strong hydrogen bonds to the protein, since there is a large loss in affinity upon deoxygenation at these positions which is not restored by fluorination or O-methylation. The slightly higher affinity observed for the binding of the 6'-deoxygen-6'-fluoro derivative to CL-14, as compared with the deoxy compound, is in principle in accordance with an additional involvement of the hydroxyl group at this position as an acceptor of a hydrogen bond from the protein. However, the affinity of the lectins for the derivatives at these two positions was too low to assess definite differences in affinity between them and therefore to comparatively evaluate quantitative aspects of the role of the hydroxyl groups as hydrogen-bond acceptors.

The \(\alpha\)-glucopyranose moiety of methyl \(\beta\)-lactoside is also involved in the recognition. Notably, the contribution to the binding seems to be different for the two lectins. The key polar interaction involves the hydroxy group at position 3. However, the loss in affinity upon deoxygenation or O-methylation at this position is significantly smaller for CL-14 than for CL-16, suggesting a stronger interaction of HO-3 with the former lectin. In addition, CL-14, but not CL-16, is able to accommodate an axial hydroxyl group at C-3 without a significant reduction in affinity as compared with the deoxy derivative. Furthermore, the hydroxyl group at C-2 is involved in a minor polar interaction with CL-16. This group does not contribute to the binding to CL-14 since the affinity is not affected by deoxygenation at C-2, as in methyl 2-deoxy-\(\beta\)-lactoside and lactal which lacks the hydroxyl groups at C-1 and C-2. Therefore, the N-acetyl—NH— group in N-acetyllactosamine may be involved in hydrogen bonding to CL-16 but not to CL-14. The higher affinity of both lectins for N-acetyllactosamine than for lactose could be the result of other interactions, possibly van der Waals interactions, between the N-acetyl group and protein residues of the combining site, as it has been proposed for bovine galectin-1.
(18). Overall, the glucose moiety of methyl $\beta$-lactoside apparently interacts more strongly with CL-16 than with CL-14. Thus, when the $K_d$ values of galactose and lactose are compared, the increase in affinity should be larger for CL-16 than for CL-14. Indeed, this predicted difference is experimentally detected (Table I).

Energetic Contribution of the Hydroxyl Groups to the Binding—The free energy contributions of individual hydroxyl groups (Table II) were estimated from the loss in affinity for the deoxy derivatives compared with methyl $\beta$-lactoside using Equation 1.

$$
\Delta \Delta G^0 = RT \ln \frac{K_{d, \text{deoxy derivative}}}{K_{d, \text{methyl } \beta\text{-lactoside}}}. \quad (\text{Eq. 1})
$$

When the affinity of the deoxy derivative was too low to assess the $K_d$ value, it was considered that the contribution of the corresponding hydroxyl group should be higher than the $\Delta \Delta G^0$ value calculated on the basis of the highest measurable $K_d$, i.e. higher than 9 kJ/mol. Since it has been reported that deletion of hydrogen bonds involving uncharged donor-acceptor pairs weakens binding by 2–6 kJ mol$^{-1}$ and that hydrogen bonds involving neutral-charged pairs can be responsible for greater $\Delta G^0$ changes (22, 23), on the basis of the calculated $\Delta \Delta G^0$ values it is possible to make assumptions about the nature of the groups of a lectin involved in hydrogen bonding. Although the $\Delta \Delta G^0$ values in Table II reflect the overall strength of hydrogen bonds donated and accepted by a given hydroxyl, the role of the key HO-4' and HO-6' groups as hydrogen bond donors to charged groups of the lectins can be stressed on the basis of the binding data obtained for the fluorodeoxy analogues at these positions, as it has been previously discussed in detail for bovine galectin-1 (18). On the other hand, the $\Delta \Delta G^0$ values associated with the removal of the other hydroxyl groups involved in the binding indicate the participation of neutral groups of the protein with the possible exception of the CL-16 partner for HO-3. In this case, the loss in affinity can well be explained by the deletion of one strong hydrogen bond with a charged group of the lectin or by the deletion of at least two weaker hydrogen bonds involving neutral pairs.

**DISCUSSION**

The salient conclusion derived from this study is that the carbohydrate-binding properties of the two chicken lectins show a number of so far unknown significant differences of possible biological relevance besides substantial similarities. First, the binding capability of the lectins is affected in a very different degree by the protein labeling and storage conditions, CL-14 appearing much more sensitive to potentially damaging conditions such as tyrosine iodination under an oxidizing environment or freezing. Second, although the main atomic features in the recognition of methyl $\beta$-lactoside are preserved, involving positions 4 and 6 of galactose and position 3 of glucose, there are notable differences in the mode of binding that suggest a different architecture of the combining site. The mode of binding of methyl $\beta$-lactoside to CL-16 is very similar to that previously observed for bovine galectin-1 (18). The only significant difference relates to the effect of the introduction of a methyl group at position 2' of the galactose unit. For bovine galectin-1 this results in an enhancement of the binding, suggesting new favorable interactions generated by the methyl group that may come into contact with hydrophobic residues at the periphery of the combining site. However, this enhancement is not observed for the chicken lectin. On the other hand, the contribution of the hydroxyl group at position 3' of galactose seems to be slightly stronger for the chicken lectin than for the bovine agglutinin. Both results suggest a somehow different orientation of the bound sugar within the combining site, but in general the hydrogen bonding and steric requirements for recognition by CL-16 appear highly homologous to those exhibited by the mammalian galectin-1.

The sequence homology between CL-16 and CL-14 does not translate into identical features of ligand recognition. Explicitly, the mode of binding observed for CL-14 shows a number of significant differences. The first one is a clearly higher affinity of this lectin for the binding of galactose as compared with CL-16 and the bovine lectin. Surprisingly, polar interactions at the galactose unit in methyl $\beta$-lactoside are apparently similar or even weaker and not sufficient to account for this increased affinity. A possible explanation could be a stronger interaction of nonpolar residues of the combining site of CL-14.

**FIG. 3.** Alignment of amino acid sequences of bovine galectin-1, CL-14, and CL-16 between residues 44 and 73. The residues of CL-14 and CL-16 that are identical to those of bovine galectin-1 are omitted. Asterisks denote residues that are directly in contact with the sugar in the crystal structure of the galectin-1N-acetylactosamine complex (16). The bars indicate residues in $\beta$-strands. The sequences are compiled on the basis of published information (6, 7, 25) for bovine galectin-1, CL-14, and CL-16.

**FIG. 4.** Stereo view of the carbohydrate-binding site of bovine galectin-1 upon substitution of Thr-57 by Leu. Coordinates for the structure of bovine galectin-1 in complex with N-acetylactosamine were taken from the Brookhaven Protein Data Bank (Reference 1SLT). In subunit A, Thr-57 was replaced by Leu using the TURBO-FRODO package, and a PDB file with these new coordinates was used for molecular dynamics simulation using the X-PLOR program (26). The resulting structure (bold lines) is shown superimposed on the x-ray crystal structure (thin lines). The arrow points out the displacement of the lateral chain of Arg-73.
with the hydrophobic surface patches of this sugar residue. The second difference is that the contribution to the binding by the glucose unit, although still important, is significantly decreased. Its hydroxyl group at C-2 in methyl β-lactoside and, by extrapolation, the N atom in N-acetyllactosamine are not measurably involved in the binding. Moreover, the contribution of hydrogen bonds at position 3 is smaller than for the other two lectins. It is noteworthy with respect to discriminatory characteristics that CL-14’s binding site can accommodate an axial hydroxyl group at this position, which is not possible for CL-16 or bovine galectin-1.

Recently, it has been proposed (24) that the combining sites of galectins can be classified into two types on the basis of the presence of conserved and variable amino acid residues involved in the recognition. According to the known fine carbohydrate-binding specificity of several galectins, the type I or conserved carbohydrate-recognition domain, found in galectin-1 from several types of mammals, shows very strict requirements for the orientation of substituents at position 3 of GlcNAc in Galβ(1,4)GlcNAc (equivalent to position 4 of GlcNAc in Galβ(1,3)GlcNAc), whereas the type II or variable positioning, found in galectin-2, -3, and -4 and in other galectins from lower vertebrates and invertebrate species, is able to accommodate an axial hydroxyl group at this position. Therefore, the type II site is apparently able to bind Galβ(1, 3)GalNAc, in which the axial HO-4 of GalNAc takes the place of the axial HO-3 in the 3-epi-methyl β-lactoside derivative. In the light of the sequence identities from residues 44 to 73, which form the carbohydrate-recognition domain, both chicken lectins have been proposed to contain type I combining sites (24). According to our results on the molecular recognition of methyl β-lactoside analogues, the mode of binding observed for CL-16 is in agreement with this prediction. However, the ability of CL-14 to bind the 3-epi derivative is, according to this classification scheme, evidently characteristic of a type II carbohydrate-recognition domain. It is presently difficult to precisely explain this particular behavior, since the residues primarily involved in ligand recognition are invariant in the sequences of bovine galectin-1 and the two chicken lectins (Fig. 3). However, there are variable residues within the sequences that encompass the carbohydrate-recognition domains, the presence of which could result in a slightly different spatial disposition of side chains directly involved in the binding. For instance, the common Thr-57 is substituted by Leu in CL-14. Molecular dynamic simulations of the possible consequences of this substitution in the combining site of bovine galectin-1 (Fig. 4) reveal a slight but significant displacement of the lateral chain of Arg-73, which is a key residue involved in interactions with the hydroxyl group at position 3 of the glucose moiety and the N-acetyl group of N-acetyllactosamine as well as in a network of salt bridges which assures the optimal orientation of other relevant amino acid residues.

In aggregate, the observed mode of binding for the panel of synthetic ligand derivatives provides information on the molecular basis of oligosaccharide-binding specificity. Thus, both lectins will bind to oligosaccharides containing the basic structure Galβ(1,4)GlcNAc or its isomer Galβ(1,3)GlcNAc. Substitution of the backbone sequence at positions 2’ and 3’ of the galactose moiety should not prevent binding by the lectins. On the contrary, binding will be hampered by substitutions at the key positions, namely positions 4 and 6 of galactose and position 3 of N-acetylgalcosamine in Galβ(1,4)GlcNAc (or position 4 in Galβ(1,3)GlcNAc). Although these general rules are valid for both CL-14 and CL-16, the observed differences in the contribution to the binding of distinct hydroxyl groups for the two lectins will most probably result in a different range of affinities for various oligosaccharides. In particular, it can be assumed that the structure Galβ(1,3)GlcNAc, which is characteristic of many glycolipid antigens specifically expressed at certain developmental stages (27), should be differentially recognized by CL-14 and CL-16. Thus, the results suggest overlapping but distinct ranges of endogenous ligands for the two lectins and in general terms illustrate the potential usefulness of this chemical-mapping approach for delineation of ligand-binding differences between homologous lectins.

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Different Architecture of the Combining Site of the Two Chicken Galectins Revealed by Chemical Mapping Studies with Synthetic Ligand Derivatives
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