Protein Engineering

Modular Control of Lectin Function: Redox-Switchable Agglutination**

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Carbohydrate–protein interactions have been shown to be crucial in a range of biological events, including cell proliferation, signaling, and regulation.[1] These interactions frequently involve lectins, which bind carbohydrates reversibly and specifically.[2] Lectins typically contain two or more

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carbohydrate recognition sites,[3] hence, their interactions are dominated by potentially multivalent connections. This gives them the ability to interact with cells that have sugars on their surface, for example, erythrocytes, and to bind these sugars in single- or multipoint-attachment modes, often initiating cross-linking between cells in a process referred to as agglutination (Figure 1).[4]

Indeed, lectin binding plays a critical role in the survival of platelets[5] and agglutination of erythrocytes (hemagglutination) is a routine method of characterizing lectins as a result of the simplicity and low material requirements of the procedure.[6] Another sensitive method of determining binding is the enzyme-linked lectin assay (ELLA),[7] a variation on the ELISA. As a consequence of the near two-dimensional ligand and protein presentation that is used, ELLA results are typically not affected by cross-linking and they therefore provide a more direct measurement of binding,[8] as opposed to cross-linking.[9] These two methods, ELLA and agglutination, can therefore be used to probe the different phenomena of direct binding (Figure 1a,b) and extended binding through cross-linked interactions (Figure 1c,d), respectively.

Using these two contrasting assays as a guide, we have sought to engineer a novel lectin system in which extended binding, as measured by agglutination, may simply be switched on or off through redox control while maintaining similar levels of direct binding (Figure 1c). Examples of lectin engineering are rare, partly as a result of some difficulties associated with establishing recombinant sources,[9] and to the best of our knowledge this is the first example of such a modular control of lectin function through engineering. Critical sites in the model snowdrop lectin Galanthus nivalis agglutinin (GNA) were chosen that would allow us to introduce a cysteine residue capable of acting as a redox-active switch for covalent bond formation across protein–protein interfaces. GNA, the first monocot mannose-binding lectin to be characterized,[10] is a useful protein for this purpose. Investigation of the binding properties of GNA have shown that it is highly specific for $\alpha$-mannosides and, in particular, for the linkages of Man($\alpha$-1,3) units.[11] The 3D structure is known and reveals three mannose binding sites, I–III, (Figure 2).[12,13] In this structure the strongest, primary binding site, I, is the one that is most frequently occupied by carbohydrates; secondary and tertiary sites II and III show weaker binding (occupancies relative to site I of 0.7 and 0.6, respectively).[12]

Accessibility is a critical parameter in establishing protein–protein interfaces.[14] Analysis of the 3D structure of daffodil lectin Narcissus pseudonarcissus agglutinin (NPA),[15] which has a strong sequence similarity to GNA and contains domain 1nplA0 that places it in the same homologous superfamily (CATH code 2.90.10.10.1[16]) as representative GNA domain 1jpc00, revealed that a putative protein–protein interface is formed in NPA that is not present in the known 3D structure of GNA. This interface forms between different lectin units through the interaction and proximity of two
residues, proline P71 and asparagine N76, with the same residues in another NPA chain (Figure 3). These residues therefore seemed to be ideally accessible positions at which to introduce cysteine into GNA to design the key interface for our redox-active switch for extended binding.

To explore the potential for protein–protein interaction in GNA, the P71 and N76 residues of GNA, which occur on the edge of binding site II, were initially mutated to aspartic acid to create P71D, N76D, and P71DN76D mutants with the aim of introducing repulsive electrostatic effects across the interface. The effects of these mutations were analyzed by the agglutination assay (Figure 4) and ELLA (Figure 5). We were encouraged by the observation of an increase in the minimum agglutination concentration values of up to 60-fold (MAC: GNA-WT 14 nm, P71D 56 nm, N76D 55 nm, P71DN76D 880 nm) whilst binding, as analyzed by ELLA, remained similar (EC50: GNA-WT 100 nm, P71D 208 nm, N76D 44 nm (IC50), P71DN76D 280 nm). Consistent with the putative model of introduced electrostatic repulsion, decreases in extended binding were greatest for the double-point P71DN76D mutant. This confirmed the critical nature of this site in primarily extended binding and not direct binding and demonstrated that for the first time extended and direct binding could be dissected by engineering a model lectin.

Next, the potentially redox-switchable P71C and N76C mutants were constructed and analyzed in the same manner (Figure 4, Figure 5). Excitingly, the P71C mutant showed a 20-fold increase in agglutination activity in comparison to the wild type (MAC: GNA-WT 14 nm, P71C 0.70 nm) whilst the binding analyzed by ELLA remained very similar (EC50: GNA-WT 100 nm, P71C 73 nm (IC50)). This observation demonstrated that not only could extended binding be enhanced through engineering but that this could also be achieved without dramatic effect on direct binding. Interestingly, mutants containing the N76C mutation gave extended binding levels similar only to the GNA wild type (MAC: GNA-WT 14 nm, N76C 15 nm) and, given the strong similarity in the GNA and NPA structures, we attribute this to the poor ability of the covalent four-atom CysCH2SSCH2Cys interface cross-linking motif (ca. 6.0 Å) to replace the natural six-atom AsnCH2CONH2NH2COCH2Asn motif (ca. 9.3 Å).

To investigate whether the observed, enhanced extended binding was a result of disulfide-bond formation and to explore the usefulness of this interaction in a novel redox switch for such binding, the GNA wild type and the P71C mutant were exposed to varying concentrations of the reductant dithiothreitol (DTT). We hypothesized that the hemagglutination activity...
of the P71C mutant would switch as the additional disulfide bond was reduced (Figure 6). Indeed, DTT had a dramatic effect on the P71C mutant and agglutination activity decreased from a disulfide-enhanced level (MAC: 3.5 nm) for this blood sample set[23] to the GNA-WT level (MAC: 56 nm; see the Supporting Information). In comparison, DTT had no effect on the GNA wild type, a result indicating that at these levels of DTT the internalized, less accessible C29–C52 disulfide bond in GNA was unaffected[23]. Again, ELLA analysis showed that these results were a consequence of the modulation of extended binding alone rather than direct binding; the EC_{50} values were all within about twofold of that for the GNA wild type. Hydrogen peroxide, oxidized glutathione (GSSG), oxidized DTT, and S-nitroso-N-acetyl-penicillamine (SNAP) were investigated for their ability to perform an oxidative “switch-back”. Although in some cases the detrimental effect (see the Supporting Information) of these oxidising agents in the blood cells proved to be a limiting factor with regard to the concentration used, pleasingly, the use of DTT followed by 1 mm GSSG (use above this concentration resulted in cell lysis) showed that it was possible to impair and then restore agglutination to a level approaching its initial prereduction level (within blood set).[22] P71C prereduction 102 nm→P71C reduced 1300 nm→P71C GSSG switch-back 218 nm). This result demonstrated that it was indeed possible to switch extended carbohydrate binding on and off in this engineered lectin simply through the use of redox conditions.

To further confirm the key role of the free thiol group in our P71C model, we performed chemical modification on this residue by using the highly thiol-specific reagent methyl methanethiosulphonate[24] to create the “methyl-capped” P71C-Me variant in which the formation of the critical interprotein cross-linking disulfide bond is prevented. Consistent with the need for a free thiol group at position 71, agglutination dropped approximately 20-fold from unmodified P71C to wild-type levels upon chemical modification to form the “methyl-capped” P71C-Me variant.

In summary, our results indicate that modular control of different modes of binding in a model carbohydrate-binding protein is possible through logical design. This work has included examples of engineered lectins in which extended binding may be electrostatically deactivated and the first examples of redox-switchable extended binding that is controlled whilst maintaining direct binding. This switchable, condition-sensitive, covalently mediated protein-interface formation offers exciting possibilities in the design of protein systems that can sense and then manipulate cell–cell interactions differently according to the redox environment, as demonstrated here by redox-switchable red-blood-cell association. Indeed, key differences in redox conditions occur in certain disease states, such as during cancer cell proliferation,[23] where such switchable cell–cell manipulation might prove useful. Interestingly, cross-interface disulfide-bond formation has recently been implicated in infectious prion protein propagation.[26] We aim to investigate these possibilities, as well as the further modulation of multivalent interactions though variations in chemical modification in this and other systems.[27]
Interestingly, proline is a common residue at position 71 in a GNA sequence numbering used throughout. GNA exists naturally in various multimeric, functional forms up to the tetramer. The site chosen for the creation of the new protein–protein interface for this noncarbohydrate-induced multimerization and is located only at the site of a carbohydrate-induced interface. Gel-filtration analysis of GNA and mutants indicates that the overall inherent multimer distribution was unaltered by mutation and compared well with a standard of extracted wild-type GNA from the plant (ca. 20% monomer, ca. 50% dimer, and ca. 30% tetramer; Amersham Pharmacia Superdex 200 16/60 column, flow rate: 0.5 mL min⁻¹, buffer: 0.25 m NaCl in 50 mM tris(hydroxymethyl)aminomethane, pH 8). For simplicity, the schematic representation in Figure 1 shows a monomeric lectin mediating binding and agglutination. In the naturally occurring dynamic noncarbohydrate-induced multimerization equilibrium that exists for GNA, it cannot be discounted that the effects that we have observed here from creating a novel protein–protein interface may be functionally affected in one or all of these multimeric forms. Analysis of the structure of wild-type GNA and its noncarbohydrate-induced multimers shows that the novel interface we create here is accessible in all.

Excitingly, during the course of this work, structures of two similar lectins that show two contrasting dimerization modes at a weaker, secondary binding site interface have been described: one shows a sugar-mediated mode, the other shows a non-covalent, nonsugar mediated mode. These findings provide a natural, partial endorsement for the general engineering approach we describe here; see: M. Hayashida, T. Fujii, M. Hamasu, M. Ishiguro, Y. Hata, J. Mol. Biol. 2003, 334, 551–565. Interestingly, initial indications in other systems suggest that a common functional theme in certain lectin types. This, in part, further highlights the suitability of these interfaces for the type of engineering we have demonstrated here. Thus, although we have shown only a slight effect of manipulating such sites upon direct binding in the GNA system, interesting additional effects upon multivalency may also be engendered.

Lack of activity in control blood types in MAC analyses (chicken, horse, sheep, human) and control polysaccharide laminarin in ELLA analyses (see the Supporting Information) demonstrated the sugar-specific nature.

All MAC analyses were conducted on the same blood sample sets in triplicate. Up to fourfold variation in absolute MAC values was observed between different blood sample sets taken from different animals on different days. Variations within the same blood sample sets were ≤ 5%. Thus, whilst set-to-set comparisons should be made with caution, those within sets show high confidence levels (p = 0.001).

Analysis of the effects of different reductants upon the noncarbohydrate-induced, inherent multimerization of GNA showed that while the agglutination and noncarbohydrate-induced multimerization state of wild-type GNA is not affected by DTT under these conditions, the use of excess 2-mercaptoethanol instead under the conditions described in M. Longstaff, K. S. Powell, J. A. Gatehouse, R. Raemaekers, C. A. Newell, W. D. O. Hamilton, Eur. J. Biochem. 1998, 252, 59–65 leads to denaturation and formation of monomeric wild-type GNA.

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