Supporting Information

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Modular Control of Lectin Function: Redox Switchable Agglutination

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Additional figures

Figure 1: Effect of DTT on GNA and P71C on MAC: While DTT has little or no effect upon the agglutination activity of GNA-WT, increasing concentrations of DTT switch the enhanced agglutination activity of P71C back to only WT levels, representing the "switch off" phase in this redox switchable system. Protein concentrations: 1: 0.006, 2: 0.01, 3: 0.02, 4: 0.05, 5: 0.1, 6: 0.2, 7: 0.4, 8: 0.8, 9: 1.6, 10: 3.1, 11: 6.3, 12: 12.5 µg/mL.

Figure 2: Graphical comparison of Figure 1, the effect of DTT on Agglutination Activity of P71C and GNA
**Figure 3**: ELLA binding analysis of wild type and mutants: 96-well microtitre plate showing results of visualization from incubation with ABTS after horse radish peroxidase-secondary antibody conjugate. Much less dramatic variation on direct binding activity as indicated by this ELLA was observed than for MAC. Measurement of $A_{410}$ from these plates allowed dose-response analysis such as that shown in Figure 5 in the manuscript. Protein concentrations: 1- 0.01µg/mL, 2- 0.02µg/mL, 3- 0.05µg/mL, 4- 0.1µg/mL, 5- 0.2µg/mL, 6- 0.4µg/mL, 7- 0.8µg/mL, 8- 1.6µg/mL, 9- 3.1µg/mL, 10- 6.3µg/mL, 11- 12.5µg/mL, 12- 25µg/mL.

**Figure 4**: Switch back of agglutination activity by oxidation: consistent with disulfide formation, the enhanced MAC of P71C is removed by treatment with reductant DTT (P71C red) and then recreated when treated with oxidant oxidised glutathione (GSSG). Protein concentrations: 1- 0.006µg/mL, 2- 0.01µg/mL, 3- 0.02µg/mL, 4- 0.05µg/mL, 5- 0.1µg/mL, 6- 0.2µg/mL, 7- 0.4µg/mL, 8- 0.8µg/mL, 9- 1.6µg/mL, 10- 3.1µg/mL, 11- 6.3µg/mL, 12- 12.5µg/mL.

**Figure 5**: Effect of chemical modification: treatment of P71C with methyl methanethiosulfonate to cap the free thiol side chains in P71C to create P71CMe removed the enhanced MAC activity of P71C and is consistent with the proposed role of this free thiol in the observed agglutination activity. Protein concentrations: 1- 0.006µg/mL, 2- 0.01µg/mL, 3- 0.02µg/mL, 4- 0.05µg/mL, 5- 0.1µg/mL, 6- 0.2µg/mL, 7- 0.4µg/mL, 8- 0.8µg/mL, 9- 1.6µg/mL, 10- 3.1µg/mL, 11- 6.3µg/mL, 12- 12.5µg/mL.
**Figure 6:** CD analysis of the WT and mutant GNA proteins: these highly similar traces (variation in ellipticity (delta e) vs wavelength) confirmed that mutation had negligible effect upon the secondary structure content of these proteins.

**Figure 7:** Detrimental lytic effects of certain oxidising conditions on red blood cells: attempts to use other oxidants or higher concentrations of oxidants to perform the oxidative leg of the redox switching system unfortunately led to cell lysis and can be seen by accompanying effervescence and/or red-to-yellow colour change.
Figure 8: Alignment, RMSD 0.24Å, of monomers of GNA (yellow) and dafoodil lectin (PNA, blue) showing close structural similarity. P71 and N76 are shown in red.

Figure 9: Immunoblotting SDS/PAGE of GNA after treatment with different reducing agents indicates strong reductant dependency: βME = after treatment with beta-mercaptoethanol according to conditions in Longstaff et al [10% beta-mercaptoethanol, 10 min boiling], DTT = after treatment with identical concentration of DTT [10 mM] instead of used for agglutination switching demonstrated here beta-mercaptoethanol; control = in the absence of reductant or boiling.
**Figure 10:** ELLA assay using control polysaccharide laminarin shows lack of activity towards non-mannose structures by GNA and highlights sugar-specific nature of observed interactions. Columns correspond to serial 2-fold dilutions starting from 25 µg/mL.
Experimental

Site-directed mutagenesis

The mature GNA coding sequence cloned into pET14b using NdeI and BamHI (kindly provided by Dr Elaine Fitches)[1] was used as the template for all site-directed mutagenesis. Mutations were produced using either the Gene Editor kit (Promega) or Quikchange kit (Stratagene) according to the manufacturers protocol. Mutations were confirmed by sequencing.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer (5′-3′)</th>
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<tbody>
<tr>
<td>P71D</td>
<td>C CCA TCG AAC AAA GAT ATT TGG GCA AGC</td>
</tr>
<tr>
<td>N76D</td>
<td>CCG ATT TGG GCA AGC GAC ACT GGA GGC</td>
</tr>
<tr>
<td>P71DN76D</td>
<td>C CCA TCG AAC AAA GAT ATT TGG GCA AGC GAC ACT GGA GGC CAA AAT GG</td>
</tr>
<tr>
<td>P71C</td>
<td>C CCA TCG AAC AAA GAC ACT TGG GCA AGC</td>
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Table 1: Primers for GeneEditor site directed mutagenesis

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer (5′-3′)</th>
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<tbody>
<tr>
<td>N76C Forward</td>
<td>G AAC AAA CCG ATT TGG GCA AGC TGC ACT GGA GGC</td>
</tr>
<tr>
<td>N76C Reverse</td>
<td>GTA ATT CCC ATT TTG GCC TCC AGT GCA GCT TGC CC</td>
</tr>
<tr>
<td>P71CN76C Forward</td>
<td>G TAC AAC CCA TCG AAC AAA TGC ATT TGG GCA AGC TGC ACT GGA GGC C</td>
</tr>
<tr>
<td>P71CN76C Reverse</td>
<td>CC ATT TTG GCC TCC ATG GCA GCT TGC CCA AAT GCA TTT GTT CGA TGG G</td>
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Table 2: Primers for Quikchange site directed mutagenesis

Protein Expression

Media components were purchased from Sigma and Fisher. All media formulations and abbreviations are taken from Molecular Cloning: A Laboratory Manual.[2]

The expression constructs were transformed into host strain BL21(DE3)pLysS (Novagen) according to the manufacturers instructions. Inoculum was prepared from a glycerol stock or single colony from an agar plate, and consisted of LB media with 50\( \mu \)g/mL carbenicillin and 34\( \mu \)g/mL
chloroamphenicol grown overnight, with shaking at 37°C. One percent of the expression culture volume of inoculum was used to prepare the expression culture along with the relevant antibiotics. The culture was grown at 37°C until the cells had reached an optical density of approx. 0.6 at 600nm at which point the cells were induced by adding IPTG to a final concentration of 0.4mM. Cultures were induced for 3h at 37°C before harvesting by centrifugation at 14,000g for 20 min.

**Protein purification**

Cell pellets were resuspended by stirring for 15min in 5x w/v denaturing binding buffer (20mM Tris, 0.5M NaCl, 5mM imidazole, 6M urea, pH 7.8). The cells were lysed by sonication using a Soniprep 150 sonicator for 3 x 1 min bursts (15 amplitude microns) and the supernatant clarified by centrifugation at 10,000g for 30min. The lysate was poured off and filtered through a 0.8μm syringe filter. Recombinant protein was isolated from the lysate and refolded by nickel affinity chromatography (His-bind resin, Novagen) using buffers with a gradual decrease in the concentration of urea. A 10mL column (Amersham Pharmacia C10/10 column) was packed with 7mL of His-bind Resin (prepared as manufacturers instructions) and equilibrated with 5 column volumes of denaturing binding buffer (20mM Tris, 0.5M NaCl, 5mM imidazole, 6M urea, pH 7.8) at 1mL/min. The filtered lysate was loaded, and the column re-equilibrated with 5 column volumes of denaturing binding buffer. Non-specifically bound protein was removed with 5 column volumes of denaturing wash buffer (20mM Tris, 0.5M NaCl, 20mM imidazole, 6M urea, pH 7.8). The
bound protein was renatured with 2 column volumes of binding buffer with decreasing concentrations of urea, 4M, 2M, 1M, 0.5M, 0.25M, 0.125M and 0M (xM urea, 20mM Tris, 0.5M NaCl, 5mM imidazole, pH 7.8), before elution of the purified protein with 4 column volumes of elution buffer (20mM Tris, 0.5M NaCl, 300mM imidazole, pH 7.8).

The eluted fraction (30mL) was dialysed against 2 portions of distilled water (6L) overnight to remove imidazole, filtered through a 0.2µm filter and concentrated using a Vivaspin 20 concentrator (Vivascience).

Assay preparation

Protein concentrations were calculated by the Bradford method (Bio-rad reagent),\textsuperscript{[3]} using BSA as a standard.

Proteins were analysed by circular dichroism to confirm the structure was unaffected by mutation. Samples were prepared to a concentration of 0.5mg/mL in deionised distilled water and analysed using a path length of 1mm (JASCO J-720 spectropolarimeter).

Chemical modification

Chemical modification was undertaken on 300µL of a 0.25mg/mL solution of P71C, to which 200µL of a 10mg/mL methyl methanethiosulphonate solution was added. The solution was mixed on an end-over-end rotator for two hours, prior to washing (3 x 500mL distilled water) and concentration using a Vivaspin 0.5mL concentrator (mwco 5,000) to yield P71C-Me.
Hemagglutination analysis

Hemagglutination assays were carried out in round bottomed microtitre plates using 25µg/mL solutions of recombinant protein. Serial two fold dilutions of the lectin solutions were made in PBS, and a 2% rabbit erythrocyte solution was prepared. 25μL aliquots of each dilution was mixed with 25μL of the erythrocyte suspension and the plate was allowed to incubate for 1h at room temperature. The lowest concentration required to completely agglutinate the red blood cells was determined visually. The effect of DTT and oxidised DTT on GNA and P71C was determined by adding the relevant amount from stock solutions of 100mM or 10mM to produce final concentrations of 0.5mM, 1mM and 10mM.

For switch back experiments, P71C was incubated with a final concentration of reduced DTT of 10mM for 15min. Oxidising agents were first screened against a 2% rabbit erythrocyte solution without protein to determine working concentrations that did not cause cell lysis. Each oxidising agent was then incubated overnight with the reduced P71C solution before the addition of 2% rabbit erythrocyte solution. See also ref [22] in main text.

ELLA analysis

ELLA assays were carried out in microtitre plates coated overnight with 50μL of 10μg/mL yeast mannan (Sigma). Plates were then washed three times with PBS before allowing the two fold serial dilutions of protein to bind for 1h. After a further three PBS washes, wells were blocked for 1h (5% marvel, 10% PBS, 0.1% Tween 20) before incubating for a further 1h with 50 μL GNA primary
antibody (1: 1500 dilution in 5% Marvel, 10% PBS, 0.01% Tween 20), wells were washed three times with 5% Marvel, 10% PBS, 0.01% Tween 20 before incubating with goat anti-rabbit horse radish peroxidase conjugate secondary antibody (1:5000 in 5% Marvel, 10% PBS, 0.01% Tween 20) for a further hour. Wells were given a final three washes with PBS before incubating with 50µL ABTS for 15min at 37°C. Absorbance was measured using a Molecular Devices SpectraMax-plus plate reader at 410nm.

