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Exploring the cross-reactivity of S25-2: complex with a 5,6-dehydro-Kdo disaccharide

The near-germline antibody S25-2 exhibits a remarkable cross-reactivity for oligosaccharides containing the bacterial lipopolysaccharide carbohydrate 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). The recent synthesis of a variety of Kdo analogues permits a detailed structural analysis of the importance of specific interactions in antigen recognition by S25-2. The Kdo disaccharide analogue Kdo-(2→4)-5,6-dehydro-Kdo lacks a 5-OH group on the second Kdo residue and has been cocrystallized with S25-2. The structure reveals that the modification of the Kdo residue at position 5 results in a rearrangement of intramolecular hydrogen bonds in the antigen that allows it to assume a novel conformation in the antibody-combining site. The cross-reactive binding of S25-2 to this synthetic ligand highlights the adaptability of this antibody to non-natural synthetic analogues.

1. Introduction

The humoral immune system has the remarkable capability of recognizing a seemingly limitless number of antigens from a limited antibody repertoire. Antibody diversity stems firstly from combinatorial rearrangement of germline genes (Hozumi & Tonegawa, 1976), and the antibodies produced in this manner can be considered to be 'germline'. Some types of antigen have the ability to stimulate mutations in these germline genes that can result in antibodies of much higher avidity through the process of affinity maturation (Jacob *et al.*, 1991); however, even the generation of such antibodies must begin with germline-recognition events, which underscores their importance. The ability of germline antibodies to adapt their combining sites to bind a wide variety of antigens has been suggested as a mechanism to enhance the diversity of the humoral immune response (Wedemayer *et al.*, 1997; Manivel *et al.*, 2000; Nguyen *et al.*, 2003).

A series of reports from our laboratories has described the molecular basis for cross-reactivity between antibodies and a panel of antigens based on chlamydial lipopolysaccharide (LPS). Antibody S25-2 and the related antibody S25-39 were remarkable in their ability to bind a range of chemically distinct antigens, which hinged on the flexible utilization of a pocket of amino-acid residues of germline origin to bind a single 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residue in each antigen (Nguyen *et al.*, 2003; Blackler *et al.*, 2011; Brooks, Blackler *et al.*, 2010; Brooks *et al.*, 2008; Gerstenbruch *et al.*, 2010; Evans *et al.*, 2011).

S25-2 remains a valuable model in the study of germline-antibody recognition because of its remarkable cross-reactivity, and the use of synthetic Kdo analogues in particular provides a unique opportunity to probe the specificity of germline antibodies. The novel Kdo disaccharide analogue Kdo-(2→4)-5,6-dehydro-Kdo (Fig. 1a; Sixta *et al.*, 2009) was synthesized in order to explore the importance of the 5-OH group on the second Kdo residue in recognition by S25-2.

2. Materials and methods

2.1. Production of antibody and Fab fragments

The mAb S25-2 was prepared by immunization of BALB/c mice with Kdo-BSA glycoconjugates as described previously (Fu *et al.*,

Table 1

Data-collection and refinement statistics for S25-2 in complex with Kdo-(2→4)-5,6-dehydro-Kdo.

Values in parentheses are for the highest resolution shell.

Resolution	19.92–1.65 (1.71–1.65)
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 45.71, b = 81.30, c = 131.42$
Observations	229551
Unique reflections	57408
Completeness (%)	95.9 (97.6)
R_{merge}	0.05 (0.253)
$\langle I/\sigma(I) \rangle$	12.4 (4.0)
No. of non-H atoms	
Polypeptide	3372
Ligand	33
Solvent	655
Ion	3
R_{work}	0.176
R_{free}	0.197
Average B factor (Å ²)	
Protein	21.9
Solvent	34.3
Ligand	17.9
Ramachandran plot, residues in (%)	
Favoured regions	98.0
Additionally allowed regions	2.0
R.m.s.d., bonds (Å)	0.008
R.m.s.d., angles (°)	1.24

1992). Fab fragments were prepared by papain digestion of S25-2 IgG using a 1:100(w:w) papain:IgG ratio for 2 h at room temperature. The reaction was quenched with 0.01 M iodoacetamide and the Fab fragments were purified from the Fc fragments by cation-exchange chromatography on a Gilson HPLC using a Shodex CM-825 column with a mobile phase of 20 mM Tris pH 8.0 and a linear 0–0.5 M NaCl gradient. Fractions containing Fab were dialyzed against 20 mM Tris pH 8.0 and concentrated to 10 mg ml⁻¹. The synthesis of the ligand used for cocrystallization, Kdo-(2→4)-5,6-dehydro-Kdo [sodium (3-deoxy- α -D-manno-oct-2-ulopyranosyl)onate-(2→4)-sodium (allyl-3,5-dideoxy- α -D-threo-oct-5-en-2-ulopyranosid)onate], has been reported by Sixta *et al.* (2009).

2.2. Crystallization

S25-2 Fab was cocrystallized with the disaccharide Kdo-(2→4)-5,6-dehydro-Kdo (25 mM). Large crystals (1 mm³) grew overnight at room temperature in PEG 4000, zinc acetate, MgCl₂, Tris pH 8.5. The inclusion of zinc acetate was critical for crystal formation, while the inclusion of MgCl₂ resulted in larger, better diffracting crystals, as reported for other crystals of S25-2 (Nguyen *et al.*, 2003; Brooks *et al.*, 2008).

2.3. Data collection and structure refinement

Crystals were flash-cooled to 113 K using an Oxford Cryostream 700 crystal cooler (Oxford Cryosystems). Data were collected using a Rigaku R-Axis IV⁺⁺ area detector (Rigaku, Japan) coupled to a MicroMax-002 X-ray generator with Osmic Blue optics. The data were processed using *CrystalClear/d*TREK* (Rigaku). The structure of Fab S25-2 in complex with the ligand Kdo-(2→4)-5,6-dehydro-Kdo was solved by molecular replacement with *PHENIX* (Adams *et al.*, 2010) using the structure of S25-2 in complex with Kdo as a search model (PDB entry 3t4y; Nguyen *et al.*, 2003), with Kdo, waters and ions being removed from the model prior to molecular replacement. *Coot* was used for manual fitting of the protein and ligand with $2F_o - F_c$ and $F_o - F_c$ electron-density maps (Emsley & Cowtan, 2004). The structure was refined using *PHENIX* (Adams *et al.*, 2010; *phenix.refine* v1.8.1069). *MolProbity* (Chen *et al.*, 2010) was used for

Ramachandran analysis. The final refinement and model statistics are given in Table 1.

3. Results and discussion

The structure of Fab S25-2 was solved in complex with the non-natural Kdo derivative Kdo-(2→4)-5,6-dehydro-Kdo (Fig. 1a), which differs from the naturally occurring Kdo-(2→4)-Kdo disaccharide found in enterobacterial and chlamydial LPS (Fig. 1b). The residues in the structure were numbered according to the Kabat numbering scheme, which provides a common system of antibody residue numbering; letters are added to indicate insertions in CDR loops

Figure 1
(a) Ligand used for cocrystallization with S25-2: Kdo-(2→4)-5,6-dehydro-Kdo. (b) Structure of chlamydial LPS composed of Kdo-(2→8)-Kdo-(2→4)-Kdo-(2→6)-GlcN4P-(1→6)-GlcN1P. The Kdo-(2→4)-Kdo that previously cocrystallized with S25-2 (PDB entry 3t77; Nguyen *et al.*, 2003) is highlighted in red. (c) $2F_o - F_c$ electron-density map contoured to 1σ around Kdo-(2→4)-5,6-dehydro-Kdo cocrystallized with S25-2.

(Kabat *et al.*, 1983). The ligand exhibited excellent electron density within the antibody-combining site (Fig. 1c).

The ligand is bound to S25-2 in the same electropositive pocket of conserved sequence as observed for other ligands (Fig. 2a), with the antibody forming a similar set of hydrogen bonds and charged-residue interactions with the terminal Kdo residue (Fig. 2b) to other liganded structures of S25-2 (Brooks *et al.*, 2008; Nguyen *et al.*, 2003). The 5,6-dehydro-Kdo is situated just outside the germline pocket,

where it forms a hydrogen bond between the 7-OH group of Kdo and the side chain of Asn53 of the antibody light chain (Fig. 2b).

The recent synthesis of Kdo analogues (Sixta *et al.*, 2009) provides a convenient tool to probe the importance and the specificity of antibody–antigen interactions in the combining site of S25-2. The structure of S25-2 in complex with the antigen Kdo-(2→4)-Kdo (Fig. 1b) has been reported (PDB entry 3t77; Nguyen *et al.*, 2003). The generation of the Kdo-(2→4)-5,6-dehydro-Kdo disaccharide

Figure 2

(a) Kdo-(2→4)-5,6-dehydro-Kdo binds in an electropositive pocket of the S25-2 combining site. (b) Interactions (green dashes) of S25-2 with Kdo-(2→4)-5,6-dehydro-Kdo. The terminal Kdo (bottom) makes a series of interactions that are conserved in other S25-2 structures (Brooks *et al.*, 2008; Nguyen *et al.*, 2003), while the 7-OH group of 5,6-dehydro-Kdo (top) forms a single hydrogen bond to residue Asn53 in the heavy chain. Amino-acid residues in the structure are numbered according to the Kabat numbering scheme for antibodies (Kabat *et al.*, 1983); (H) refers to residues on the heavy chain and (L) refers to residues on the light chain.

Figure 3

(a) Overlay of binding sites of S25-2 in complex with Kdo-(2→4)-5,6-dehydro-Kdo (yellow; interactions shown in green) and Kdo-(2→4)-Kdo (white; interactions shown in red). In Kdo-(2→4)-Kdo the 5-OH group of the second Kdo residue forms a hydrogen bond to Asn53 (heavy chain) orienting the Kdo to form a salt bridge with Arg27f (light chain). The lack of the 5-OH group in the second Kdo of Kdo-(2→4)-5,6-dehydro-Kdo results in different interactions in the binding site. (b) Stereoview of Kdo-(2→4)-5,6-dehydro-Kdo (yellow) and Kdo-(2→4)-Kdo (white), which exhibit different orientations in the binding site. Amino-acid residues in the structure are numbered according to the Kabat numbering scheme for antibodies (Kabat *et al.*, 1983); (H) refers to residues on the heavy chain and (L) refers to residues on the light chain.

allows direct examination of the importance of the 5-OH group on the second Kdo (Figs. 1*a* and 1*b*). In the structure with the Kdo-(2→4)-Kdo disaccharide, a single hydrogen bond is formed from the 5-OH group on the second Kdo residue to residue Asn53 in the heavy chain of S25-2 (Fig. 3*a*; Nguyen *et al.*, 2003). The ligand 5,6-dehydro-Kdo is unable to form this interaction and only forms the hydrogen bond between the 7-OH group and the side chain of Asn53 (Figs. 2*b* and 3*a*). An overlay of S25-2 in complex with Kdo-(2→4)-Kdo and with Kdo-(2→4)-5,6-dehydro-Kdo reveals no significant movement (r.m.s.d. on C α atoms of 0.2 Å) of the protein backbone and side chains in the combining site (Fig. 3). However, the two ligands are in different conformations, with a 55° rotation of the Kdo ring occurring about the glycosidic bond (Figs. 3*a* and 3*b*), with the effect that the 5,6-dehydro-Kdo does not form the salt bridge to residue Arg27f on the light chain of the antibody that is observed in the Kdo-(2→4)-Kdo disaccharide structure (Fig. 3*a*). Kdo-(2→4)-Kdo exists as a mixed population of two major conformers. Interestingly, S25-2 does not bind to either of these conformers, but rather binds to an energetically unfavourable conformation that does not exist in solution (Haselhorst *et al.*, 1999). This specific bound orientation of Kdo-(2→4)-Kdo is created by a salt bridge between the carboxyl group of the second Kdo and Arg27f which twists the sugar ring in the binding site. This strong interaction does not form in the 5,6-dehydro-Kdo structure. The side chain of Arg27f is disordered in the 5,6-dehydro-Kdo structure, suggesting that the moiety is highly mobile. Initial correct positioning of the ligand in the binding site may be required to form the salt bridge and restrict the mobility of Arg27f. Thus, the interaction of the antibody with the 5-OH group on the second Kdo acts as an anchor and appears to be crucial in the formation of the salt bridge to Arg27f and hence the differences in the conformations of the Kdo-(2→4)-Kdo and 5,6-dehydro-Kdo disaccharides observed in the S25-2 binding sites (Haselhorst *et al.*, 1999; Nguyen *et al.*, 2003). The Kdo-(2→4)-Kdo disaccharide binds to S25-2 with a K_d of 1.1×10^{-6} M, which is 15 times stronger than the binding of the Kdo monosaccharide ($K_d = 15 \times 10^{-6}$ M), indicating the importance of the interactions of the second Kdo in the generation of high-affinity binding to S25-2 (Brooks *et al.*, 2008). Although we do not have binding data for 5,6-dehydro-Kdo, we can hypothesize that the altered interactions to the modified second Kdo residue will result in a significantly lower affinity, similar to those observed for other Kdo analogue structures (Brooks *et al.*, 2008).

This structure firstly underscores the importance of intermolecular hydrogen bonds in maintaining antigenic conformations, which certainly contribute to the affinity of binding of the Kdo-(2→4)-Kdo

ligand. Secondly, this structure again demonstrates the remarkable ability of S25-2 to adapt and bind to the synthetic 5,6-dehydro-Kdo by forming new and different interactions in the antibody-combining site and highlights the diversity of the ability of the immune system to respond to novel antigenic challenges.

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References

- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Blackler, R. J., Müller-Loennies, S., Brooks, C. L., Evans, D. W., Brade, L., Kosma, P., Brade, H. & Evans, S. V. (2011). *Biochemistry*, **50**, 3357–3368.
- Brooks, C. L., Blackler, R. J., Sixta, G., Kosma, P., Müller-Loennies, S., Brade, L., Hiram, T., MacKenzie, C. R., Brade, H. & Evans, S. V. (2010). *Glycobiology*, **20**, 138–147.
- Brooks, C. L., Müller-Loennies, S., Brade, L., Kosma, P., Hiram, T., MacKenzie, C. R., Brade, H. & Evans, S. V. (2008). *J. Mol. Biol.* **377**, 450–468.
- Chen, V. B., Arendall, W. B., Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S. & Richardson, D. C. (2010). *Acta Cryst.* **D66**, 12–21.
- Emsley, P. & Cowtan, K. (2004). *Acta Cryst.* **D60**, 2126–2132.
- Evans, D. W., Müller-Loennies, S., Brooks, C. L., Brade, L., Kosma, P., Brade, H. & Evans, S. V. (2011). *Glycobiology*, **21**, 1049–1059.
- Fu, Y., Baumann, M., Kosma, P., Brade, L. & Brade, H. (1992). *Infect. Immun.* **60**, 1314–1321.
- Gerstenbruch, S., Brooks, C. L., Kosma, P., Brade, L., MacKenzie, C. R., Evans, S. V., Brade, H. & Müller-Loennies, S. (2010). *Glycobiol.* **20**, 461–472.
- Haselhorst, T., Espinosa, J. F., Jiménez-Barbero, J., Sokolowski, T., Kosma, P., Brade, H., Brade, L. & Peters, T. (1999). *Biochemistry*, **38**, 6449–6459.
- Hozumi, N. & Tonegawa, S. (1976). *Proc. Natl Acad. Sci. USA*, **73**, 3628–3632.
- Jacob, J., Kelsoe, G., Rajewsky, K. & Weiss, U. (1991). *Nature (London)*, **354**, 389–392.
- Kabat, E. A., Wu, T. T., Bilofsky, H., Reid-Miller, M. & Perry, H. (1983). *Sequences of Proteins of Immunological Interest*. Bethesda: National Institutes of Health.
- Manivel, V., Sahoo, N. C., Salunke, D. M. & Rao, K. V. (2000). *Immunity*, **13**, 611–620.
- Nguyen, H. P., Seto, N. O., MacKenzie, C. R., Brade, L., Kosma, P., Brade, H. & Evans, S. V. (2003). *Nature Struct. Biol.* **10**, 1019–1025.
- Sixta, G., Wimmer, K., Hofinger, A., Brade, H. & Kosma, P. (2009). *Carbohydr. Res.* **344**, 1660–1669.
- Wedemayer, G. J., Patten, P. A., Wang, L. H., Schultz, P. G. & Stevens, R. C. (1997). *Science*, **276**, 1665–1669.