Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of an anti-LewisX Fab fragment with and without its LewisX antigen

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Received 28 March 2003
Accepted 7 May 2003

LewisX-containing glycoconjugates are abundantly expressed by schistosomes and are assumed to be of prime importance for the survival of the parasite within the human host. Monoclonal antibody 291-2G3-A, which was generated from mice infected with schistosomes, was found to interact with monomers, dimers and trimers of the LewisX trisaccharide. The Fab fragment of monoclonal antibody 291-2G3-A has been crystallized and soaked with its LewisX antigen. X-ray data sets were recorded for the different Fab crystals with and without LewisX. Crystals grown from 25% polyethylene glycol 3350, 0.17 *M* ammonium sulfate and 15% glycerol belong to the triclinic space group *P*1, with unit-cell parameters a = 67.4, b = 71.6, c = 104.8 Å, α = 86.5, β = 71.3, γ = 83.3° for the native crystals and with slightly different unit-cell parameters a = 67.3, b = 72.4, c = 104.8 Å, α = 85.8, β = 71.3, γ = 83.3° for the crystals containing bound LewisX. Crystals grown from 14% PEG 3350, 50 m*M* Tris pH 8 and soaked with LewisX also belong to the triclinic space group *P*1, but with different unit-cell parameters a = 45.1, b = 60.8, c = 91.6 Å, α = 96.0, β = 95.4, γ = 101.8°.

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1. Introduction

Schistosoma species are trematode worms that cause a chronic, debilitating and sometimes fatal tropical disease called schistosomiasis. These parasites have a complex life cycle involving a freshwater snail intermediate host and a vertebrate definitive host. Adult schistosomes, which reside in the blood vessels of infected individuals, release relatively high amounts of circulating cathodic antigen (CCA) from their gut. CCA, which is thought to be important in the modulation of the host's immune response, has an immunoreactive part predominantly consisting of polymers of LewisX trisaccharides [→3Galβ1–4(Fucα1–3)-GlcNAcβ1→; Fig. 1] (Ko *et al.*, 1990; Srivatsan *et al.*, 1992; van Dam *et al.*, 1994).

LewisX is not a schistosome-specific carbohydrate, as it is one of the carbohydrate blood-group antigens and plays an important role in cell adhesion *via* both protein–carbohydrate and carbohydrate–carbohydrate interactions (Eggens *et al.*, 1989). On cell membranes it can be present on glycoproteins or glycosphingolipids (Cooling *et al.*, 1997) and is expressed as stage-specific embryonic antigen 1 (SSEA-1) on mouse embryonic cells (Fenderson *et al.*, 1984). Furthermore, it is expressed on numerous tumour cells, which might contribute to tumour metastasis *via* increased adherence to vascular endothelium and platelets (Hakomori *et al.*, 1984). Moreover, *Helicobacter pylori* produces LewisX on its cell-surface lipopolysaccharides and this has been implicated as playing a role in autoimmunity, cell adhesion and colonization of this pathogen (Sherburne & Taylor, 1995).

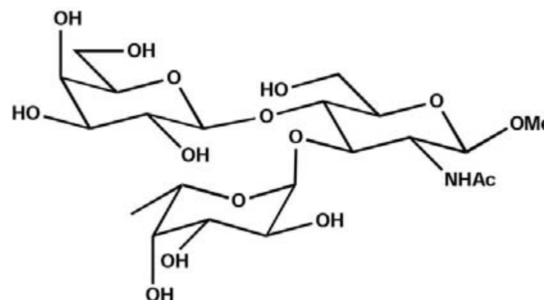

Figure 1
β-*O*-Methyl glycoside of LewisX used in soaking experiments.

In individuals infected with schistosomes the presence of anti-LewisX antibodies has been demonstrated and these antibodies are thought to play a role in the immunology of the disease (Nyame *et al.*, 1996; Richter *et al.*, 1996). It was found that anti-LewisX antibodies can mediate granulocyte lysis *in vitro* (van Dam *et al.*, 1996); however, it remains to be established whether this effect plays a significant role in the immunomodulation *in vivo*.

Previously, a large panel of monoclonal antibodies (Mabs) was generated from mice with a schisto-

somal infection (Deelder *et al.*, 1996). These Mabs are important immunodiagnostic tools in the detection of schistosomiasis (van Lieshout *et al.*, 2000), with a large number of these antibodies being reactive towards a variety of carbohydrate structures, including both parasite-specific structures and endogenous structures (van Remoortere *et al.*, 2000; Hokke & Deelder, 2001). A selected group of Mabs was screened for interaction with the LewisX epitope by surface plasmon resonance detection using BIAcore technology (van Roon *et al.*, 2003). For these screens, chemically synthesized monomeric, dimeric and trimeric LewisX were each conjugated to BSA and immobilized on a BIAcore chip. Mab 291-2G3-A, IgG3$_{\kappa}$, was found to interact with monomeric, dimeric and trimeric LewisX. The elucidation of the X-ray structure of the Fab fragment of Mab 291-2G3-A in complex with the LewisX trisaccharide (Fig. 1) will give us further insight into the structural requirements for the binding of this antibody to the LewisX epitope. Moreover, in view of the limited number of structures investigated so far, it will also provide additional information concerning carbohydrate recognition by antibodies in general. Currently, the structures of Fab fragments in complex with an oligosaccharide published include the Fab fragment of Se155-3, which recognizes *Salmonella* bacterial membrane oligosaccharides (Cygler *et al.*, 1991; Bundle *et al.*, 1994; Zdanov *et al.*, 1994), the Fab fragment of antibody BR96, which recognizes LewisY but not LewisX (Jeffrey *et al.*, 1995), and the Fab fragment of S20-4, which also binds bacterial oligosaccharides (Villeneuve *et al.*, 2000).

Here, we describe the production and purification of the Fab fragment of anti-LewisX monoclonal antibody 291-2G3-A, the method of crystallization, soaking with LewisX and the preliminary X-ray diffraction analysis of both the Fab fragment and the Fab fragment soaked with LewisX.

2. Materials and methods

2.1. Protein production and purification

The murine Mab 291-2G3-A was purified from hybridoma cell-culture supernatant using protein A affinity chromatography (Protein A Sepharose, Amersham). Serum-free culture medium was used to prevent the introduction of non-specific IgG molecules. The cell-culture supernatant was loaded onto the protein A column. After extensive washing of the column with a buffer containing 1.5 *M* glycine, 3 *M* NaCl pH 8.8, the IgG was eluted from the column with 0.1 *M* citrate pH 3. Fab fragments were obtained by papain digestion with immobilized papain agarose beads (Pierce) following the manufacturer's instructions. The digestion buffer contained 25 m*M* phosphate, 5 m*M* EDTA and 10 m*M* cysteine at pH 7.2 and the Mab was digested at 310 K for 2.5 h. After digestion, the Fab fragments were separated from undigested protein and the Fc fragments by protein A affinity chromatography. This was followed by a concentration step with a cation-exchange column using 20 m*M* citrate pH 3.5 as a running buffer and a 0–1 *M* NaCl gradient to elute the protein. At this stage, standard reducing SDS–PAGE showed two closely adjacent bands at 25 kDa, as expected for the light chain and the Fab portion of the heavy chain. Analysis on an isoelectric focusing gel revealed four different isoforms of the Fab fragment to be present in the mixture, with the isoforms having pIs of 9.3, 8.2, 7.4 and 6.9. Therefore, the protein mixture was extensively dialysed against 20 m*M* Tris–HCl pH 8.8, 0.02%(*w*/*v*) NaN_3 and loaded onto a UNO Q-1 anion-exchange column (Biorad). The different Fab isoforms were eluted with a 0–1 *M* NaCl gradient. The flowthrough of the column, which contained the isoforms with pIs 9.3 and 8.2, was dialysed again and ion-exchange column chromatography under the same conditions yielded a single pure isoform with a pI of 9.3, which was used in crystallization trials. This purification scheme is similar to those used for other Fab fragments; however, depending on the pI of the desired product the ion-exchange chromatography step has to be optimized for each individual Fab fragment.

The sample was concentrated to 8 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.8 using an Ultrafree-0.5 Centrifugal Filter Unit (Millipore). Prior to crystallization, the samples were filtered through a low-protein-binding 0.22 µm filter (Millipore) to remove dust particles and protein aggregates. The amino-acid sequence of this Fab fragment will be published elsewhere together with the BIAcore study (van Roon *et al.*, 2003). Determining the sequence of the Fab fragment was not straightforward and a detailed description of this procedure is beyond the scope of this crystallization paper.

2.2. Crystallization trials

Crystallization trials were carried out using the sitting-drop vapour-diffusion method at 295 K using equal volumes of protein and reservoir solutions. Crystals suitable for X-ray crystallography were grown from 1 µl of protein solution mixed with 1 µl of reservoir solution, which contained 23–26% polyethylene glycol 3350, 0.17 *M* ammonium sulfate and 15% glycerol. Crystals of a similar size could also be obtained from 15–18% polyethylene glycol 3350 in 50 m*M* Tris pH 8.

In order to grow crystals of the Fab fragment in complex with LewisX, crystallization trials were set up around the crystallization conditions for the native Fab fragment but now in the presence of 10–20 equivalents of LewisX. In almost all crystallization trials phase separation occurred and no crystals could be found. Therefore, crystals grown in the absence of LewisX were soaked for varying times in their crystallization buffer containing 50 m*M* LewisX. The crystals were either transferred to a new freshly equilibrated droplet containing 50 m*M* LewisX or 1 µl of a 100 m*M* LewisX solution was added directly to the droplet containing the Fab crystals prior to measuring X-ray diffraction data.

2.3. Data collection

The X-ray diffraction analysis of the Fab crystals was carried out at the X-ray crystallography beamline ID19 at Argonne National Laboratory, Argonne, Illinois using an ADSC Q310 CCD detector and at the European Synchotron Radiation Facility at beamline ID14-1 using an ADSC Q4 CCD detector. Crystals were either grown in the presence of cryoprotectants so they could be mounted directly from the crystallization droplet in cryoloops (Hampton Research) or were soaked for 5 s in a solution containing a cryoprotectant prior to mounting, followed by flash-freezing in a nitrogen-gas stream at 100 K. Data were collected using 1° oscillations and 240 images were recorded per data set. The collected data were indexed, integrated and scaled with *HKL*2000 (Otwinowski & Minor, 1997).

3. Results and discussion

The first attempts to crystallize the Fab fragment of monoclonal antibody 291-2G3-A used material that had only been purified on a protein A column after the papain digestion. Previously, it had been shown that the low ionic strength PEG 3350 screen as described by Harris *et al.* (1995) is a good starting point to find crystallization conditions for intact monoclonal antibodies and their fragments (Valjakka *et al.*, 2000). Therefore, this screen was set up and sea-

urchin-like clusters of crystals were found using 14–20% PEG 3350 and 50 m*M* Tris pH 8. However, after more extensive purification of the Fab fragment by anion-exchange chromatography, single crystals of dimensions up to 0.15 × 0.15 × 0.10 mm (Fig. 2*a*) could be grown using the same crystallization conditions. At the same time, Hampton Crystal Screen Cryo was set up and the condition containing 25.5% polyethylene glycol 4000, 0.17 *M* ammonium sulfate and 15% glycerol yielded small crystals. Replacement of PEG 4000 by PEG 3350 and varying its concentration between 23 and 26% yielded single crystals after several days (Fig. 2*b*). After two months, one crystal with a size of 0.35 × 0.3 × 0.25 mm was found that had grown from 26% PEG 3350, 0.17 *M* ammonium sulfate and 15% glycerol. From this crystal, a data set was recorded to 2.05 Å resolution. The Fab had crystallized in space group *P*1 (for a summary of the data processing, see Table 1).

Crystallization trials with the Fab fragment in the presence of 10–20 equivalents of LewisX resulted in phase separation. Various conditions in combination with Additive Screen 1 (Hampton Research) failed to co-crystallize the Fab fragment with LewisX. However, soaking experiments with LewisX proved more successful. Crystals grown from 25% polyethylene glycol 3350, 0.17 *M* ammonium sulfate and 15% glycerol were soaked in a pre-equilibrated crystallization condition containing either 50 or 100 m*M* LewisX. Although the quality of the crystals deteriorated, probably owing to the soaking, X-ray diffraction could still be measured from a crystal soaked overnight in 50 m*M* LewisX and the data could be processed. A summary of the statistics is given in Table 1. The unit-cell parameters of the crystal with LewisX are very similar to those of the crystal without LewisX except for a more elongated *b* axis, which is most likely to be a result of the soaking, as the crystal was grown under similar conditions as the native Fab fragment. The calculated V_M (Matthews, 1968) of the native Fab is 2.2 $Å^3\,Da^{-1}$ and that of the Fab–LewisX is 2.4 $Å^3\,Da^{-1}$, which corresponds to a solvent content of about 50%, assuming there to be four molecules in the asymmetric unit.

Furthermore, 100 m*M* LewisX solution was added to a droplet containing crystals that had grown from 14% PEG 3350 and 50 m*M* Tris pH 8. X-ray diffraction was measured from a crystal that was soaked with LewisX for half an hour. This crystal diffracted to 1.8 Å resolution and differed in all unit-cell dimensions compared with the crystals grown from 25% polyethylene glycol 3350, 0.17 *M* ammonium sulfate and 15% glycerol (Table 1). Since all the axes are smaller, the crystal only contains two Fab molecules in the asymmetric unit. The calculated V_M (Matthews, 1968) of this Fab–LewisX crystal is 2.5 $Å^3\,Da^{-1}$; this corresponds to a solvent content of 50%.

Optimization of the crystallization conditions for the Fab fragment, the soaking method and time led to a high-resolution data set of the Fab fragment of Mab 291-2G3-A in complex with its LewisX antigen.

The structure of the Fab fragment of Mab 291-2G3-A with and without LewisX was determined by molecular replacement using the Fab fragment of an anti-LewisY antibody (Jeffrey *et al.*, 1995) as a model. Clear density can be found for LewisX in the binding site in case of the Fab fragments that were soaked with its antigen. Model building and refinement are in progress.

We are very grateful to Mr René van Zeyl for the supply of large quantities of Mab 291-2G3-A. We are grateful for the support of Dr Norma Duke of the APS at Argonne, IL, USA during data collection. Use of the Argonne National Laboratory Structural Biology Center beamlines at the Advanced Photon Source was supported by the US Department of Energy, Office of Energy Research under contract No. W-31-109-ENG-38. Furthermore, we acknowledge the European Synchrotron Radiation Facility at Grenoble, France for provision of synchrotron-radiation facilities and we would like to thank Dr Pierre Legrand for assistance in using beamline ID 14-1.

Table 1
Crystal and diffraction data of the 291-2G3-A Fab fragment with and without LewisX.

Values for reflections in the last resolution shell are shown in parentheses.

	291-2G3-A Fab, unliganded	291-2G3-A Fab with LewisX	291-2G3-A Fab with LewisX
Crystallization conditions	26% PEG 3350, 0.17 *M* $(NH_4)_2SO_4$, 15% glycerol	25% PEG 3350, 0.17 *M* $(NH_4)_2SO_4$, 15% glycerol	14% PEG 3350, 50 m*M* Tris pH 8
Beamline	APS ID19	APS ID19	ESRF ID14-1
Wavelength (Å)	1.03	1.03	0.934
Space group	Triclinic *P*1	Triclinic *P*1	Triclinic *P*1
Unit-cell parameters			
a (Å)	67.4 ± 0.0004	67.4 ± 0.0020	45.1 ± 0.0003
b (Å)	71.6 ± 0.0006	72.4 ± 0.0019	60.8 ± 0.0005
c (Å)	104.8 ± 0.0008	104.8 ± 0.0046	91.6 ± 0.0009
α (°)	86.5 ± 0.0004	85.8 ± 0.0010	96.0 ± 0.0006
β (°)	71.3 ± 0.0007	71.3 ± 0.0007	95.4 ± 0.0002
γ (°)	83.3 ± 0.0002	83.3 ± 0.0035	101.8 ± 0.0002
Resolution (Å)	45–2.05 (2.12–2.05)	45–3.2 (3.31–3.20)	45–1.8 (1.86–1.80)
Measured reflections	256292	64458	951604
Unique reflections	103313	27423	87852
Average $I/\sigma(I)$	19.7 (2.1)	6.1 (1.6)	11.1 (2.2)
R_{merge}† (%)	4.6 (32.9)	14.6 (46.9)	9.4 (38.6)
Completeness (%)	88.1 (38.9)	87.0 (43.4)	92.9 (72.0)

† $R_{merge} = \sum(I - \langle I \rangle)/\sum(I)$ (*SCALEPACK* output).

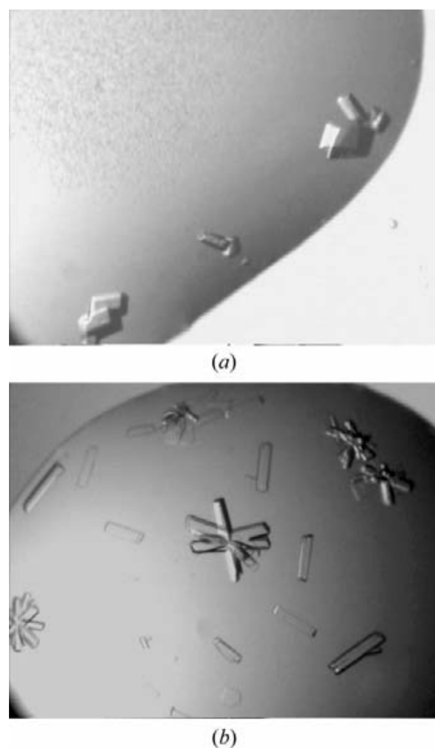

Figure 2
(*a*) Crystals of the Fab fragment grown in 14% PEG 3350, 50 m*M* Tris pH 8. (*b*) Crystals of the Fab fragment grown in 23.5% PEG 3350, 15% glycerol and 0.17 *M* ammonium sulfate.

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