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Structure of glutathione *S*-transferase 1 from the major human hookworm parasite *Necator americanus* (Na-GST-1) in complex with glutathione

Glutathione *S*-transferase 1 from *Necator americanus* (Na-GST-1) is a vaccine candidate for hookworm infection that has a high affinity for heme and metal porphyrins. As part of attempts to clarify the mechanism of heme detoxification by hookworm GSTs, co-crystallization and soaking studies of Na-GST-1 with the heme-like molecules protoporphyrin IX disodium salt, hematin and zinc protoporphyrin were undertaken. While these studies did not yield the structure of the complex of Na-GST-1 with any of these molecules, co-crystallization experiments resulted in the first structures of the complex of Na-GST-1 with the substrate glutathione. The structures of the complex of Na-GST-1 with glutathione were solved from pathological crystalline aggregates comprising more than one crystal form. These first structures of the complex of Na-GST-1 with the substrate glutathione were solved by molecular replacement from data collected with a sealed-tube home source using the previously reported apo structure as the search model.

1. Introduction

Human hookworm infection is a neglected tropical disease (NTD) that remains a major cause of anemia, malnutrition, generational poverty and underdevelopment for about one sixth of the world's population (Hotez, 2008, 2009, 2010; Musgrove & Hotez, 2009; Brooker *et al.*, 2008). Hookworm infection leads to lifelong health problems including neonatal prematurity, low birth weight and stunted physical and intellectual development in childhood (Musgrove & Hotez, 2009; Hotez, 2009; Brooker *et al.*, 2008). Current deworming and sanitation programs have not effectively controlled hookworm infection because of the high rates of reinfection following drug treatment, emerging drug resistance and the inadequate coverage of global treatment (Bundy *et al.*, 1995; Albonico *et al.*, 1995, 2003; Knopp *et al.*, 2012). As part of ongoing efforts to control hookworm infection, recombinant multivalent vaccines for hookworm infection are being investigated (Zhan *et al.*, 2005). Adult-stage vaccine antigens include the cytosolic glutathione *S*-transferases (GSTs).

In addition to having antigenic properties, hookworm GSTs have been shown to have a high affinity for heme and metal porphyrins. This is considered to be an adaptation related to the blood-feeding activity of hookworms. Hookworm GSTs belong to a superfamily of isoenzymes that catalyze the detoxification of various compounds and protect against peroxidative damage (Armstrong, 1991). GSTs function as homodimers that inactivate and extrude electrophilic substrates by the nucleophilic addition of reduced glutathione (Ketterer, 1988; Ketterer *et al.*, 1988). GSTs are the major detoxification system of hookworms, which lack cytochrome P-450 dependent enzymes (Brophy & Barrett, 1990; Precious & Barrett, 1989*a,b*). We previously solved the structures of the three GSTs from *Necator americanus* (Asojo *et al.*, 2007; Kelleher *et al.*, 2013). In order to understand the mechanism of heme detoxification by hookworm GSTs, we initiated co-crystallization and soaking studies with heme-like molecules, porphyrin and hematin. While our studies did not yield the structure of the complex of Na-GST-1 with any of these

molecules, co-crystallization experiments resulted in the first structures of the complex of Na-GST-1 with the substrate glutathione.

2. Materials and methods

2.1. Crystallization experiments

The recombinant expression and purification of Na-GST-1 were as reported previously (Zhan *et al.*, 2010). Crystals were grown as described previously (Asojo *et al.*, 2007). Briefly, the protein was concentrated to 15 mg ml⁻¹ in phosphate-buffered saline (PBS) or Tris-HCl pH 7.4 and crystals were grown at 298 K by vapor diffusion in sitting drops. Drops were prepared by mixing 3 µl protein solution with 1.5 µl reservoir solution consisting of 0.1 M sodium acetate pH 4.6, 30% PEG 400, from which crystals were obtained within two weeks. For soaking experiments, potential ligands were dissolved in the reservoir solution at a final concentration of at least 100 µM. Crystals were transferred into the soaking solution and incubated for a week. The compounds screened were protoporphyrin IX disodium salt, hematin and zinc protoporphyrin (Sigma-Aldrich). Soaking studies resulted in either a complete loss of diffraction or in apo structures of Na-GST-1 at a significantly lower resolution than those previously reported. The soaked crystals belonged to a different space group than that reported earlier (Asojo *et al.*, 2007) and an example is described in the Supporting Information¹. Consequently, all structural analyses were performed on co-crystals.

The best diffracting crystals were obtained from co-crystallization experiments with protoporphyrin IX disodium salt in sitting drops by mixing 3 µl protein solution with 1.5 µl reservoir solution consisting of 0.1 M sodium acetate pH 4.6, 40% PEG 400. The protein solution was 15 mg ml⁻¹ Na-GST-1 in 50 mM Tris-HCl pH 7.4, which was incubated with 100 µM protoporphyrin IX disodium salt for one week at 277 K. Within a week, what appeared to be inseparable stacked crystalline aggregates of at least two crystals were produced; the sample selected for data collection appeared to be two crystals with approximate dimensions of 0.035 × 0.21 × 0.25 and 0.035 × 0.18 × 0.46 mm.

2.2. Data collection

Data-collection experiments on the crystalline aggregates were carried out at 100 K using an Agilent SuperNova diffractometer equipped with a four-circle kappa platform, a 135 mm diagonal Atlas CCD detector and a high-brilliance sealed-tube (Cu) X-ray micro-source, with crystal-to-detector distances and exposure times that were optimized and determined using the *CrysAlis^{Pro}* strategy (v1.171.36.28; Agilent Technology, Oxford, England). Data were collected to ensure spot separation using four scan runs at a 115 mm crystal-to-detector distance with 0.5° frame width and exposure times of 40 s per frame. The four scan runs were collected at different kappa values, which were selected to ensure 100% completeness in the triclinic space group (see below). A total of 618 frames were collected over a total elapsed experiment time of 14 h 3 min. The longer than expected data-collection time was because the data collection included frame correlation, background correction and overflow re-measurements.

¹ Supporting information has been deposited in the IUCr electronic archive (Reference: SX5114).

3. Results and discussion

3.1. Data processing

Co-crystallization with protoporphyrin IX disodium salt resulted in crystalline aggregates that appeared to be distinct units, which always grew stacked on top of each other. The diffraction images suggest that there are reflections from multiple crystal forms in the crystal aggregate, and indexing in one lattice does not capture a significant subset of the reflections (Fig. 1). Three of these crystal forms account for two-thirds of all of the diffraction spots and could be indexed, whereas 32.4% of all of the diffraction spots could not be indexed and were rejected. The crystal forms were identified and data for each form were processed separately using the *CrysAlis^{Pro}* software package (Agilent Technologies, Oxford, England). All data were integrated using *CrysAlis^{Pro}*, which automatically processed and deconvoluted overlapping spots during integration. The deconvoluted reflections were not rejected.

Two of these crystal forms were triclinic, while the third belonged to the monoclinic space group C2. Additionally, the major crystal forms appear to be approximately aligned along their *c* axes. Crystal form 1 has unit-cell parameters $a = 45.2$, $b = 47.8$, $c = 103.1$ Å, $\alpha = 80.5$, $\beta = 78.7$, $\gamma = 62.0^\circ$. This crystal form comprised 39.1% of the total diffraction reflections (19.8% of the separated reflections and 19.4% of the overlapped reflections). The R_{merge} for this crystal form was 7%. Crystal form 2 is also triclinic and has unit-cell parameters $a = 45.0$, $b = 47.9$, $c = 104.9$ Å, $\alpha = 80.2$, $\beta = 78.3$, $\gamma = 61.8^\circ$. Crystal form 2 comprised 21% of the total diffraction reflections (6.3% of the separated reflections and 14.7% of the overlapped reflections). Although the unit cell of crystal form 2 was similar to that of crystal form 1, the R_{merge} for crystal form 2 is 21%, which makes it unsuitable for structure determination. The third crystal form identified from the crystalline aggregate is the monoclinic crystal form 3, which has unit-cell parameters $a = 84.6$, $b = 45.2$, $c = 113.8$ Å, $\beta = 112.1^\circ$. Crystal form 3 accounted for 34.9% of the total diffraction reflection spots (16.9%

Figure 1
Sample diffraction image from the crystal aggregates. The diffraction image reveals reflections that belong to more than one crystal form. Predictions for the triclinic space group are superposed on the image.

Table 1
Intensity statistics.

(a) Monoclinic crystal form.

Resolution (Å)	Reflections			Completeness (%)	Multiplicity¶	⟨I⟩	⟨I/σ(I)⟩	R _{int} ††	R _σ ‡‡
	Measured†	Theory‡	Unique§						
∞–5.65	3677	1217	1076	88.4	3.4	2588.40	20.47	0.087	0.040
5.75–4.58	3313	1150	1076	93.6	3.1	3430.31	19.17	0.082	0.046
4.58–4.00	3070	1126	1076	95.6	2.9	4754.90	19.91	0.088	0.045
4.00–3.65	2793	1110	1076	96.9	2.6	4102.25	15.89	0.090	0.057
3.65–3.39	2614	1113	1076	96.7	2.4	3490.59	12.67	0.091	0.068
3.39–3.19	2630	1094	1076	98.4	2.4	2541.32	10.06	0.098	0.087
3.19–3.04	2612	1093	1076	98.4	2.4	2026.58	8.37	0.103	0.105
3.04–2.91	2443	1091	1076	98.6	2.3	1533.70	6.81	0.112	0.131
2.91–2.80	2341	1095	1076	98.3	2.2	1273.59	5.82	0.112	0.150
2.80–2.70	2215	1116	1083	97.0	2.0	1067.40	5.00	0.124	0.174
∞–2.70	27708	11205	10767	96.1	2.6	2783.72	13.25	0.093	0.072

(b) Triclinic crystal form.

Resolution (Å)	Reflections			Completeness (%)	Multiplicity¶	⟨I⟩	⟨I/σ(I)⟩	R _{int} ††	R _σ ‡‡
	Measured†	Theory‡	Unique§						
∞–5.65	3808	2230	1737	77.9	2.2	2778.04	17.37	0.063	0.048
5.65–4.47	3502	2262	1737	76.8	2.0	3719.43	16.33	0.065	0.050
4.47–3.93	3167	2167	1737	80.2	1.8	4730.81	16.07	0.070	0.052
3.93–3.58	2783	2146	1737	80.9	1.6	4188.94	12.55	0.072	0.064
3.58–3.34	2521	2053	1737	84.6	1.5	3317.15	9.65	0.073	0.080
3.34–3.16	2364	1869	1737	92.9	1.4	2422.36	7.39	0.084	0.102
3.16–3.02	2264	1870	1737	92.9	1.3	1974.68	6.22	0.087	0.118
3.02–2.90	2210	1911	1737	90.9	1.3	1534.73	5.16	0.098	0.148
2.90–2.79	2136	1940	1737	89.5	1.2	1281.86	4.43	0.109	0.162
2.79–2.70	2119	2038	1745	85.6	1.2	1071.18	3.85	0.125	0.185
∞–2.70	26874	20488	17378	84.8	1.5	2872.81	10.94	0.072	0.071

† Total number of observed reflections. ‡ Total number of possible unique reflections. § Number of measured unique reflections. ¶ Multiplicity = measured/unique. †† $R_{int} = [\sum |F_o - F_o(\text{mean})|] / (\sum F_o)$. ‡‡ $R_\sigma = [\sum \sigma(F_o^2)] / (\sum F_o^2)$.

of the separated reflections and 18.0% of the overlapped reflections) and has an R_{merge} of 7%. The redundancy of the monoclinic form is lower than expected for 309° of data because while the data collection was optimized to maximize completeness for one of the triclinic crystal forms, it was not necessarily oriented for the other components of the crystalline aggregate. Although the reduced cells for the triclinic and monoclinic forms are similar, attempts at indexing the triclinic form in monoclinic diffraction symmetry resulted in an R_{merge} of 24% with unit-cell parameters $a = 84.5$, $b = 45.2$, 106.3 Å, $\alpha = 90$, $\beta = 108.5$, $\gamma = 90^\circ$. The intensity statistics for crystal forms 1 and 3 are shown in Table 1.

3.2. Structure determination and refinement

As previously reported, the expressed protein has 206 amino-acid residues (Asojo *et al.*, 2007). The structures of both crystal forms were solved by molecular replacement with *Phaser* (McCoy *et al.*, 2005; Storoni *et al.*, 2004). The molecular-replacement search model was a monomer of *Na-GST-1* (PDB entry 2on7) stripped of all ligands and water molecules (Asojo *et al.*, 2007). Molecular replacement was followed by iterative cycles of manual model building with *Coot* (Emsley *et al.*, 2010) and structure refinement with *REFMAC5* (Murshudov *et al.*, 2011) within the *CCP4* package (Winn *et al.*, 2011). The refined coordinates were subjected to *PDB-REDO* (Joosten, Salzemann *et al.*, 2009; Joosten, Womack *et al.*, 2009; Joosten *et al.*, 2012) to optimize the model and this was followed by several additional cycles of manual building to fix any poorly fitting regions that were introduced during *PDB-REDO*. The data for each crystal form are of sufficient quality to yield clear and unambiguous crystal structure solutions. The resulting structures did not have proto-

porphyrin IX ligand bound; instead, both crystal forms are complexes with glutathione.

All main-chain atoms have visible electron-density maps in the triclinic structure except for the N-terminal residue in two chains, while some of the side chains are disordered. In the monoclinic structure the loop connecting residues Gly111 through Asp116 is disordered as well as the N-terminal residue in one chain. Overall, the triclinic structure is of better quality than the monoclinic structure, as reported in Table 2. The refinement statistics are consistent with the low resolution of the data sets as well as the pathology of the crystalline aggregates used for data collection. Despite this, there is good agreement of the refined models with the electron-density maps (Fig. 3). While the monoclinic crystal form has a dimer in the asymmetric unit, the triclinic crystal has two dimers in the asymmetric unit (Fig. 2). After taking into account the unit-cell translations as well as symmetry operators, a dimer of dimers can be generated from symmetry mates of the monoclinic crystal form that can be superposed with the triclinic crystal form. When both dimers of dimers are compared, it appears that the greatest difference is in the loop connecting Gly111 through Asp116, which is partly disordered, perhaps owing to conformational flexibility (Fig. 2c). We previously reported multiple conformations of this loop in our structures of *Na-GST-1* and *Na-GST-2* (Asojo *et al.*, 2007).

3.3. Glutathione binding and the active site

Although no glutathione (GSH) was added to the crystallization mixture, unambiguous density for a glutathione molecule was observed in the GSH-binding site (G-site) of *Na-GST-1* (Fig. 3). Surprisingly, glutathione binding of *Na-GST-1* was only observed in the structures obtained from co-crystals and not those from the soaks

Table 2

Crystallographic data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Data set	Monoclinic C2	Triclinic P1
PDB code	4ofn	4ofm
Unit-cell parameters		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	84.55, 45.2, 113.76	45.20, 47.84, 103.12
α , β , γ (°)	90, 112.11, 90	80.54, 78.70, 61.99
Resolution (Å)	50–2.7 (2.8–2.7)	50–2.7 (2.8–2.7)
<i>R</i> _{merge} [†] (%)	7.1 (17.4)	7.2 (18.5)
No. of measured reflections	27708 (2119)	27708 (2119)
No. of unique reflections	10767 (1083)	17378 (1745)
Completeness (%)	96.1 (97.0)	84.8 (85.6)
Multiplicity	2.6 (2.0)	1.5 (1.2)
<i>I</i> / σ (<i>I</i>)	13.2 (5.0)	10.9 (3.8)
Refinement statistics		
Resolution (Å)	50–2.7 (2.8–2.7)	50–2.7 (2.8–2.7)
<i>R</i> _{cryst} [‡] (%)	29.2 (32.1)	22.1 (28.5)
<i>R</i> _{free} [§] (%)	33.0 (35.2)	26.7 (35.4)
No. of atoms in model		
Protein	3340	6728
Water	25	120
GSH	40	60
Mean <i>B</i> factor (Å ²)	10.7	13.5
R.m.s. deviation from ideal		
Bond lengths (Å)	0.006	0.012
Bond angles (°)	0.971	1.608
Chiral (Å ³)	0.055	0.110
Ramachandran plot (%)		
Favored regions	95.1	96.1
Allowed regions	4.4	3.3
Outlier regions	0.5	0.6

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ are the intensity of measurement *i* and the mean intensity of the reflection with indices *hkl*, respectively. [‡] $R_{\text{cryst}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$, where F_{obs} are observed and F_{calc} are calculated structure-factor amplitudes. [§] The R_{free} set uses a randomly chosen 5% of the reflections.

or from native crystals (Asojo *et al.*, 2007). The native crystals of the other *N. americanus* GSTs, *Na*-GST-2 (Asojo *et al.*, 2007) and *Na*-GST-3 (Kelleher *et al.*, 2013), had glutathione bound but no exogenous glutathione was added to any of the proteins. It is possible that glutathione was usurped during the fermentation process for all three enzymes. Overall, both the monoclinic and triclinic structures are similar to the previously reported orthorhombic apo crystal form of *Na*-GST-1 (Asojo *et al.*, 2007). When the main chains of the biological dimers of the structures are superposed with each of the previously reported *Na*-GST-1 dimers, the r.m.s.d. is approximately 0.6 Å. The largest differences between all of the structures are in loop regions as well as in the active sites to accommodate glutathione binding. The ability to bind glutathione is prevented by Gln50 in the apo *Na*-GST-1 structure (Asojo *et al.*, 2007). However, in the monoclinic and triclinic structures Gln50 adopts an alternative conformation which allows glutathione binding (Fig. 3). The interactions of the G-site residues with glutathione in *Na*-GST-1 are similar to those observed and reported in *Na*-GST-2 (Asojo *et al.*, 2007) and *Na*-GST-3 (Kelleher *et al.*, 2013). This is as expected since the residues forming the G-site are highly conserved in the three proteins (Kelleher *et al.*, 2013).

4. Concluding remarks

The active form of *Na*-GST-1 is a homodimer and all homodimers from the reported crystal structures are very similar. The pairwise superposition r.m.s. deviation for all main-chain atoms does not exceed 0.6 Å. Attempts at soaking and co-crystallizing *Na*-GST-1 with several ligands did not result in the binding of any of these ligands. Co-crystallization studies instead yielded the first structures

of *Na*-GST-1 in complex with the substrate glutathione. We have previously reported that *Na*-GST-1 is an efficient detoxifier of many molecules (Goud *et al.*, 2012; Asojo *et al.*, 2007), which may contribute to the inability of the tested ligands to stay bound to the enzyme. We noted significant structural similarity of the three *N. americanus* GSTs, *Na*-GST-1, *Na*-GST-2 and *Na*-GST-3. Since the *in vitro* enzymatic activity of *Na*-GST-2 and *Na*-GST-3 are lower than that of *Na*-GST-1 (Asojo *et al.*, 2007) these may be more suitable enzymes

Figure 2

Asymmetric unit of the monoclinic and triclinic crystal forms. Cartoon diagrams are shown of the crystallographic asymmetric unit of the (a) monoclinic and (b) triclinic crystal forms. In the monoclinic dimer one monomer is colored green while the second is colored cyan. The monomers are colored green, cyan, yellow and magenta for the triclinic tetramer. Glutathione (GSH) is shown as sticks in all monomers. (c) The symmetry-generated dimer of dimers from the monoclinic form (shown in green and aquamarine) is superposed with the dimer of dimers from the triclinic form in gray and reveals that the major difference lies in the loop region indicated by a red arrow.

Figure 3 Glutathione binding. Refined model of GSH placed into $F_o - F_c$ maps calculated from molecular-replacement phases at the 2.5σ contour level for (a) one of the monomers of the monoclinic structure and (b) one of the monomers of the triclinic structure with electron-density maps for bound GSH in blue. The fit of the refined model in the $REFMAC5$ -generated $2F_o - F_c$ maps calculated from the refined model in gray contoured at 1σ is shown for one of the monomers of (c) the monoclinic and (d) the triclinic structures. (e) Superposed G-site residues of structures of Na-GST-1 in complex with GSH (green) and without bound GSH (gray) reveal the conformational flexibility of Asn50 that occludes GSH binding; GSH from the former structure is colored magenta.

for structural studies of heme binding in hookworm GSTs. Alternative approaches may include generating ligands that will covalently bind to the active site.

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