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Crystallization and preliminary X-ray analysis of *Drosophila* glutathione S-transferase-2

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The σ-class glutathione S-transferase-2 (GST-2) from *Drosophila melanogaster* is predominantly found within the indirect flight muscles (IFMs), where it is bound to the 'heavy' subunit of the IFM thin filament troponin complex (Tn-H). An N-terminal extension found in GST-2 is unique within the σ GST class and may be involved in its interaction with Tn-H or modulate its enzymatic function. The recombinant protein has been crystallized at room temperature using ammonium sulfate as precipitant. Synchrotron radiation was used to measure a complete native data set to 1.75 Å resolution from flash-cooled crystals. The crystals belong to one of the trigonal space groups $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 89.7$, $c = 131.8$ Å. The self-rotation function is consistent with a GST-2 dimer in the asymmetric unit.

1. Introduction

Glutathione S-tranferases (GSTs) are a family of multifunctional enzymes which are mainly involved in the detoxification of harmful physiological and xenobiotic compounds. Based on their sequence and substrate specificity, at least six mammalian cytosolic classes have been identified and termed α, μ, π, θ, ζ (Hayes & Pulford, 1995; Board *et al.*, 1997) and ω (Board *et al.*, 2000). Additional classes, which may have specialized functions, are found in plants, fungi, fish, yeast and bacteria. σ-class GSTs are an intriguing group whose members are largely unstudied. They include the functional S-transferases from squid (Tomarev *et al.*, 1993), parasitic helminths (Hayes & Pulford, 1995) and vertebrates (Kanaoka *et al.*, 2000), as well as the enzymatically inactive S-crystallins from the eye lens of cephalopods (squid and octopus; Tomarev & Zinovieva, 1988).

Insect GSTs fall into two main classes: θ, which contains most isoforms, and σ. The sequence identity between members of the same insect class is usually high (>80%), whereas it is low (<30%) when compared with their mammalian homologues. Insect θ-class GSTs have been implicated in detoxification processes, in particular in conferring resistance towards various insecticides (Prapanthadara *et al.*, 2000; Syvanen *et al.*, 1994; Fournier *et al.*, 1992). The function of insect σ-class GSTs is less clear, although they have also been implicated in the acquisition of pesticide resistance (Cochrane *et al.*, 1992).

In *D. melanogaster*, the σ-class GST named glutathione S-transferase-2 (GST-2) has marginal sequence homology (less than 20%) to the θ isoform (GST-1), suggesting that the two classes have discrete biological functions. GST-2 from the housefly (*Musca domestica*) is almost 90% identical to the *Drosophila* protein and is found mainly in the indirect flight muscles (IFMs) of the thorax (Franciosa & Berge, 1995). The same sub-cellular localization was established by Clayton *et al.* (1998) for the GST-2 of *D. melanogaster*. The same study showed that approximately 90% of GST-2 is located in the IFMs *in vivo*, bound to the thin filaments *via* an interaction with Tn-H ('heavy troponin'; Bullard *et al.*, 1988), an insect-specific subunit of the troponin complex which regulates muscle contraction. Intriguingly, both Tn-H and GST-2 contain hydrophobic proline- and alanine-rich extensions with a similar repetitive motif. It is suspected that these extensions mediate the interaction of GST-2 and Tn-H. Alternatively, it is possible that Tn-H modulates the enzymatic activity of GST-2 *in vivo*, as in the case of hydrophobic polycyclic compounds whose binding affects the enzymatic activity of several other GSTs (Van der Jagt *et al.*, 1983; Boyer & Vessey, 1987; Nishihira *et al.*, 1992).

In an effort to gain insight into the function of GST-2 in the muscle, we have set out to determine the three-dimensional structure of GST-2. As an initial step, we have expressed, purified and crystallized GST-2 and subjected the crystals to X-ray crystallographic analysis.

2. Materials and methods

2.1. Protein expression and purification

A DNA fragment containing the GST-2 sequence originally cloned by Beall *et al.*

(1992) was kindly provided by Dr E. Fyrberg. The GST-2 coding sequence was modified by PCR using the primers DmGST2-F1, 5′-tag aag ctt **cat ATG** GCC GAT GAA GCA CAA GC-3′, and DmGST2-R1, 5′-gag ctt aa**g gat cc**T TAG ACC TCG GTG ACG CCC CG-3′ (upper case, coding sequences; bold, introduced sites), which introduced unique *Nde*I and *Bam*HI sites. The resulting fragment was then cloned into the *Nde*I and *Bam*HI sites of the expression vector pET-3a (Novagen). The sequence of the resulting clone matched that of the GST-2 locus in the recently completed *D. melanogaster* genome (249 amino acids; SPTREMBL accession code Q9V7Y4). This sequence differs from that originally published by Beall *et al.* (247 amino acids; SWISS-PROT accession code P1043) as a result of two frameshifts. The clone was used to transform *Escherichia coli* strain BL21(DE3) pLysS. For a typical production batch, freshly transformed bacteria were grown in 12 l of minimal medium supplemented with a casein amino-acid hydrolysate (Difco; recipe available upon request) at 310 K under well aerated conditions until they reached mid-log phase (OD_{600nm} = 0.6–0.8). GST-2 expression was induced with 0.4 m*M* IPTG (isopropyl β-D-thiogalactopyranoside) for 1.5 h. The level of protein expression was extremely low for a GST (usually of the order of 0.2–0.3 mg l^{-1}) despite repeated attempts to increase the yield. Cell pellets were resuspended in 200 ml of buffer *A* (20 m*M* sodium phosphate pH 7.0, 2 m*M* β-mercaptoethanol) plus 1 m*M* PMSF on ice and lysed using a French press. Lysates were centrifuged at 20 000*g* and the supernatant mixed in batch mode with 5 ml of glutathione agarose (Sigma) prewashed in buffer *A*. To ensure complete GST-2 binding on the column, the suspension was left rotating for 16 h at 277 K. Agarose beads were collected by centrifugation and washed extensively with 200 ml of buffer *A*. GST-2 was eluted with three column volumes of elution buffer [buffer *A* plus 15 m*M* reduced GSH (tripeptide glutathione, γ-Glu-Cys-Gly; Sigma)]. The pooled fractions contained pure protein as judged by SDS–PAGE and Coomassie blue staining. Prior to crystallization, GST-2 in elution buffer was concentrated by ultrafiltration (VivaSpin concentrators with a 10 kDa cutoff, VivaScience).

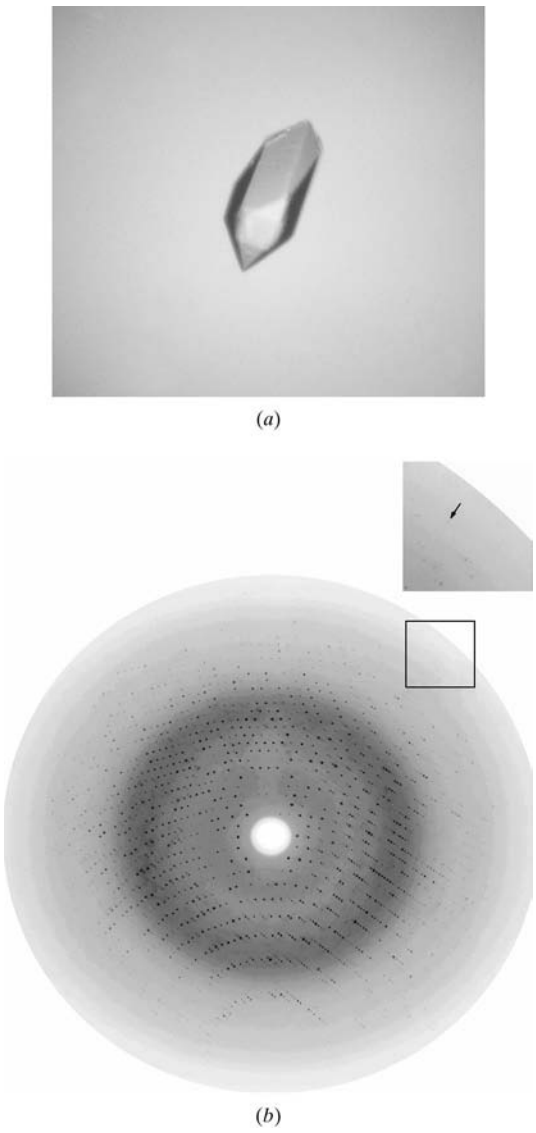

Figure 1
(*a*) A typical GST-2 crystal of dimensions 0.24 × 0.1 × 0.1 mm. (*b*) 1° oscillation diffraction image from a single GST-2 crystal recorded on a MAR Research 345 imaging plate. The crystal was measured on the EMBL X11 beamline (Hamburg). The arrow in the inset shows a reflection at 1.75 Å resolution.

2.2. Crystallization and data collection

The hanging-drop vapour-diffusion method was used to crystallize GST-2. Crystals were grown at room temperature (294 ± 2 K) by equilibrating mixtures containing 2 µl protein solution (7 mg ml^{-1} protein in 20 m*M* sodium phosphate pH 7.0, 2 m*M* β-mercaptoethanol, 15 m*M* GSH) and 2 µl reservoir solution (1.6–1.9 *M* ammonium sulfate, 0.1 *M* sodium phosphate pH 6.0–6.5) against 1 or 0.5 ml reservoir solution. Two native data sets were collected.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Data set	Nonius (294 ± 2 K)	X11 (100 K)
Space group	$P3_121$ or $P3_221$	$P3_121$ or $P3_221$
Unit-cell parameters (Å)	$a = b = 91.0$, $c = 133.2$	$a = b = 89.7$, $c = 131.8$
Resolution (Å)	3.1	1.75
No. of measured reflections	48239	623131
No. of unique reflections	11907	62202
Completeness (%)	98.9 (99.5)	99.5 (99.9)
$\langle I/\sigma(I)\rangle$	6.8 (2.54)	27.6 (9.14)
$I > 2\sigma(I)$ (%)	77.5 (53.1)	96.1 (85.6)
R_{sym}†	16.9 (50.4)	3.9 (23.8)

† $R_{sym} = \sum(\sum_i |I_i - \langle I\rangle|)/\sum\langle I\rangle$, where $\langle I\rangle$ is the mean of the intensity measurements I_i and the summation extends over all reflections.

For the first native data set, a single crystal was mounted in a capillary and data were collected at ambient temperature on an 18 cm MAR Research image plate attached to a Nonius rotating-anode generator providing Cu $K\alpha$ radiation. A search for suitable cryoprotectants was performed using this in-house setup, except that crystals were now transferred into solutions of candidate cryoprotectants, mounted on loops and flash-cooled in a nitrogen-gas stream at 100 K (Oxford Cryosystems). The best cryoprotectant was determined to be mother liquor saturated in sucrose.

The second native data set was collected at cryogenic temperatures (100 K) on the EMBL X11 beamline for macromolecular crystallography at the DESY synchrotron, Hamburg, Germany. The wavelength of the X-rays was 0.91 Å. The X-ray diffraction data were recorded on a MAR345 image plate at an oscillation range per image of 1°. Crystal mosaicity was 0.3°. Both native data sets were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

GST-2 crystals were obtained within one week and typically had a trapezoidal appearance (Fig. 1*a*). The X-ray data statistics for the two native data sets are given in Table 1. In-house diffraction patterns from GST-2 crystals mounted on capillaries at

ambient temperature were observed to a diffraction limit of 3.1 Å. The space group was determined to be $P3_121$ or its enantiomorph $P3_221$ using *DENZO* autoindexing and inspection of the systematic absences after data processing with *SCALEPACK*. The unit-cell parameters were $a = b = 91.0$, $c = 133.2$ Å. In the highest resolution shell, the data had $I/\sigma(I) = 2.54$ and a rather high $R_{\rm sym}$ of 50.4%. The unit-cell volume was 955 251 Å^3.

To improve the quality of the X-ray diffraction data obtained from GST-2 crystals, we collected data with synchrotron radiation at cryogenic temperatures using the EMBL X11 beamline (Table 1). The observed diffraction limit was 1.75 Å (Fig. 1*b*), substantially higher than that observed in-house. The data displayed a mean $I/\sigma(I)$ of 27.6 and an $R_{\rm sym}$ of 3.9, falling to 9.14 and 23.8, respectively, for the highest resolution shell (Table 1). The unit-cell parameters were $a = b = 89.7$, $c = 131.8$ Å, resulting in a unit-cell volume of 918 792 Å^3. These values correspond to a reduction of 3.97% in the unit-cell volume of GST-2 crystals upon flash-cooling.

GSTs are usually dimeric in solution; assuming two GST-2 molecules (249 residues per monomer with a calculated MW of 27613 Da) in the asymmetric unit, the $V_{\rm M}$ is 2.77 Å^3 Da^{-1} and the solvent content is 55.3% by volume, values which are well within the range observed in protein crystals (Matthews, 1968). The self-rotation function calculated from the cryogenic data using *POLARRFN* (Collaborative Computational Project, Number 4, 1994) at $\kappa = 180^\circ$ showed four peaks at 43% of the crystallographic peak height falling on the diagonals between the crystallographic *a* and *b* axes (Fig. 2). This self-rotation function is consistent with a non-crystallographic twofold axis at a projection angle of approximately 30° from axis *a* (or *b*), suggesting a GST-2 dimer in the asymmetric unit. The extra peaks [on the diagonals and one in the centre ($\omega = 0, \varphi = 0^\circ$; Fig. 2)] arise as the product of the local dyad with the crystallographic symmetry. Similar peaks were obtained from self-rotation functions calculated on the room-temperature data (not shown). No non-crystallographic peaks were found at $\kappa = 120^\circ$.

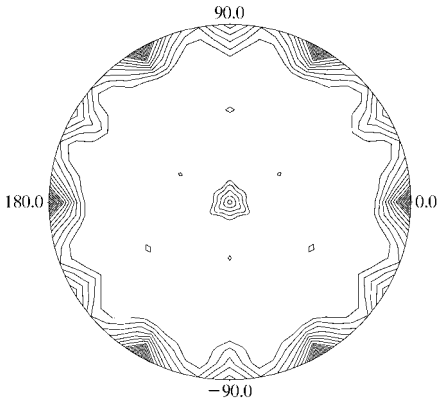

Figure 2
A stereographic plot of the self-rotation function from the GST-2 synchrotron native data set (25–4 Å) at $\kappa = 180^\circ$. Peaks corresponding to twofold non-crystallographic axes can be seen on the diagonals between the origin peaks. The plot was contoured at 5σ levels above 20σ.

To determine the structure, the molecular-replacement method was employed using the coordinates of squid σ GST (Ji *et al.*, 1995). The cross-rotation function calculated using the programs *AMoRe* (Collaborative Computational Project, Number 4, 1994) or *CNS* (Brunger *et al.*, 1998) failed to produce solution peaks significantly higher from noise. Nevertheless, the first 100 peaks from the cross-rotation were subjected to translation-function searches. To account for the known interdomain angular variability observed in GSTs, the orientation of the N- and C-terminal domains of the model was optimized using the PC-refinement option in *CNS* before translation runs. However, both *AMoRe* and *CNS* with PC refinement did not produce identifiable solutions. The use of the same model with loops trimmed or as polyalanine did not improve the molecular-replacement results. Therefore, we have initiated structure solution by the multiple isomorphous replacement method. Determination of the GST-2 structure, which would be the first insect σ GST structure to be reported, will undoubtedly help to clarify its putative roles in the IFM structure and function.

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