SHORT COMMUNICATIONS

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Expression, crystallization and preliminary X-ray analysis of ligand-free human glutathione S-transferase M2–2

LARYSA N. PATSKOVSKA, ALEXANDER A. FEDOROV, YURY V. PATSKOVSKY, STEVEN C. ALMO AND IRVING LISTOWSKY* *at Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, USA. E-mail: irving@aecom.yu.edu*

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Abstract

Human glutathione-S-transferase M2–2 (hGSTM2–2) was expressed in *Escherichia coli* and purified by GSH-affinity chromatography. The recombinant enzyme and the protein isolated from human tissue were indistinguishable based on physicochemical, enzymatic and immunological criteria. The catalytically active dimeric hGSTM2–2 was crystallized without GSH or other active-site ligands in two crystal forms. Diffraction from form *A* crystals extends to 2.5 Å and is consistent with the space group $P2_1$ (a = 53.9, b = 81.5, c = 55.6 Å, β = 109.26 Å) with two monomers in the asymmetric unit. Diffraction from form *B* crystals extends to 3 Å and is consistent with a space group $P2_12_12_1$ (a = 57.2, b = 80.7, c = 225.9 Å) with two dimers in the asymmetric unit. This is the first report of ligand-free mu-class GST crystals, and a comparison with liganded complexes will provide insight into the structural consequences of substrate binding which are thought to be important for catalysis.

1. Introduction

Glutathione S-transferases (GST's, E.C. 2.5.1.18) are a group of enzymes that catalyze glutathione (GSH) conjugation to various xenobiotics and natural metabolites including some mutagens, carcinogens and certain antitumor drugs. As a result of the formation of GSH conjugates, reactive compounds are detoxified and excreted (Hayes & Pulford, 1995). Based on sequence homologies and other biochemical similarities, the mammalian GST's have been classified as alpha, pi, mu, theta and microsomal forms (Mannervik *et al.*, 1992). Five different subtypes of human mu-class GST's subunit [M1(*a* or *b*), M2, M3, M4, and M5] have been described in the literature (Hayes & Pulford, 1995). Mammalian GST's usually exist in the form of homo- or heterodimers assembled between subunits of the same class but not between subunits of different classes. Despite overlapping substrate specificities and homologies in their tertiary structure, each subunit type has distinct catalytic properties that may reflect differences in the structure of their substrate-binding sites. GST's naturally form homo- or heterodimers in solution, but each subunit of the dimer appears to be catalytically independent.

Members of the pi, mu, alpha and theta classes of GST's have been crystallized in the presence of glutathione or certain GSH conjugates (Dirr *et al.*, 1994; Wilce *et al.*, 1996; Wilce & Parker, 1994; Sinning *et al.*, 1993; Wilce *et al.*, 1995). The structures of unliganded human GSTA1–1 and GST from *Schistosoma japonica* have been published recently (Cameron *et al.*, 1995; McTigue *et al.*, 1995). For the mu class, only the liganded structures of the rat GST3–3 (currently named the rGSTM1–1) (Ji *et al.*, 1992; 1994; Xiao *et al.*, 1996) and a mutant form of human hGSTM2–2 (F214W) (Raghunathan *et al.*, 1994) have been studied. The crystal structure of the mutant hGSTM2–2 (F214W) complexed with glutathione has been solved in three orthorhombic crystal forms containing a monomer, dimer or tetramer per asymmetric unit to resolutions of 1.85, 3.5 and 2.5 Å, respectively (Raghunathan *et al.*, 1994). To date, there are no published reports of crystal structures of mu class GST's without GSH or substrate analogues bound, so it remains unclear how substrate binding may affect the conformation of the binding sites of GST's.

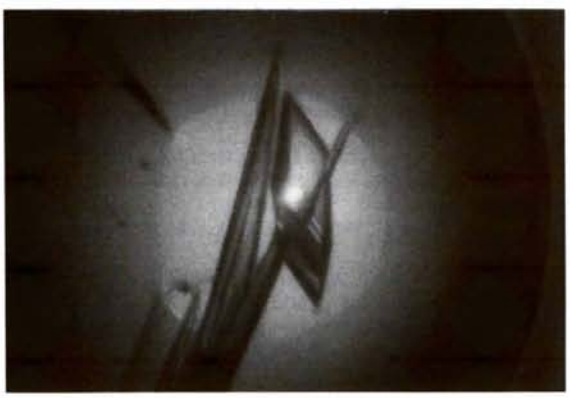
We report the crystallization of the unliganded hGSTM2–2 in two different crystal forms; the monoclinic form *A* with two monomers in the asymmetric unit and the orthorhombic form *B* with two dimers in the asymmetric unit. These crystals diffract to a resolution of 2.5 and 3.0 Å, respectively, and a comparison with ligand complexes will allow for a detailed description of structural rearrangements which occur upon ligand binding.

2. Experimental

The hGSTM2–2 cDNA was amplified using RT-PCR with the following specific primers: sense CCA ACC AGC CAT ATG CCC ATG ACA CTG GGG TAC, containing a restriction site for *Nde*I, and reverse GCC TTC AGG ATC CTA CTT GTT GCC CCA GAC AGC CA, carrying a *Bam*HI restriction site. Total RNA was purified from HeLa cells using a QIAGEN purification kit according to the manufacturer's instructions. The reverse oligo served as a primer for reverse transcriptase (RAV-2, Amersham) and the ensuing cDNA was used as a template in the PCR procedure (367 K, 1 min; 328 K, 1 min; 345 K, 1 min; 35 cycles). The purified PCR product as well as the pET3a expression vector were digested with *Nde*I/*Bam*HI, ligated and transformed into *E. coli* strain BL21(DE3) (Novagen). Clones expressing hGSTM2–2 were identified by dot-hybridization using hGSTM2 specific antiserum (Rowe *et al.*, 1997). Recombinant plasmid DNA was then purified and sequenced in both directions using an Applied Biosystems automated sequencer (Perkin-Elmer). An *E. coli* clone carrying the pET3a–GSTM2 recombinant plasmid was used for expression of recombinant hGSTM2–2. In brief, the overnight culture of bacterial cells was diluted in a ratio 1:100 with fresh LB medium and grown until an OD_{540} = 0.1–0.2 was

attained. The synthesis of the recombinant GST was induced by addition of 1m*M* IPTG and then the incubation was prolonged for 6 h by shaking at 310 K. *E. coli* cells were collected by centrifugation and disrupted by sonication in the loading buffer. The expressed protein as well as a cytosolic fraction of human brain GST's were purified by GSH affinity chromatography as described earlier (Campbell *et al.*, 1990). The purity of the recombinant hGSTM2–2 was tested by non-denaturing and denaturing electrophoresis in 12% polyacrylamide gels, or by isoelectrofocusing (pH 3–7) ready-to-use 5% polyacrylamide gels (Novex). Gels were either stained with Coomassie Blue or prepared for immunoblotting. The hGSTM2–2 enzymatic activity was measured using 1m*M* GSH and 1m*M* 1-chloro-2,4-dinitrobenzene (CDNB) as substrates (Jenson *et al.*, 1985). The recombinant hGSTM2–2 as well as total cytosolic GST's isolated from a human brain were analyzed by reversed-phase high-pressure liquid chromotography (HPLC) using a Hewlett Packard HPLC 1090 system and a Vydac C4 column, as recently described (Rowe *et al.*, 1997). HPLC fractions were collected manually and used directly for mass spectrometry or for western-blot analyses. Mass spectral analysis was performed using an API-III triple-quadropole mass spectrometer (PE-SCIEX, Ontario, Canada).

(*a*)



(*b*)

Fig. 1. (*a*) Diamond-shaped form *A* crystals under polarized light. The size of the largest crystal in the photograph is approximately 0.3 × 0.2 × 0.2 mm. (*b*) Photograph of rod- and needle-shaped form *B* crystals. The size of the central crystal is approximately 0.4 × 0.2 × 0.2 mm.

Crystals of hGSTM2–2 were grown by hanging-drop vapor diffusion. Frozen samples of the enzyme were thawed and concentrated with Filtron MICROSEP 10K concentrators (Filtron Tech.Corp., Northborough, MA) to 15–31 mg ml^{-1} in 10 m*M* Tris–HCl (pH 7.5) containing 0.2 m*M* DTT. 2 µl of this solution was mixed on a cover slip with an equal volume of a precipitant solution and inverted over sealed reservoirs containing 1 ml of the precipitant solution. A preliminary broad crystallization screen was performed using a commercially available Hampton Crystal Screen I kit (Hampton Research, Laguna Hills, CA). Preliminary studies identified several distinct crystallization conditions under which small needle-like protein crystals were observed over pH ranges from 4.6 to 8.5, in the presence of PEG 4000 and PEG 8000 as precipitants. Based on these findings, additional experiments were conducted using 0.1 *M* Tris–HCl (pH 8.5) with 18–24% PEG 4000 as a precipitant, or 0.1 *M* Na-cacodylate buffer (pH 6.5) containing 12–16% PEG 8000 as a precipitant at 289 K. In the first set of buffer solutions small (not larger than 0.03–0.05 mm) diamond-shaped form *A* crystals usually appeared after 3–5 d and slowly continued to increase the size (up to 0.1 mm of length) for 2 weeks. The optimal condition for the growth of form *A* crystals contained buffer with 22% of PEG 4000, but the average size of crystals was not larger than 0.05 × 0.1 × 0.1 mm. The small crystals were used for seeding procedures as follows: individual crystals or several crystals together were transferred to fresh drops, each containing 2 µl of the protein solution (22–31 mg ml^{-1}) and 2 µl of a precipitant solution (0.1 *M* Tris–HCl, pH 8.5; 22% PEG 4000, 0.1% NaN_3). Form *A* crystals continued to increase in size (usually up to 0.5 mm of length) for 3–7 d (see Fig. 1*a*) and retained their shapes and sizes for at least two months after the seeding procedure.

The second set of conditions [0.1 *M* Na-cacodylate (pH 6.5), 12–16% PEG 8000, 0.1% NaN_3, 289 K] produced needle- and rod-shaped form *B* crystals (see Fig. 1*b*) which usually appeared after 1–3 d and grew to their final size (about 0.1 × 0.1 × 0.5 mm) over an additional 2–3 d. These crystals are stable for 1–2 months at constant ambient temperature.

The stability of the crystallized hGSTM2–2 protein was tested by electrophoretic analyses and by enzymatic activity measurements of protein obtained from the crystals that were washed with their reservoir solutions and then dissolved by addition of 10 m*M* Tris–HCl (pH 8.5) buffer, containing 0.2 m*M* DTT (dithiothreitol).

3. Results and discussion

The molecular mass of the recombinant hGSTM2 subunit determined by ESI-MS was identical to that purified from human tissue (25 617 ± 2) and to the predicted value based on the sequence deduced from the cDNA (Vorachek *et al.*, 1991). Thus, there are no natural post-translational modifications of the hGSTM2 subunit. No differences between the expressed and natural enzyme have been observed in electrophoretic mobilities on PAGE, retention times on HPLC, determined values of isoelectric points (p*I* 5.3) and specific activities with CDNB as a substrate (328 ± 42 µmol min^{-1} mg^{-1}). The recombinant hGSTM2–2 may thus be considered to be identical to the authentic human enzyme. The protein also had no residual CDNB conjugating activity in the absence of GSH even if the assay was conducted with high concentrations of

enzyme (up to 1 mg ml^{-1}). Thus, virtually all residual GSH was eliminated, allowing for the unliganded form of the protein to be crystallized. The form *A* crystals were found to be stable after over two months at room temperature in the presence of 0.2 m*M* DTT and 0.1 m*M* NaN_3 which prevents the oxidation and degradation of the enzyme. Under these conditions, the recombinant hGSTM2-2 retained enzymatic activity, and had the same electrophoretic mobility (data not shown).

Diffraction data to 2.5 Å were collected for form *A* crystals at room temperature using a Rigaku RU-200 rotating-anode X-ray source operating at 50 kV and 80 mA, coupled to a Siemens X-1000 multiwire area detector. All data were reduced (R_{merge} = 5.1%) with *XDS* (Kabsch, 1988). According to the analysis of X-ray diffraction patterns, the form *A* monoclinic crystals have the unit-cell parameters a = 53.9, b = 81.5, c = 55.6 Å, β = 109.26°. In the highest resolution shell the completness of the data was 48% and $I/\sigma(I)$ was 4.2. The analysis of the data integrated in a low-symmetry space group *P*1, showed that the pattern of systematically absent reflections was consistent with the space group $P2_1$. The unit-cell volume was 228 125 $Å^3$. The molecular weight of each subunit is 25 617, and assuming two monomers per asymmetric unit, a V_m ratio (volume/protein molecular weight) of 2.23 $Å^3$ Da^{-1} was obtained, corresponding to a solvent content about 45%. These values fall within the expected range for globular proteins (Matthews, 1968). The presence of two molecules per asymmetric unit was confirmed by calculating a self rotation function using the programs *GLRF* (Tong & Rossman, 1990) and *X-PLOR* (Brünger, 1990). The solution for self-rotation is a single peak with polar angle φ = 165, ψ = 42, and κ = 180°. Attempts are under way to solve the monoclinic crystal structure by molecular replacement using human GSTM2-2(F214W) models available from the previously described orthorhombic crystal form of the complex with glutathione (Raghunathan *et al.*, 1994).

Form *B* crystals grew much faster than form *A* and usually appeared on the second day after initiation of the crystallization procedure in the form of long (up to 1 mm) rods and thick needles with an average size 0.1 × 0.1 × 0.5 mm (see Fig. 1*b*). Diffraction data to a 3.0 Å resolution were collected (R_{merge} = 9.4%), and in the highest resolution shell the completness was 39% and $I/\sigma(I)$ was 3.4. Following the same procedure of analysis described above, diffraction from form *B* crystals is consistent with space group $P2_12_12_1$ with unit-cell parameters a = 57.2, b = 80.7, c = 225.9 Å. A similar orthorhombic crystal form with four monomers in the asymmetric unit, was previously described for a hGSTM2-2(F214W) glutathione complex. The parameters (a = 56.9, b = 79.7 and c = 220.1 Å) (Raghunathan *et al.*, 1994) of the mutant complex crystals show significant differences compared to the ligand-free crystals reported here. It is anticipated that conformationally responsive elements in the enzyme will be identified by comparison of the unliganded structure with enzyme-inhibitor complexes.

The reported crystal structure of the hGSTM2-2(F214W) glutathione complex was found to have high temperature-factor values determined for C-terminal portion of the protein (residues 202–217), possibly because the point mutation Phe214→Trp affected this part of the protein (Raghunathan *et al.*, 1994). It had been also predicted from the crystal structure of rGSTM1-1 complexes (Xiao *et al.*, 1996) that the C termini of mu class GST's are involved in the formation of the substrate-binding site, especially for aromatic compounds. Previously reported data on the crystal structures of the C-termini of hGSTM2-2(F214W) and rGSTM1-1 may reflect different structures of their substrate-binding sites. It is, therefore, important to compare the structure of the ligand-free enzyme with those obtained in the presence of substrates or analogues. The solution of the present crystal structures should help to resolve these issues.

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