

Preliminary crystallographic studies on glutamine synthetase from *Mycobacterium tuberculosis*

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The etiologic agent of tuberculosis, *Mycobacterium tuberculosis*, has been shown to secrete the enzyme glutamine synthetase (TB-GS) which is apparently essential for infection. Four crystal forms of a recombinant TB-GS were grown. The one chosen for synchrotron X-ray data collection belongs to space group $P2_12_12_1$ with unit-cell dimensions $208 \times 258 \times 274 \text{ \AA}$, yielding 2.4 \AA resolution data. A Matthews number of $2.89 \text{ \AA}^3 \text{ Da}^{-1}$ is found, corresponding to 24 subunits of molecular mass 1300 kDa in the asymmetric unit. From earlier work, the structure of *Salmonella typhimurium* GS, which is 51% identical in sequence to TB-GS, is known to be dodecameric with 622 symmetry. Self-rotation calculations on the TB-GS X-ray data reveal only one set of sixfold and twofold axes of symmetry. A Patterson map calculated from the native X-ray data confirms that there are two dodecamers in the asymmetric unit, having both their sixfold and twofold axes parallel to one another.

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1. Introduction

Tuberculosis (TB) is the leading cause of death by a single infectious agent, each year emerging as an active disease in eight million humans and causing three million deaths (Kochi, 1991). Existing antimycobacterial drugs are becoming less effective because of the rise in resistant strains (Centers for Disease Control, 1991). Discovery of novel drug targets is complicated by the need to penetrate the thick cellular envelope of mycobacteria. However, pathogenic mycobacteria may have an Achilles heel: they secrete enzymes into their local environment, which may be essential for growth (see Young *et al.*, 1990, for a review). One enzyme of particular interest is glutamine synthetase (TB-GS), product of the *glnA* gene from *M. tuberculosis* (Harth *et al.*, 1994). TB-GS is secreted into the medium, where it is thought to play a role in the synthesis of poly-(L-glutamine-L-glutamate) chains (Harth *et al.*, 1994), a constituent unique to pathogenic mycobacterial cell walls, which comprise 10% of the bacterial mass (Hirschfield *et al.*, 1990). These chains have been reported to be tightly associated with the peptidoglycan layer of the cell wall. Thus, inhibition of secreted TB-GS may disrupt normal cell-wall development and consequently the organism's growth, suggesting that secreted TB-GS is an attractive target for TB-specific drugs.

In microorganisms, glutamine synthetase (GS) is typically a cytoplasmic enzyme central to nitrogen metabolism, catalyzing the ATP-dependent condensation of ammonia and

glutamate to form ADP, glutamine and free phosphate (Woolfolk *et al.*, 1966). The X-ray crystal structure of GS from *Salmonella typhimurium* has been reported at 3.5 \AA resolution (Almasy *et al.*, 1986) and subsequently refined to 2.9 \AA (Liaw & Eisenberg, 1994). *S. typhimurium* GS molecules are dodecamers, having two face-to-face hexameric rings, with 12 symmetrically related active sites, one formed between every pair of subunits within a ring. Protein-sequence alignments show a 51% identity between *S. typhimurium* GS and TB-GS, suggesting a common tertiary fold for the two molecules. As the first step in the

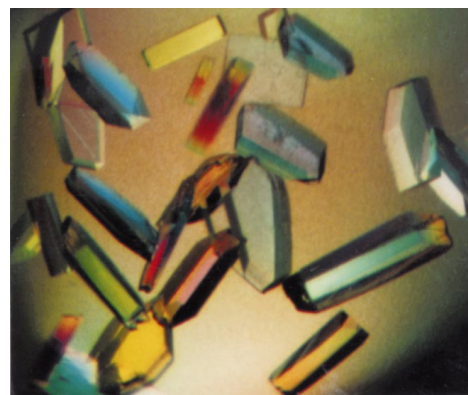


Figure 1

Crystals of recombinant TB-GS. Two crystal forms of rTB-GS grew in a single crystallization drop. The multi-sided chunky crystals belong to space group $P2_12_12_1$ (form 2) and the rod-shaped elongated crystals can belong either to space group $P2_12_12_1$ or space group $P2_1$ (form 2 or 1). Dimensions vary from 0.5 to 1 mm in each dimension.

determination of the three-dimensional structure of TB-GS, we report here the crystallization of a recombinant (r) TB-GS and show that rTB-GS has the same fold and adopts the same quaternary structure.

2. Materials and methods

2.1. Purification of rTB-GS

TB-GS was expressed in an *Escherichia coli* recombinant system, as will be described elsewhere. The rTB-GS was purified by the procedure described in Woolfolk *et al.* (1966) for *E. coli* GS isolation, omitting the following steps: heat treatment, acetone

precipitation and the second ammonium sulfate precipitation. An Affigel-blue (Biorad 100–200 mesh) affinity column was used as the final purification step, as described by Janson *et al.* (1984) for *S. typhimurium* GS isolation. This was followed by dialysis against 15 mM imidazole (pH 7) and 2.2 mM MnCl₂ until all nucleotide from the elution buffer was removed, as judged spectrophotometrically (Stadtman *et al.*, 1979).

2.2. Crystallization of rTB-GS

TB-GS crystals used in data collection were grown at room temperature in 3–4 d by

the hanging-drop vapor-diffusion method. The volume of the well solution was 1 ml and the solution consisted of 6.5% (w/v) polyethylene glycol 4000 (EM Science 9727-2), 100 mM sodium citrate, 1 M sodium chloride. The total volume of each drop was 4 µl and contained an equal mixture of well solution and a 70–110 mg ml⁻¹ stock of enzyme, having a final pH of 5.5. These crystallizing conditions are distinct from the conditions used for crystallizing *S. typhimurium* GS (Liaw & Eisenberg, 1994).

2.3. X-ray data collection and processing

Cryogenic data from the four crystal forms were initially recorded on a R-AXIS IV detector with a rotating-anode X-ray generator. Synchrotron-generated data were subsequently obtained from crystal form 2 (see Table 1) at the National Synchrotron Light Source (NSLS) X12B beamline using the same frozen crystal which had been used for data collection on the rotating-anode generator. The data used in this work were recorded with the (2 × 2) Quantum 4 CCD detector (Area Detector Systems Corporation) and processed with an updated version of the program *MOSFLM* (Leslie, 1992). The oscillation angle was 0.5° and each frame was exposed for 2 min. The crystal-to-detector distance was set at 220 mm. A 90° oscillation yielded complete data within 23 h.

3. Results and discussion

3.1. Crystal forms of rTB-GS

Four crystal forms of rTB-GS were grown and characterized, as shown in Table 1. Crystal form 3 has the smallest unit-cell volume but only diffracted to 3.3 Å resolution, with weak signal-to-noise ratios for the intensity measurements. Consequently, we focused on crystal forms 1 and 2 (Fig. 1). Upon optimization of growth conditions, crystal form 2, with two molecules per asymmetric unit in space group *P*₂₁₂₁, was

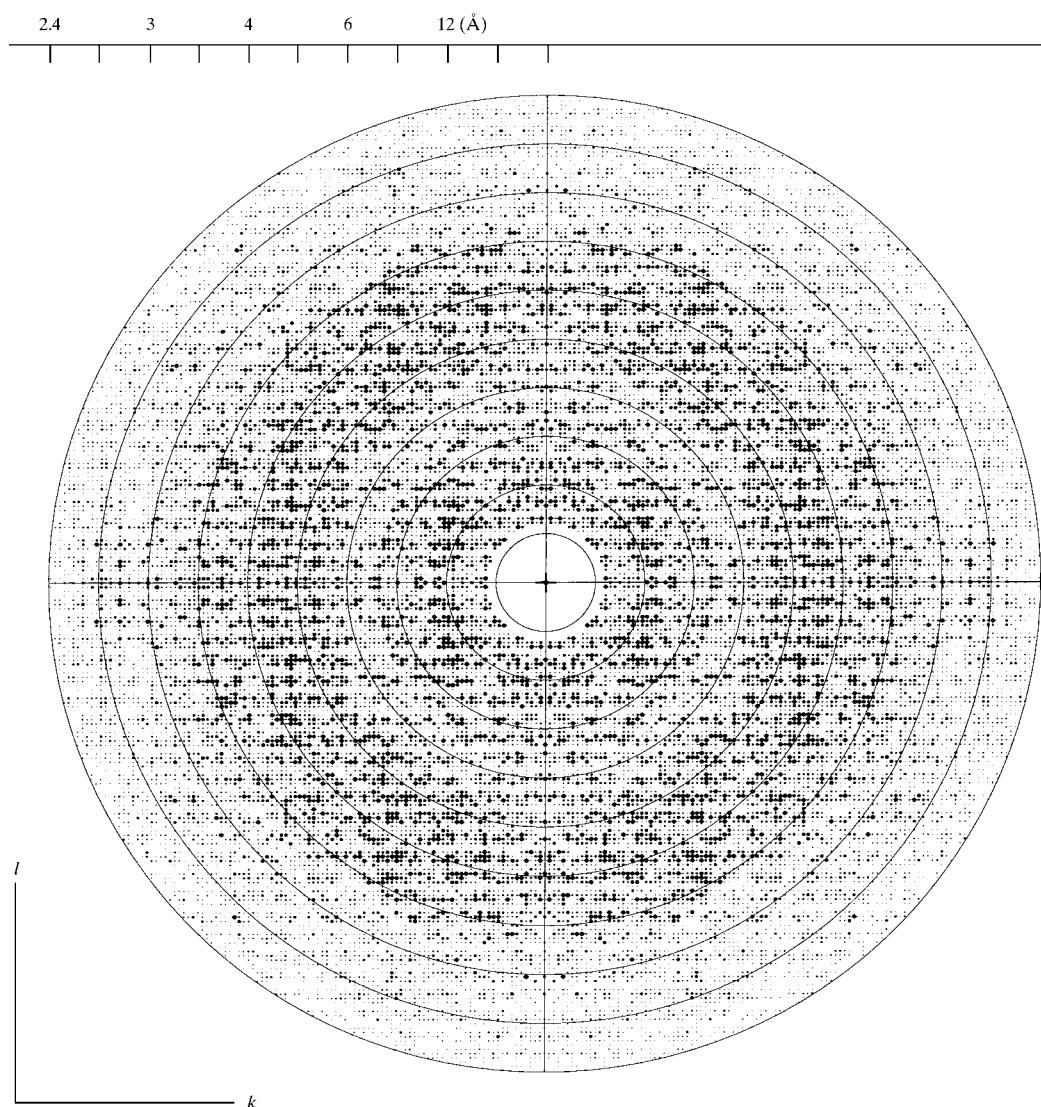


Figure 2

0kl section of the reciprocal lattice. The *c** axis is vertical; the *b** axis is horizontal. This section was simulated with the program *PLOTHKL* (Pfuegl, 1993) from full three-dimensional data. X-ray diffraction data to 2.4 Å resolution were collected from a single form 2 crystal at 90 K. The data set is 99.6% complete and consists of 2 600 000 observations which reduced to 566 370 unique reflections with an R_{sym} [$R_{\text{sym}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$], where $I_i(hkl)$ is the *i*th measurement and $\langle I(hkl) \rangle$ is the mean of all measurements of $I(h)$ for Miller indices h, k, l] of 7.5% and an average signal of 4.5σ above the background. The *0kl* section contains 37 974 reflections and suggests by its strength at the edge that the diffraction extends well beyond 2.4 Å resolution. The screw axes of the *P*₂₁₂₁ space group are shown by the observed systematic absences of the form $2n + 1$ along the *0k0* and *00l* lines.

Table 1
Characterization of rTB-GS crystal forms.

Crystal form	Space group	Resolution (Å)	Cell parameters (Å, °)	V_M (Å ³ Da ⁻¹) (n)†
1	$P2_1$	2.8	$120 \times 259 \times 239$, $\beta = 94.4$	2.9 (2)
2	$P2_12_12_1$	2.8 (2.4‡)	$208 \times 258 \times 274$	2.9 (2)
3	$C2$	3.3	$131 \times 224 \times 194$, $\beta = 91.7$	2.2 (1)
4	Rhombohedral	5	$420 \times 420 \times 420$	3.2 (4)§

† n is the number of dodecameric molecules in the asymmetric unit; V_M is Matthews number (Matthews, 1968). ‡ Refers to data collected at the National Synchrotron Light Source X12B Beamline. § Assumes $R3$ space group with hexagonal axes.

chosen for data collection. The data-collection statistics are given in Fig. 2.

3.2. Self-rotation function

If the quaternary structure of rTB-GS is in fact the same as *S. typhimurium* GS, which has 622 symmetry, we expect peaks for a self-rotation function at κ sections of 60, 120 and 180°. The self-rotation function (Rossmann & Blow, 1962) indeed contains strong

non-crystallographic peaks for κ values of 60 and 120°, with φ and ω values of 0 and 90°, respectively (Fig. 3). The peaks for the sixfold and threefold rotation axis are 6.3σ above the mean, with the highest noise peak being 1.7σ . Since we find peaks only at $\kappa = 60, 120, 180^\circ$ for rotation about the a axis, the relationship among the protein chains is a sixfold symmetry. We also find six dyads perpendicular to a hexad, approximately equally spaced on or near to the bc plane.

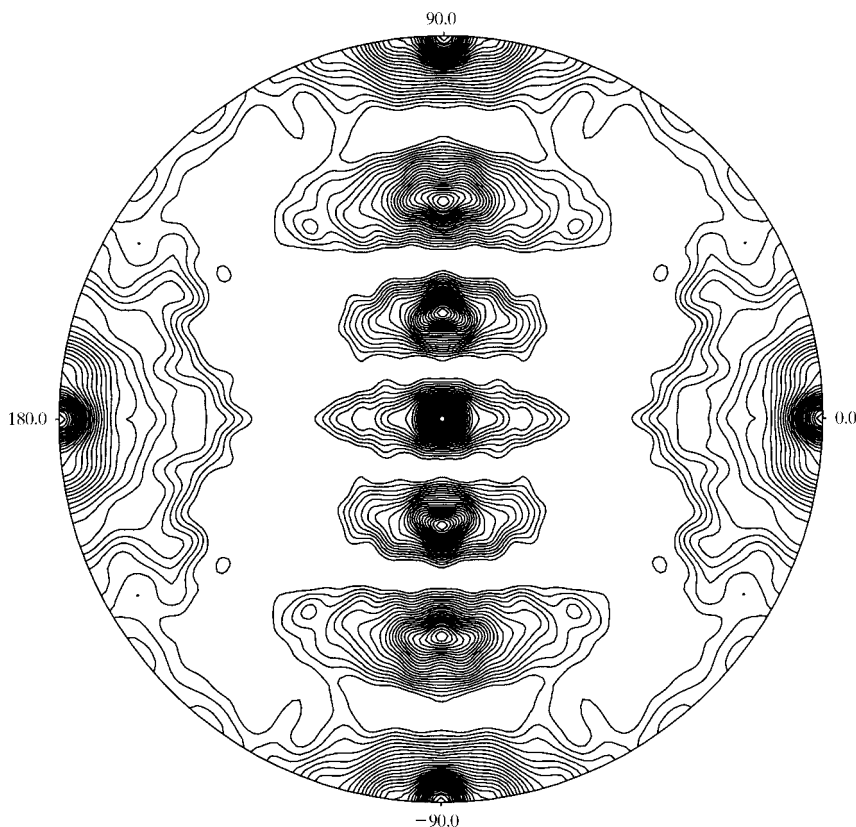


Figure 3

The $\kappa = 180^\circ$ section of the self-rotation function. Self-rotation functions (Rossmann & Blow, 1962) were calculated with the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994). The self-rotation function is described in terms of the rotation angle κ and the spherical polar coordinates ω and φ . φ is the angle between the x axis and the projection of the rotation axis onto the xy plane and ω is the angle between the rotation axis and the z axis, with x horizontal and y vertical. A resolution range of 15–4 Å and Patterson space integration radii of 5, 10, 15, 20, 25, 30, 35, 40 and 50 Å were explored, and the rotation angle κ was sampled every 5°. The Patterson integration radius of 50 Å gave stronger peaks than smaller Patterson integration radii. Six apparent dyads are seen in the figure, lying along a great circle, and spaced approximately 30° from one another in ω . One noncrystallographic twofold axis at $(\omega, \varphi) = (90, 0)$ is orthogonal to each dyad, and is masked by one of the crystallographic twofold axes.

These six dyads correspond to six twofold axes of symmetry of rTB-GS, thereby revealing the entire 622 symmetry.

3.3. The packing of rTB-GS molecules in the crystal

The molecular arrangement within the asymmetric unit can be deduced from the number of dodecamers per asymmetric unit and by the self-rotation function. The data in Table 1 indicate two GS dodecamers with a combined mass of 1300 kDa per asymmetric unit for crystal form 2; the Matthews number (Matthews, 1968) of $2.89 \text{ \AA}^3 \text{ Da}^{-1}$ is typical of other GS crystals ($2.5 \text{ \AA}^3 \text{ Da}^{-1}$ for *S. typhimurium* GS). A single GS dodecamer in the asymmetric unit would give a value of $5.8 \text{ \AA}^3 \text{ Da}^{-1}$, which would lie considerably outside the usual range of $1.6 \text{ \AA}^3 \text{ Da}^{-1}$ for densely packed crystals and $3.6 \text{ \AA}^3 \text{ Da}^{-1}$ for moderately loosely packed crystals (Matthews, 1968). We conclude that there are two dodecamers in the asymmetric unit, both having their sixfold axes parallel or nearly parallel to the a axis. This means that the twofold axes lie in the bc plane. However, from the self-rotation function, only one set of dyads and only one hexad seem to be present. Because we find only a single set of twofold axes on or near the bc plane, the simplest explanation is that the two GS molecules have their twofold axes parallel to each other. However, there is a second possibility: because each GS molecule with 622 symmetry has two distinct sets of twofolds perpendicular to the sixfold axis, 30° apart, the molecules could be rotated by 30° from one another. This second possibility is ruled out by a native Patterson map calculated from the X-ray data, which shows there is a second dodecamer which is translationally related to the first one: there is only one very large peak (103, 0, 60 Å), corresponding to a shift of 119 Å, with a signal of 10σ above the mean, with the highest noise peak being 4σ above the mean. Since there is only one large Patterson peak, the two dodecamers must have the same orientation.

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