

**Experiment title:**

Kinetic crystallographic studies on glutathione synthetase

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**Report:**

Glutathione synthetase (GSHase) catalyzes the synthesis of glutathione from MgATP,  $\gamma$ -L-glutamyl-L-cysteine ( $\gamma$ -Glu-Cys) and glycine. The reaction is thought to proceed through the formation of an acyl-phosphate intermediate,  $\gamma$ -Glu-Cys phosphate, followed by nucleophilic attack of glycine to yield glutathione. In the light of the reaction mechanism, we synthesized a transition state analogue (TSA), in which the C-terminal carboxyl group is replaced with a tetrahedral phosphinyl group with the 2-carboxyethyl moiety mimicking the incoming glycine. It is tightly-bound ( $K_i = 21\text{nM}$ ) to GSHase in the presence of MgATP. X-Ray diffraction analysis has shown that the tight-binding species is phosphoryl-TSA produced at the enzyme active site. The features of the tight-binding and catalytic single turn over allow us to apply time-resolved crystallographic analysis to this system. We started rapid diffraction data collection studies by Laue diffraction to capture the ternary complex structure just before phosphorylation (GSHase:ATP:TSA complex). The structure that mimics productive Michaelis complex provides us the implication for the catalytic mechanism of the first step in which  $\gamma$ -phosphate of ATP is transferred to the C-terminal carboxylate of  $\gamma$ -Glu-Cys. Kinetics of the reaction in the crystals have indicated that its half life ( $t_{1/2}$ ) is to be 0.31 min at 20 °C, and 0.75 min at 0 °C.

Hexagonal crystals (1.0 x 1.0 x 0.2 mm) were grown at 20 °C by batch methods in 0.8 M Li<sub>2</sub>SO<sub>4</sub> solution including 2 mM caged-ATP and 5 mM TSA in the dark. The crystals belong to a space group P6<sub>2</sub>22 with cell dimensions, a=b= 87.15, c= 169.8 Å. Flash photolysis was achieved with a dye laser of ID9 or Xe flash lamp developed by Gert. Rapp. Photolytic efficiency was determined by HPLC from the contents of the caged- and uncaged-nucleotides in the crystals. After a variable delay period (3.5 to 10 sec) from the photolysis, the crystal was exposed to a 2 to 5 msec white X-ray from ID9, ESRF and 10 to 14 images were collected on the ESRF X-ray Image Intensifier detector at intervals of 13 sec. As a control, the diffraction images of unphotolyzed crystals were also collected. One hundred thirty-four crystals were used for the X-ray exposures. Temperature of the crystals mounted was controlled with a FTS air-jet cooler. The images were indexed by LAUEGEN of CCP4 suite and the diffraction spots including spatially overlapped ones were integrated by PROW, a program written by D. Bourgeois.. The wavelength normalization and merging of the data were processed by LAUENORM, AGROVATA, and ROTAVATA of the CCP4 suite. As an example, merging six different images from an unphotolyzed GSHase crystal with 9191 independent reflections yielded 61.8 % completeness (100 to 2.4 Å resolution) with 3.9 redundancy. An Fo-Fc electron density map from this data showed clear electron density for the ligands omitted from the structural model used for the calculation (Fig 1). As for the time-resolved data, two sets of data at each time points were obtained at room temperature and -1 °C. Crystallographic different analyses of these time-resolved data are in progress.

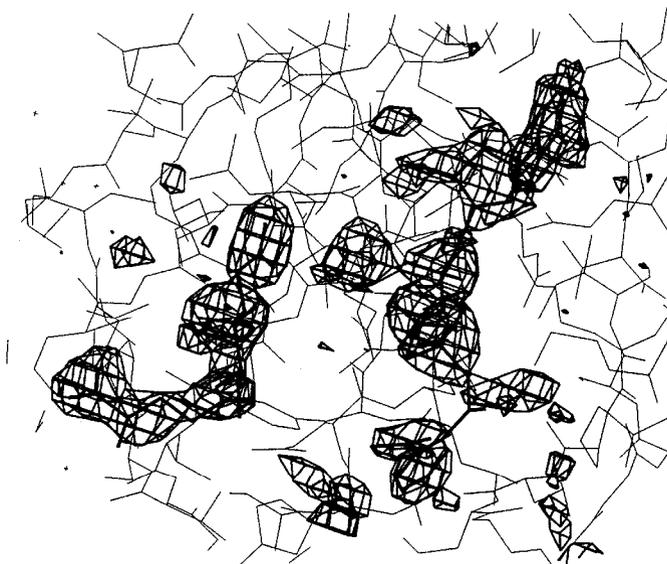


Fig. 1