First attempts to crystallize a non-homogeneous sample of thioredoxin from *Litopenaeus vannamei*: What to do when you have diffraction data of a protein that is not the target?

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ABSTRACT

The importance of sample homogeneity and purity in protein crystallization is essential to obtain high-quality diffracting crystals. Here, in an attempt to determine the crystal structure of thioredoxin 1 from whiteleg shrimp *Litopenaeus vannamei* (L/Trx), we inadvertently crystallized the hexameric inorganic pyrophosphatase of *Escherichia coli* (E-PPase) from a non-homogeneous sample product during the initial over-expression steps and partial purification of L/Trx. The structure determination and identification of the crystallized protein were derived from several clues: the failures in the Molecular Replacement (MR) trials using L/Trx coordinates as a search model, the unit cell parameters and space group determination, and essentially by the use of the program BALBES. After using the previously deposited E-PPase structure (PDB entry 1mjw) as a search model and the correct space group assignation, the MR showed an E-PPase complexed with SO₄²⁻ with small changes in the sulfate ion binding region when it compares to previously deposited E-PPases in the PDB. This work stresses the importance of protein purity to avoid the risk of crystallizing a contaminant protein or how pure need to be a protein sample in order to increase the possibility to obtain crystals, but also serves as a reminder that crystallization is by itself a purification process and how the program BALBES can be useful in the crystal structure determination of previously deposited structures in the PDB.

1. Introduction

To determine the three-dimensional coordinates of proteins by crystallography, it is necessary first to purify and then generate crystals of suitable size and quality for X-ray diffraction experiments. Although significant work has been performed to develop protein crystallization methodologies, protein crystallization remains as a bottleneck for structural determination [1–4]. Purity is the first variable that is essential to accomplish to obtain protein crystals [5–7] since contaminants within a protein batch may alter the crystal packing of a growing crystal [8–10]. Studies on the effect of macromolecular impurities on protein solubility and crystallizability are limited. However, Skouri et al. in 1995 [6] measured the effect of 2% (w/v) ovalbumin on lysozyme solubility over a concentration of 3–8% (w/v) NaCl. They also conducted similar experiments exploring the effect on lysozyme solubility using 1% (w/v) ovalbumin, 1% (w/v) conalbumin, and 1% (w/v) bovine serum albumin. However, no significant effects were observed in the crystalline packing. *Escherichia coli* is the most used bacterial expression systems due to the in-depth knowledge of this microorganism and for the high amounts of heterologous proteins that can be produced. However, despite its many advantages, particular conditions such as incubation time or other stresses during the culture stage may favor the expression of *E. coli* native proteins more than the heterologous protein expression. Such is the case of E-PPase expressed in conditions where the energetic source is compromised to keep the culture alive [11]. In this work, we describe the expression, purification, crystallization, structural determination and coordinates analysis of the E-PPase complexed with SO₄²⁻ as a result of crystallization experiments using a non-homogeneous partially purified L/Trx sample.
2. Materials and methods

2.1. Expression and purification

The *E. coli* PPass was similarly purified by previously published studies of whiteleg shrimp thioredoxin 1 from *Litopenaeus vannamei* (LvTrx) [11–13]. Cells of the host strain *E. coli* BL21 (DE3) transformed with plasmid pET11a/LvTrx were grown on Luria-Bertani (LB) agar plates [1% (v/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl] containing 200 μg ml⁻¹ ampicillin at 310 K. A single colony was picked and grown for plasmid isolation. A colony was inoculated into 50 ml LB broth containing 200 μg ml⁻¹ ampicillin and incubated for 12 h at 310 K; part of the culture (25 ml) was used to inoculate 1 L of LB medium with 200 μg ml⁻¹ ampicillin. The culture was grown to an absorbance of 0.6 at OD₆₀₀. IPTG (isopropyl-β-D-thiogalactoside) was added to the broth to a final concentration of 0.4 mM and grown for an additional 12 h. The cells were then harvested by centrifugation (24,000g, 30 min, 277 K) using a Beckman JA-14 rotor centrifuge (Beckman Coulter, CA, USA) and washed in 0.9% NaCl (100 mM Tris–HCl, pH 7.5) containing Complete EDTA-free® protease-inhibitor cocktail (Roche Molecular Biochemical, USA) and sonicated with three pulses per step. A solution containing 50 ml LB broth containing 200 µg ml⁻¹ ampicillin was resuspended in buffer (10 mM Tris–HCl, pH 8.0) containing Complete EDTA-free protease-inhibitor cocktail (Roche Molecular Biochemical, USA) and sonicated with three pulses per step at 50% and 85% ammonium sulfate saturation. The precipitate formed with plasmid pET11a/LvTrx was not found among the presence of different protein populations. After the ion exchange chromatography step had been performed, a denaturing electrophoresis (15% SDS-PAGE) was applied, revealing different protein populations and proportions (Figs. 1a, 1b).

2.2. Protein crystallization

Crystallization trials were performed using Crystal Screen and Crystal Screen II kits from Hampton Research (Aliso Viejo, CA, USA) by the hanging drop vapor-diffusion method at 291 K. The drops were prepared manually in 24-well crystallization plates by mixing the enzyme (2 μl) at 30 mg ml⁻¹ with the reservoir solution (2 μl) containing 0.1 M sodium acetate, pH 4.6 and 2.0 M ammonium sulfate. DTT was added to a final concentration of 5 mM directly in the crystallization droplets. Suitable crystals for diffraction were obtained in one month. Crystals for data collection were then flash-cooled by immersion in liquid nitrogen using 30% (v/v) glycerol into the mother solution as a cryoprotectant.

2.3. Data collection and structure determination

Diffraction data were collected on beamline X6A of the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (BNL), USA, using an ADSC Quantum 270 detector. The X-ray diffraction data were collected from a single crystal at 100 K (incident wavelength, λ=0.975 Å). The diffraction images were integrated using XDS [15] and scaled with SCALA from the CCP4 suite (Collaborative Computational Project, Number 4) [16]. Molecular replacement was done with a cross-rotational search followed by a translational search using the coordinates of E-PPase was performed using the program PHASER [17] to obtain initial models and phases (LiG=8383, RFZ=9.9, TFZ=11.9, being a trimer found in the asymmetric unit). The models were improved based on manual inspection of the 2Fₐ−Fᵢ map after rigid-body refinement and geometric constraint performed in REFMAC [18]. All further refinement was done using the PHENIX suite [19]. The final model was completed and refined using the programs PHENIX and COOT to a final Rwork/Rfree of 19.3/23.5% [20]. Data collection and refinement statistics are summarized in Table 1.

### Table 1

| Parameter                          | E-PPase PDB (4UM4) |
|------------------------------------|--------------------|
| **Data collection statistics**     |                    |
| X-ray source                       | BNL NSLS Beamline X6A |
| Wavelength (Å)                     | 0.975              |
| Space group                        | C 1 2 1            |
| **Unit-cell dimensions**           |                    |
| a, b, c (Å)                        | 120.0, 108.9, 81.0 |
| α, β, γ angles (°)                 | 90.0, 97.0, 90.0   |
| Resolution range (Å)               | 19.10–2.65         |
| No. of reflections                 | 61,030             |
| No. of unique reflections          | 27,508             |
| Completeness (%)                   | 92.0 (94.0)        |
| Rwork/Rfree (%)                    | 6.0 (43)           |
| Rmerge (%)                        | 7.9 (56)           |
| I/σ(I)                            | 10.3 (2.3)         |
| Multiplicity                       | 2.2 (2.2)          |
| Asymmetric unit content            | Trimer             |
| **Refinement statistics**          |                    |
| Rwork/Rfree (%)                    | 19.3/23.5          |
| B-value (Å²)                       | 40.8               |
| Protein                            | 83                 |
| Ion/Ligand                         | 38                 |
| Water                              | 50.8               |
| Wilson plot B-value (Å²)           | 45.8               |
| **RMSD from ideal stereochemistry**|                    |
| Bond lengths (Å)                   | 0.017              |
| Bond angles (°)                    | 1.88               |
| Coordinate error (Maximum-Likelihood Base) | 0.12               |
| Ramachandran plot (%)             | 92.3               |
| Most favored regions               | 4.8                |
| Additional allowed regions         | 4.8                |
| Disallowed regions                 | 2.9                |

1. Rwork=Σhkl | |h|/|Σhkl|, Rfree=Σhkl | |h|/|Σhkl|, where |h| is a redundancy-independent version of Rwork, Rfree = Σhkl | |h|/|Σhkl|, where |h| is the number of unique measurements and the corresponding mean values. The summation is over all unique measurements.
2. Rmerge is a redundancy-independent version of Rmerge = Σhkl | |h|/|Σhkl|−1 | |Σhkl|−1 | |h|, where |h| is the number of unique measurements and the corresponding mean values.

3. Results and discussion

3.1. Co-expression between a heterologous enzyme and E-PPase host enzyme

During the initial attempts to establish a protocol for expressing and purifying recombinant enzyme *LiuTrx* [13], high levels of the over-expressed *LiuTrx* were not found among the presence of *E. coli* proteins as a result of the long incubation period. After the ion exchange chromatography step had been performed, a denaturing electrophoresis (15% SDS-PAGE) was applied, revealing different protein populations and proportions (Figs. 1a, 1b).

During the purification process and despite several attempts to...
improve it, we were unable to obtain a homogeneous sample. Therefore, we decided to perform crystallization trials with the sample as it was, which resulted in the E-PPase crystallization (after this experiment the conditions to effectively over-express LeTrx were obtained and published) [13]. A densitometric analysis of the SDS-PAGE band profile presented in Fig. 1b estimates that 21% and 16% of the sample used for crystallization correspond, respectively, to E-PPase and LvTrx. Additionally, E-PPases and LvTrx are 2 among 18 species which generate a discrete band in the SDS-PAGE profile.

A likely explanation for why the E-PPase was favored throughout the expression, purification, and the crystallization process is that the purification protocol of E-PPase is very similar to the protocol for recombinant LeTrx, specifically the heating step and ammonium sulfate precipitation steps [22]. Taking into account that the purification protocol of E-PPase requires 12 h for over-expression, this may explain the co-expression with the recombinant LeTrx because 12 h of expression was, in fact, an additional stress condition for the bacterial culture. This long expression time reminds us that E-PPase is a constitutively expressed protein that plays an important role in macromolecular biosynthesis under stress conditions and is essential for the viability of E. coli in the last stages of bacterial culture [11,23].

During the purification protocol of the recombinant LeTrx, the sample was heated to a temperature of 343 K for 20 min. At this temperature the LeTrx protein is stable, but it is also known that the active conformation of bacterial PPases is stabilized by interactions between monomers in a hexameric arrangement, being the thermo-

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**Fig. 1.** a) Coomassie blue stain of SDS-PAGE of the sample that was crystallized. The first lane shows the markers (M). The second lane shows the non-homogenous sample used for crystallization trials. The arrows indicate the identification of the LeTrx and E-PPase enzymes according to their molecular mass (12 kDa and 20 kDa, respectively). b) The relative concentration of LeTrx and E-PPase were estimated using a densitometric analysis of the SDS-PAGE sample band profile. The densitometric analysis was performed by using the program ImageJ [21].

**Fig. 2.** a) E-PPase trimer complexed with SO₄²⁻ in ribbon representation. b) Superposition of chain A, B and C, showing the SO₄²⁻ bound to the E-PPase. The images were generated by CCP4mg and PDBsum, respectively [31,32].
stability of bacterial PPases a primary property of the oligomer [24,25]. Thus, the E-PPase is stable at 343 K, maintaining LvTrx and the E-PPase structurally stable during the purification process. Furthermore, it has been shown that E-PPase is a common contaminant enzyme in expression assays [26,27]. Retrospectively, including a gel filtration purification step could help to separate the hexameric E-PPase from the dimeric LvTrx.

In regards to the crystallization process, the reported crystallization condition for recombinant LvTrx [13] is very similar to those for E-PPase, potentially resulting in the favoring of the nucleation and crystal formation process of E-PPase more than LvTrx due to E-PPase thermal stability and probably also because E-PPase represent the highest population, 21% of the total sample, among 18 species detected in the SDS-PAGE profile of the sample used for crystallization (Fig. 1b). Our results in other published works [12,13] conclude that the incubation time for the over-expression of LvTrx was key in obtaining the recombinant enzyme completely pure (5 h instead of 12 h), without the presence of the E-PPase.

### 3.2. Molecular replacement and refinement

Originally, we were unable to determine the crystal structure of LvTrx with the X-ray diffraction data collected and described in this work. After we successfully determined the crystal structure of LvTrx [13], we decided to go back to the X-ray diffraction data described here. At initial stages of the phase determination process by MR using R 3 2 or H 3 2 as a space group, no solution could be found using the LvTrx recombinant enzyme coordinates, even after trying different unit cell parameters, and space groups (P 1; C 2; R 3 or H 3) or other average search coordinate tricks (such as partial or multiple coordinates, use of poly-Ala or poly-Gly models, among several approaches). After failing with several trials of MR and exhausting all possible combinations using the LvTrx coordinates for phase determination, we decided to consider that the crystallized protein could be another one coming from E. coli.

As an approximation to identify the protein, we decided to use the space group H 3 2, due to the apparent better statistics concerning the other space groups and unit cell candidates attempted while using LvTrx coordinates.

The amplitudes from space group H 3 2 and the sequence from LvTrx (sequence reference from UniProtKB was B1PWB9) were submitted to the CCP4i BALBES interface server, which found a solution structure by MR without user intervention [28]. The best solution found by BALBES was using the PDB coordinates from entry 1mjw [29] with a space group H 3 2 and refinement Rwork/Rfree values of 35/42%, which correspond to the inorganic pyrophosphatase from E. coli. In fact, if we had not reduced the data in the space group H 3 2 and used it in BALBES, we would not have been able to estimate the phases as readily. After the MR solution had appeared, we were able to corroborate that approximately 95% of the crystallographic structures of E-PPases deposited in the PDB belong to space group H 3 2, with unit cell parameters very similar to the parameters determined in this work.

The initial phases were obtained using the E-PPase coordinates from E. coli as the best search model. However, the parameters of the MR process resulted in a solution demonstrating an overlapping between the crystallographic neighbor copies. To address the problem, we used all the possible combinations of space groups and unit cell parameters.

![Fig. 3. a-c) Representation of SO₄²⁻ interactions among the E-PPases reported in the PDB (entries1mjx, 1mjw, 1jfd). d) E-PPase structure derived in this work (PDB entry 4um4) showing a different position of SO₄²⁻ interacting with residues Lys-104 and Tyr-55 at 2.6 Å and 3.0 Å, respectively. Figure prepared with LigPlot [35].](attachment:image.png)
parameters determined previously in this work. The best solution belonged to space group C 1 2 1, with unit cell parameters a = 120.0 Å, b = 108.9 Å, c = 81.0 Å; α = 90.0°, β = 97° and γ = 90.0°. Additionally, POINTLESS [30] clearly supported the final space group selection. It is important to remark, that space group C 1 2 1, is a novel space group for E-PPases. The final model of the E-PPase consisted of a trimer in the asymmetric unit in complex with SO₄⁻ located in each monomer active site at 2.65 Å resolution (Table 1 and Figs. 2a, 2b).

3.3. E. coli PPhase crystal structure

The crystal asymmetric unit contained three subunits, each including 175 amino acid residues with all the residues clearly visible in the final electron density map (2Fo–FF map). The biological hexamer is generated using crystallographic symmetries. In the active site crevice, we found fourteen amino acids residues limiting the cavity: Asp-42, generated using crystallographic symmetries. In the active site crevice, eubacterial aspartyl-tRNA synthetase crystals, Acta Crystallogr. D Biol. Crystallogr. 65 (2009) 1062–1073.

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