**Project ID**: NCCAT-TP1

**Project name:** Embedded user training for microED data collection of protein crystals

**Primary username:** Vesna de Serrano

**Institution:** North Carolina State University

**Submission date:** 06/30/2024

**Summary Statement:**

I am applying to NCCAT for the embedded user training program (TP1) to gain expertise in single particle analysis and microED methods covering all essential techniques, including sample preparation, data acquisition, and processing methodologies using grid preparation and cryo-TEM instruments. My goal is to become independent in these procedures, enabling me to collect and process data both at your national facility and also in cryo-EM facilities in Research Triangle, NC area until we obtain our own instrument here at North Carolina State University.

## Training Goals:

My goals are to obtain training to independently carry out structure determination by primarily electron diffraction, referred to as microcrystal electron diffraction (microED) and 3D electron diffraction (3DED), as well as to obtain training in single particle cryo-EM. In reality, I would appreciate it to be trained for both these methods at the same time, if possible, because presently we want to determine the electron diffraction structure of difficult DHP-substrate complexes, but we also need to be able to use SPA cryo-EM methods because we are additionally in the process of exploring new proteins with activities similar to DHP, that we would like to include in our research projects.

## Training Plan:

1. Learn to prepare grids with my crystals personally, using Vitrobot and Leica GP2 rapid cryo plungers. So far I have only been able to observe grid preparation, collaborating with a group at NIEHS in RTP, North Carolina
2. Learn to operate the cryo-TEM, with the goal to be able to screen and evaluate the grids myself, select the microcrystal targets in diffraction mode for subsequent data collection (so far I have only observed the screening of the grids prepared with my crystals), and learn to set up for tilt series diffraction data collection in continuous rotating mode, using low electron dose rates, with the overall low accumulative dose of about 2-6 $e^-/Å^2$.
3. Learn to convert and use multiple collected data sets in normal macromolecular crystallography software.
4. Obtain training in the analogous procedures that are necessary in single particle cryo-EM, including grid preparation, screening, data collection, and in particular data processing, for all of which I do not have much experience.

5. In addition, to learn all these procedures that are practiced at NCCAT in order to be able to later on collect both micoED and SPA data at your national cryo-EM facility, as North Carolina State University still does not have a cryo-EM core facility, although several electron microscope instruments, not operated under liquid nitrogen, are available in the material science department.

**Resources Requested:**

I am not certain how much time is necessary to learn all these procedures to the point of becoming an independent cryo-TEM user. I have seen the instrument being operated, but I have never been trained to use it independently or permitted to work with it myself. I assume that the entire training procedure may take at least a week if not much longer, but for the true assessment I will have to depend on NCCAT training staff.

**Background and History:**

I am currently working in the group of Dr. Reza Ghiladi at the Department of Chemistry at NC State University. Our group uses primarily X-ray crystallography to study structural aspects of the multifunctional enzyme dehaloperoxidase (DHP), the coelomic hemoglobin from the marine worm *Amphitrite ornata* which shows five different activities including oxygen transport, peroxides, peroxygenase, oxidase and oxygenase functionality. We have determined many crystallographic structures of this protein in complex with its smaller substrates, such as various halophenols. However, it was proven difficult to observe some larger substrates in the enzyme binding cavity. In order to be able to determine these difficult substrate complex structures a couple of years ago we started looking to incorporate microED/3DED methods to increase the probability of observing the mode of binding of these larger substrates to DHP. So far, we were able to prepare and screen grids, and presumably being trained in using the instrument and learning how to collect electron diffraction data properly, we will be able to collect publishable diffraction data sets. In addition, we have started looking for enzymes that are homologous to DHP with similar catalytic properties, and for one of them we have already done some introductory screening on cryo-TEM, and would need to learn SPA in order to study structure of this new enzyme (molecular weight in the vicinity of 60-80 kDa). In all this regard, I have already had some exposure to negative staining, and the rest of it is virtual participation in several workshops offered by Stanford-SLAC Cryo-Electron Microscopy Center ($S_2C_2$ ) as well as virtual Cryo-EM Course at LBMS, in addition to studying the on-line course by Grant Jensen.

**Geographical Demographics:**

As a member of the Ghiladi group in the Chemistry Department at North Carolina State University, I am the first to attempt incorporating the microED/3DED cryoEM method to determine the structure of proteins in complex with larger polycyclic substrates, addressing their diffusion problems within larger crystals. Additionally, being among the first ones who are interested in this methodology in this area, I am involved in developing the microED/3DED method at several cryo-EM facilities in the Research Triangle Park area in North Carolina. Moreover, complete training in this methodology would enable me to assist my coworkers who may also be interested in using this method. Since North Carolina State University is further away from NCCAT, I assume I would need to spend extended periods of time at the NCCAT facility to complete the training.

**Determinig structure of DHP in complex with larger, polycyclic substrates utilizing micoED/3DED**

As a model for probing the protein structure-function correlation in the hemoprotein superfamily in humans, our work has focused on the enzyme dehaloperoxidase (DHP), the coelomic hemoglobin from the marine worm *Amphitrite ornata*. The enzyme employs several forms of enzymatic activity and we have established that the binding of the substrate itself is determinant for the form of DHP activity that the enzyme will carry out in the process of modification/degradation of that particular substrate. Exhibiting the variety of enzymatic activities such as peroxidase, peroxygenase, oxidase and oxygenase, this enzyme has been shown to play a role of a detoxifying agent for *A. ornata* to survive harsh environments by performing the oxidation of marine pollutants that are produced by other organisms in its living environment. We have also shown (1,2) that great many of existing as well as emerging pollutants of anthropogenic origin, such as bi- and bisphenols that have been linked to adverse health effects in humans and are commonly monitored in the environment, have turned out to be substrates for DHP. Therefore, it is particularly important to determine their mode of interaction with DHP and their subsequent modification by the one of the activities of this enzyme. For illustration, the reaction of BPA with DHP in presence of the co-substrate $H_2O_2$ is shown in Figure 1, also showing products of this reaction.

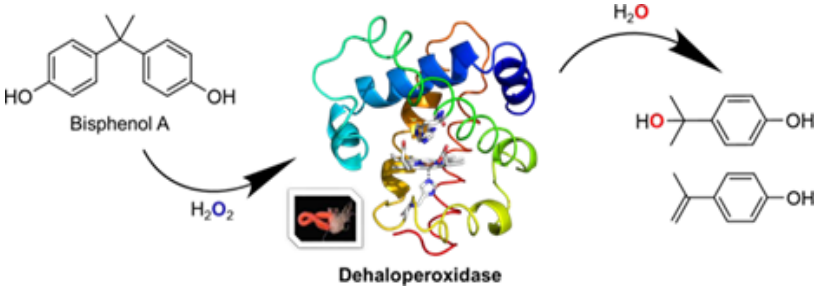


Figure 1. The reaction of BPA with DHP, shown as its 3D structure, in presence of $H_2O_2$ leads to its oxidation products generated by DHP's peroxidase activity (*Amphitrite ornata* is also shown).

In general, we have been able to determine structures of DHP in complex with many of its substrates using X-ray crystallography. However, many larger polycyclic substrates have been recalcitrant to both co-crystallization and crystal soaking and although we are able to determine the substrate binding affinities (using UV-Vis spectroscopy) we were not able to determine structures of very important DHP-substrate complexes, such as BPA (Figure 1) utilizing X-ray crystallography. Therefore, we want to use the cryo-EM method microED since we will be able to use sub micrometer size crystals to increase the likelihood of diffusion of these larger substrates into the crystal.

For the microED structure determination that we want to explore when the substrates are too large for easy diffusion into a preformed crystal, we have optimized the preparation of small, micron to submicron size crystals by varying volume ratio of protein (30 mg/ml) and mother liquor containing 40% PEG 4000 as the crystalizing component using 0.6 ml Eppendorf tubes and incubating one or two days at 4 °C (Figure 2A and 2B)

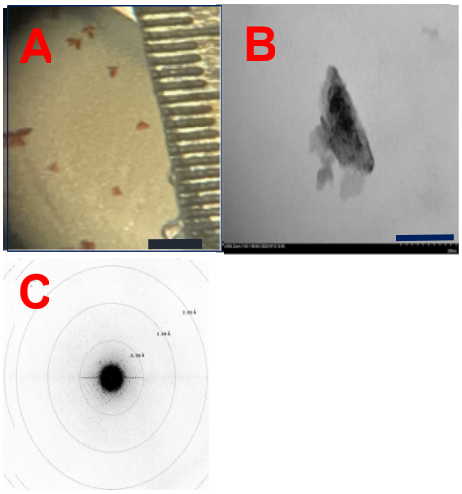


Figure 2. A) Sizes of crystals grown for microED experiments in batch crystallization mode as described (scale bar 100 μ), B) size of crystal on TEM grid selected for a microED experiment (scale bar 200 nm), C) diffraction pattern recorded in the initial trials of collecting electron diffraction of DHPb crystal in the cryo-EM core facility at UNC-CH: recorded on K3 camera in counting mode using Thalos Arctica electron microscope operating at 80 K and acceleration voltage of 200 kV.

According to the initial experiments I was able to observe the electron diffraction of DHP crystals, but I am still working on the improvement of the crystal electron diffraction, trying to reduce viscosity of the crystallization solution in order to further reduce the thickness of ice in which the crystals are embedded. In the latest developments I was able to prepare grids using Leica GP2 cryo plunger at NIEHS cryo-core facility, from which it is easier to observe crystals in the cryo-TEM screening, shown in figure 3, although to identify crystals with more precision I still need to perform diffraction screening (not yet available at NIEHS facility) on selected areas in order to eventually be able to collect the whole, publishable electron diffraction data sets, and process them using established macromolecular X-ray crystallography

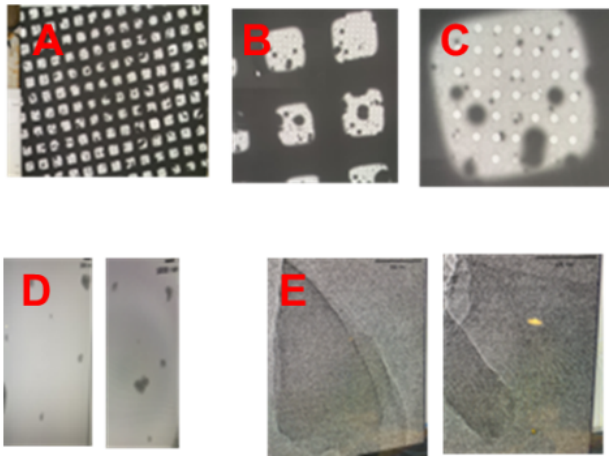


Figure 3. A) Grid atlas at 65.5 X magnification, B) atlas zoomed in, C) grid square at 1250 X magnification, D) grid square zoomed in showing probable crystal, E) crystals at 36000 X magnification.

**References**

1. Yun, D. et al. J. Inorg. Biochem. 238, 112020, 2022
2. Aktar, M.S. et al. J. Inorg. Biochem. 247, 112332, 2023

**BIOGRAPHICAL SKETCH**

Provide the following information for the Senior/key personnel and other significant contributors.

Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: de Serrano, Vesna S.

eRA COMMONS USER NAME: n/a

POSITION TITLE: Adjunct Associate

Research Professor

EDUCATION/TRAINING

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | FIELD OF STUDY |
|---|---|---|
| University of Sarajevo, Sarajevo, Bosnia & Herzegovina | BS | Chemistry |
| University of Notre Dame, Notre Dame, IN | MS | Biochemistry |
| University of Notre Dame, Notre Dame, IN | PhD | Biochemistry |

**A. Personal Statement**

My present research focuses on the structural aspects of the enzyme dehaloperoxidase (DHP) from the marine organism Amphitrite ornata. This enzyme has developed various oxidation activities as a defense mechanism against its toxic environment. These diverse activities are induced by interactions with different substrates. My goal is to define the structural differences

of these substrate complexes to better characterize the binding interactions that ultimately induce different activities in the enzyme.

To achieve this, we are using macromolecular crystallography and neutron diffraction, along with a series of methods to study the activity and product formation. These methods include kinetics using UV-Vis spectrometry and HPLC coupled with mass spectrometry to identify reaction products and quantify product formation.

My main contributions toward understanding the various activities of this enzyme include determining the structures of DHP in complex with cresol substrates, which invoke both peroxidase (electron transfer) and peroxygenase (oxygen transfer) activities. This work has helped elucidate the interactions of these substrates with the enzyme, as well as the interactions of larger substrates, such as bi- and bis-phenols, that can bind in the enzyme's binding cavity. This research on defining structural differences that lead to changes in the activity of DHP has generated several publications, some of which are listed below.


1. Malewschik, T.; de Serrano, V.; McGuire, A. H.; Ghiladi, R. A.The Multifunctional Globin Dehaloperoxidase Strikes Again:Simultaneous Peroxidase and Peroxygenase Mechanisms in the Oxidation of EPA Pollutants. *Arch. Biochem. Biophys.* 2019, *673*, No. 108079.
2. Yun, D.; de Serrano, V.; Ghiladi, R. A. Oxidation of BisphenolA (BPA) and Related Compounds by the Multifunctional Catalytic Globin Dehaloperoxidase. J. Inorg. Biochem. 2023, 238, No. 112020.
3. Aktar, M. S.; De Serrano, V.; Ghiladi, R.; Franzen, S.Comparative Study of the Binding and Activation of 2,4-Dichlorophenol by Dehaloperoxidase A and B. *J. Inorg. Biochem* 2023, *247* (July), No. 112332.
4. McGuire, A. H.; Carey, L. M.; De Serrano, V.; Dali, S.; Ghiladi, R. A. Peroxidase versus Peroxygenase Activity: Substrate Substituent Effects as Modulators of Enzyme Function in the Multifunctional Catalytic Globin Dehaloperoxidase. *Biochemistry* 2018, *57* (30),4455−4468.


In general, my interests and goals are primarily in structural aspects of protein interactions and activities, geared toward potential drug developments, and toward that goal I have worked with the group of Dr. Edwin Pozharsky at the University of Maryland Medical school in Baltimore on projects involved in studies of crucial proteins from Helicobacter pylori, such as NikR, transcription regulator involved in regulation of nickel uptake, as well as phosphorylation regulation of signal regulated kinase ERK2, as well as with the group of Dr. Carla Matos at the Molecular and Structural Biochemistry at North Carolina State University in research related to Ras GTPase, a small GTP-binding protein, also involved in signaling pathways, which is in its mutated forms most frequently associated with various human cancers. Through that work I have acquired an expertise in X-ray crystallography, which has significantly contributed to my current and long-term research interests.

The extensive list of my publications can be found in MyBibliography:
https://www.ncbi.nlm.nih.gov/myncbi/1lAEG8aOhfeQF/bibliography/public/

**B. Positions and Honors**

**Positions and Employment**

2022-present Adjunct Associate Research Professor, Department of Chemistry, North Carolina State University

2017-2022 Adjunct Assistant Research Professor, Department of Chemistry, North Carolina State University

My ORCID: 0000-0002-8316-2907