Methods for structure acquisition of small proteins; Cryogenic Electron Microscopy and Time Resolved Wide-Angle X-ray Scattering

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ACKNOWLEDGEMENT
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DEDICATION

To my son.

ABBREVIATIONS

Abbreviation	Meaning
AsLOV2	*Avena sativa* Light Oxygen Voltage domain 2
CryoEM	Cryogenic Electron Microscopy
DNA	Deoxyribonucleic Acid
DARPins	Designed Ankyrin Repeat Proteins
FSA	Fructose-6-phosphate aldolase
QAAS	Glutamine, Alanine, Alanine, Serine
PNK	polynucleotide kinase
PCR	Polymerase Chain Reaction
XL1-Blue	"extra-low protease" 1-blue *E. coli* strain
BL21	B strain of *E. coli,* Low protease level
araBAD	Ara-arabinose, araB enzyme for arabinose phosphorylation, araA enzyme for catalysing arabinose conversion to D-xylulose-5-phosphate and araD an enzyme that catalyzes the metabolism of the product.
DLS	Dynamic Light Scattering
NADH	Nicotinamide Adenine Dinucleotide Hydrogenated (reduced form)
PDB	Protein Data Bank
TRWAXS	Time Resolved Wide Angle X-ray Scattering
SAXS	Small Angle X-ray Scattering
RELION	REgularized LIkelihood OptimizatioN
Cryo-SPARC	CryoEM Single-Particle *ab initio* Reconstruction and Classification
MBP	Maltose-Binding Protein
KDEL	Lysine-Aspartic Acid-Glutamic Acid-Leucine
FMN	Flavin Mononucleotide
GPCRs	G protein coupled receptors
SARS-COV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SDS	Sodium Dodecyl Sulfate
PAGE	Polyacrylamide Gel Electrophoresis
TIM	Triose phosphate isomerase

MHz	Megahertz
XFELs	X-ray Free Electron Lasers
PDB	Protein Data Bank
NMR	Nuclear Magnetic Resonance

FEATURED PUBLICATIONS

Manuscript 1. New fusion strategy for visualizing small proteins in CryoEM; Fructose-6-phosphate aldolase (FSA) as a carrier for <100 kDa domains. (Submitted in Acta Crystallographica D, under review) I performed all experiments including gene cloning, protein production, CryoEM data collection and analysis.

Paper 2. Microsecond time-resolved X-ray scattering by utilizing MHz repetition rate at second-generation XFELs (Konold, P.E., Monrroy, L., Bellisario, A. *et al.* Microsecond time-resolved X-ray scattering by utilizing MHz repetition rate at second-generation XFELs. *Nat Methods* **21**, 1608–1611 (2024). https://doi.org/10.1038/s41592-024-02344-0)

In this paper, I attended the beamtime, and helped in lab techniques such as sample dilution and assembly of cylindrical reservoirs that deliver the sample to the X-ray interaction point. The protein was purified by Patrick Konold and his teach from the Department of Cell and Molecular Biology; Molecular Biophysics, Uppsala University.

ABSTRACT

Small proteins (< 100 amino acids) and microproteins (< 50 amino acids) are common in the proteome of living organisms. They are translated from small open reading frames and can occur as single or modular domains in larger proteins. They play crucial roles in signal transduction, gene expression, metabolism, enzyme functioning and post-translational modification of larger proteins. Previously, these proteins were difficult to identify due to limitations of genome annotation methods. Improvement of gene sequencing and proteome profiling advances have led to identification of small open reading frames coding for these proteins and are curated in different databases. Despite these improvements, structure acquisition of small proteins lags behind, there are fewer 3D structures solved as compared to available annotated sequences. Traditionally structure acquisition has been limited to NMR and X-ray crystallography for small proteins. Development and technical advances in CryoEM provide a more affordable method for protein structure studies. In this thesis I discuss one applicable method that can be used to enable structure acquisition of small proteins and micropeptides in CryoEM. I also highlight a new method of using high energy X-ray repetition data acquisition scheme using TRWAXs, as an advanced method for identifying structure variation in a signalling event in small proteins.

To achieve the objectives, the first manuscript describes methods used in the CryoEM study including selection of model proteins (*E. coli* fructose 6-phosphate aldolase, FSA, and the AsLOV2 domain), peptide linker design, molecular cloning, protein expression and purification, together with analyses of function (e.g. catalysis) and biophysical characteristics.

The first paper discusses protein constructs, protein production and characterization, together with identified challenges in applying CryoEM for structure analysis. The structure analysis suggested a monomer of the protein to be compared to the decameric structure of the carrier protein FSA. The second paper discusses identification of transition states in the AsLOV2 domain during a signalling event. This was possible due to MHz data acquisition scheme applied at the XFEL during TRWAXS method. The high data volumes acquired and advanced processing highlight significantly reduced noise levels in the data compared to previous application of traditional acquisition methods. The method is recommended for future applications in reaction dynamic studies that involve small proteins.

The methods discussed in this thesis are relevant in improvements of studies of structure and function of proteins. Recommendations drawn from various challenges are highlighted to overcome limitations of CryoEM to guide design and construction of suitable scaffolds that enable solving tertiary structures of small proteins.

INTRODUCTION

Structure characterization of proteins

Proteins are crucial functional units of folded polypeptide chains after translation of corresponding gene transcripts(Han et al., 2007). Proteins may consist of a single folded polypeptide chain, although larger proteins are often composed of multiple polypeptide chains interacting together to adopt supermolecular (quarternary) structures. Smaller proteins consisting of <100 amino acids are classified as those resulting from translation of small open reading frames(Steinberg & Koch, n.d.). They play different roles in conveying signals in and out of the cells, in growth and different metabolic processes(Su et al., 2013). Methods for structural studies, X-ray crystallography, Nuclear Magnetic Resonance (NMR) and Cryogenic Electron Microscopy (CryoEM) are used to decipher structural information in protein structures, where the latter is less well suited for studies of small proteins.

Methods for acquiring structural information in small proteins

Crystallography is the gold standard of protein structure determination and has remained on the forefront since its discovery in 1912(Barends et al., 2022). The majority of protein structures deposited in the Protein Data Bank (PDB) are contributed from X-ray crystallography studies. The principle is based on diffraction patterns acquired after illuminating well-ordered protein crystals with X-rays. X-ray crystallography can provide atomic level details of protein folding in space such as bond lengths, angles, ligand binding sites and overall structure conformations(Kubota et al., 2018). Over the years, tremendous improvement in crystallography such as use of X-ray free lasers, different methods for crystal acquisition and computational methods for diffraction dataset processing have pushed boundaries in drug discovery research(Gotthard et al., 2023). The process of crystallography data acquisition is preceded by steps such as protein expression, purification, screening salt precipitants and crystals size optimization in time-resolved crystallography. It can be tedious before acquiring well diffracting crystals.

NMR is an alternative method of solving structures of protein structures based on magnetic field of atomic nuclei. In NMR spectroscopy the structural information of a macromolecule is obtained from measurement of signals of the labeled atoms that are transformed into spectra(Lysak et al., 2023). The method is highly sensitive in providing atomic insights, chemical shifts and solving structures of small protein domains (Bystrov et al., 1978). Limitations of the method include requirement of high protein concentration which is a challenge in heterologous protein expression for example in production of membrane proteins. The steps from sample preparation to data analysis are intensive, labor consuming and expensive as compared to other methods.

Solution scattering methods, Wide/Small angle X-ray scattering (WAXS and SAXS), are also utilized in structural probing of macromolecules. In SAXS the information of protein structure organization is determined by measuring the intensity or the scattered X-rays verses the angle of scattering which is determined by the atom arrangements(Jeffries et al., 2021). WAXS on the other hand focuses on measuring wide angles of scattered X-rays relative to the focused X-ray beam. The data obtained from WAXS is very precise in computing structural differences as well as similarities of protein folding(Makowski, 2010). It has an advantage over other structural methods due to high sensitivity in conveying slightest changes in protein domain movements and loop opening as described later in this thesis.

CryoEM as a method for small protein structure determination

CryoEM has emerged as an alternative method in structure determination complementing deficits of previous methods(Nogales, 2016). The advantages of the method are the ability to obtain structural information without the need to crystallize the proteins, lesser sample consumption and ability to acquire structural data within a short time frame when compared to other methods(Nogales, 2016). The time taken for processing CryoEM data has greatly been shortened due to invention of automated programs leading to output of structure data. CryoEM single-particle *ab initio* reconstruction and classification (Cryo-SPARC) is one of the developed softwares that enable 3D reconstruction of protein structures automatically from particle picking. The algorithm computes structure averaging from micrographs without relying on an input model. It is user friendly (Punjani et al., 2017). On the other hand, RELION (REgularized LIkelihood OptimizatioN), enables computation of high resolution structures from single-particles by using a Bayesian approach(Scheres, 2012). A recently developed program called Warp has an integrated algorithm that is able to correct errors during real-time data recording(Tegunov & Cramer, 2019). The algorithm is able to correct image distortions and automate particle picking using graphics processing unit. It gives the user ability to observe micrograph patterns, skip any bad grid squares and automatically exclude any low-quality imaging making the entire data acquisition process highly efficient and was reported to improve overall resolution of previously published influenza virus hemagglutinin.

The majority of protein structure resolutions in CryoEM are within 3-4 Å, with a good number of structures within 2-3 Å and fewer reported to be below 2 Å(Yip et al., n.d.). The highest resolution recorded thus far was apoferritin structure at 1.54 Å(Yip et al., 2020). Improvements such as camera focus and electron sources have contributed to improvement of protein structure resolution below 2Å(Cheng, 2015; Nakane et al., 2020). Attained resolutions between 1.5 to 2Å have enabled acquisition of more biologically relevant information from protein structures such as residue conformations, presence of solvent molecules as well as bond coordinates(Nakane et al., 2020; Yip et al., 2020). While majority of structure determination by CryoEM lean towards high molecular weight multimeric proteins, recent advancements in the field have looked into utilizing the method for small-proteins structure determination(Nygaard et al., 2020; Scapin et al., 2018). Imaging small proteins using CryoEM faces limitations due to low signal-to-noise ratio as well as few physical features necessary for particle alignment. To overcome these limitations and push boundaries of CryoEM different strategies of embedding small proteins onto large molecular weight scaffolds have been engineered(X. Wu & Rapoport, 2021). In 2016, the structure of Maltose-Binding Protein (MBP) with a molecular weight of 40 kDa, and considered as a “small” protein in the context of CryoEM analysis, was solved at sub-nanometer resolution using CryoEM. This was possible by the scaffolding approach, where by researchers linked MBP onto glutamine synthetase (a homododecamer)(Coscia et al., 2016). The junction between MBP and the scaffold protein was a rigid linker of optimized length, allowing the protein to fold into a stable symmetric particle. Another approach utilized to image small proteins was use of an assembled “legobody” of 120 kDa. The legobody was assembled by attaching a target-binding nanobody to a fab (fragment antigen binding) of an antibody specific for the nanobody. The other domain of the legobody was a nanobody binding protein A fragment fused to MBP and fab-binding domains. The complex (120 kDa) had unique symmetry easily recognizable in CryoEM thus facilitating particle picking, alignment and image computations. The approach was used to visualize the structures of KDEL (Lysine-Aspartic Acid-Glutamic Acid-Leucine) receptor 23 kDa membrane protein to a 3.2 Å electron density map resolution. The method was also used to demonstrate the structure of receptor binding domain of SARS-COV-2 (Severe Acute Respiratory Syndrome Coronavirus 2), spike protein resulting in a map of approximately 3.6

Å resolution(Coscia et al., 2016). Liu *et al.*, reported a 3.8 Å map resolution of green fluorescent protein (GFP, 26 kDa) solved using a scaffold of fused terminal helices of a nanocage protein and an adapter protein (Liu et al., 2018). Their design used designed ankyrin repeat proteins (DARPins) 14 kDa, with high affinity to bind target proteins as adapters between the symmetric protein carrier and GFP. Similar strategy was applied by Yao *et al.*, whereby a scaffold of tetrameric rabbit muscle aldolase fused through the N-terminal extension to the C-terminal helix of DARPins was constructed. The scaffold offered wide surface area for binding GFP. The resulting GFP-DARPin-aldolase chimeric protein was successfully expressed, purified and structure of GFP solved at 4-5Å using CryoEM(Yao et al., 2019). Other small proteins solved via fusion strategy to CryoEM stable carriers include G protein coupled receptors (GPCRs) fused to a split luciferase via protein-protein interaction strategy called nanobit. Other exploited strategies include use of epitope tags and two point insertion into single particle assisted carriers which enhanced imaging of GPCRs like adenosine receptor to <4 Å(Wentinck et al., 2022). The approach of using homo-oligomerizing scaffold for binding small proteins improves target symmetry for 3D refinement during CryoEM data processing. The number of particles required for data analysis is largely reduced and one can readily visualize the target scaffold in negative stain Electron Microscopy images. Small proteins occupy the largest part of the human proteome yet less than 2% of their structures are solved(Wentinck et al., 2022). This approach of creating highly symmetric and stable chimeric proteins for CryoEM is a promising approach in bridging this gap and providing structural details for small proteins that cannot be studied using other available methods.

Protein linkers

Protein domain linkage is a technique used in biotechnology to fuse gene products from different organisms to express chimeric proteins of biotechnological importance. Researchers came up with linking strategies to engineer recombinant proteins through use of inter-domain linkers that can enhance proper folding, protein solubility, increased yield as well as constructing bifunctional enzymes (Aalbers & Fraaije, 2019; Amet et al., 2009; Hennig & Schäfer, 1998). Engineering stable protein linkers forming helical structures was reported to be determined by choosing the composition of ingoing amino acids(Chakrabartty et al., 1994). It was established that different amino acids contribute entropically to formation and stability of alpha helical structures. The frequency of occurrence of different amino acids forming helix connections in multi-domain proteins was analyzed and used as basis for engineering synthetic helical linkers. Alanine, threonine, glycine, serine, glutamine, asparagine, phenylalanine and lysine were some of the most occurring residues within the analyzed linkers(Arai, 2021). Alanine based linkers were previously reported to form stable and rigid alpha helical linkers which could link different protein domains in a construct.[23,](Arai, 2021; Arai et al., 2001). The small methyl group side chain as well as hydrophobic nature enable close packing of the alanine in the helix formation. Hydrophilic amino acids such as glutamine and serine were also highlighted in different studies as key residues for alpha helical linkers(Jeong et al., 2016; Lee et al., 2013). The polar side chains can contribute to the stability of the helix through hydrogen bonding. To engineer a rigid alpha helical linker for fusion proteins, length, flexibility and solubility of the primary sequence are considered as critical optimization points. In this research, different lengths of QAAS peptide repeats (short, medium and long) were tested for their capacity to form rigid alpha helices between two protein domains.

Fructose-6-phosphate aldolase (FSA) as a carrier for small proteins in CryoEM

Fructose-6-phosphate aldolase (FSA) from *Escherichia coli*, is an enzyme presumably involved in the pentose phosphate pathway, although a definitive physiological role has not been conclusively determined. FSA belongs to the class I aldolases catalyzing aldol addition and retro aldol cleavage reactions and is of high interest in synthetic chemistry(Schürmann & Sprenger, 2001). Structurally it is composed of ten identical subunits forming $\alpha_8\beta_8$-barrel structures in each subunit(Cornelius et al., 2023). It was selected as scaffold protein firstly, because the structure is highly symmetric which is a merit for CryoEM data analysis. Secondly, there is an alpha helix sticking out at the C-terminal of each subunit to the next. The helix forms a basis for attaching a small protein via a helical linker.

In this research we used a small domain from a plant phototropins, the Light Oxygen Voltage domain of *Avena sativa* (AsLOV2) PDB ID: 7PGY, UniprotKB; 049003, 404-546 amino acid range (Dietler et al., 2022). The LOV2 domains occur as modular domains in plant phototropins. They are a subclass of the Per-Arnt-Sim (PAS) domains which respond to blue light and changes in redox environments.(Flores-Ibarra et al., 2024; Zayner et al., 2019a). The selected AsLOV2 domain binds the FMN cofactor in its chromophore which absorbs blue light(Dietler et al., 2022; Flores-Ibarra et al., 2024). Illumination with blue light triggers structural rearrangements in the chromophore which start with formation of a reversible thiol-adduct bond between FMN and a conserved cysteine residue(Iwata et al., 2002; Swartz et al., 2001). The photocycle has been extensively studied and has led to selection of LOV domain in optogenetic studies(Kalvaitis et al., 2019; Pudasaini et al., 2015). The AsLOV2 domain has previously been a model for time resolved crystallography studies and different structures of the domain are solved via crystallography as well (Berntsson et al., 2017; Halavaty & Moffat, 2007). In addition, the domain has an N-terminal helix that could be linked to the C-terminus of the scaffold carrier. The chromophore gives the protein a visible yellow color which is a physical characterization of the sample. The FSA/LOV pair was thus considered a good model system for methods development as described in the featured research articles summary below.

OBJECTIVES

1. To enhance structural data acquisition of small proteins using CryoEM.
2. To improve structure resolution in the use of Time Resolved WAXS as a method of studying transient states of small proteins during a signaling event.

PUBLICATION SUMMARIES

PAPER 1.

Background

In this work, a strategy for imaging small proteins using Cryo electron microscopy by combining a stable carrier protein scaffold with a smaller protein is discussed. Small proteins can be described as those with less than 100 kDa, that occur as single or modular domains as well as microproteins of ≤50 amino acids in the context of CryoEM. Traditionally, structures of small proteins (<100 kDa) have been acquired through X-ray crystallography or NMR. Some of these proteins present challenges during structure acquisition due to occurrence of flexible loops. Strategies to overcome molecular weight limits to enable imaging of also smaller proteins in CryoEM has caught researchers' attention recently. Some of the invented strategies include the use of target specific affinity tags in the form of Fabs, synthetic antibodies and DARPins to link a smaller protein of interest to the carrier scaffold(S. Wu et al., 2012; X. Wu

& Rapoport, 2021; Yao et al., 2019). More recently, the approach to use exposed terminal alpha helices in the target and carrier proteins have taken lead. Here, we applied this strategy using a carrier protein (FSA, decamer of ~220 kDa) with extended C-terminal helices, and connected a protein domain (AsLOV2, 18 kDa). The FSA-AsLOV2 complexes were connected via helical linkers between the protein domains.

Selecting inter-domain linkers

A peptide unit was selected for linking the two proteins. The peptide sequence, QAAS, was chosen based on the α-helix forming propensity of included amino acids, hypothetically leading to a relatively rigid linkage. Alanine has been reported to contribute to stability of alpha helical interdomain linkers due to its small methyl side-chain which enhanced proper folding without interfering with other stabilizing salt bridges or hydrogen bonds in the linkers(Arai et al., 2001; Chakrabartty et al., 1994). Glutamine and serine, at positions *i* and *i*+3, were included to enhance helix stability through their hydrophilic side chains, allowing for solvent interactions and solubility. The QAAS peptide unit was assembled in tandem to generate short^{QAAS}, medium^{QAASQAAS} and longer ^{QAASQAASQAASQAAS} linker lengths. A previous study defined short alpha helix protein linkers as those with five amino acids and below, medium linkers were defined to have higher than five residues and below ten. Long linkers completely separating protein domains were defined to have upto twenty amino acids(Chen et al., 2013).

Chimeric protein constructs

Five different fusion constructs were constructed. FSA-AsLOV2, in which the smaller protein was attached directly to the C-terminal amino acid without a linker. FSA-TS-AsLOV2, in which the linker consisted of only two amino acid residues (Thr-Ser), FSA-QAAS-AsLOV2, FSA-(QAAS)2-AsLOV2 and FSA-(QAAS)4-AsLOV2, in which 1.1, 2.3 and 4.6 (assumed) helical turns separated the FSA carrier and the AsLOV2 domain.

In the construct design, a custom synthesized AsLOV2 gene flanked with correct restriction sites at the N and C-terminal was used. Restrictions sites used here were *Spe*I endonucleases (from *Sphingomonas paucimobilis)* whose restriction site is 5'-ACTAGT-3' and *Hin*dIII (from *Haemophillus influenzae)* which recognizes 5'-AAGCTT-3'(ThermoFisher Scientific, n.d.-b, n.d.-a). The enzymes selection was guided by the vector plasmid map which contained the palindromic sites for the two restriction enzymes sites at the C-terminal of the FSA sequence where the linkers and the AsLOV2 gene was to be inserted. Restriction enzymes creating sticky ends are widely preferred in generating recombinant DNA due to easier repair activity by DNA ligase(Di Felice et al., 2019; Pingoud et al., 2005). Cleaved plasmids were purified in agarose gel, excised and extracted using a commercial kit (Qiagen). DNA fragments for the linkers and the AsLOV2 gene were amplified using PCR and in *E. coli* XL1 blue competent cells. Ligation was performed in the presence of T4 DNA ligase that catalyzes the ATP dependent formation of phosphodiester bonds in 3'-hydroxyl group of one nucleotide to the 5'-phosphate group of another, repairing nicks in DNA strands. The ligated plasmids were amplified in *E. coli* XLI-Blue and the constructed gene fusions were confirmed by DNA sequencing. In the case of the FSA-AsLOV2 plasmid the ligation was performed using Phusion PCR in combination with polynucleotide kinase.

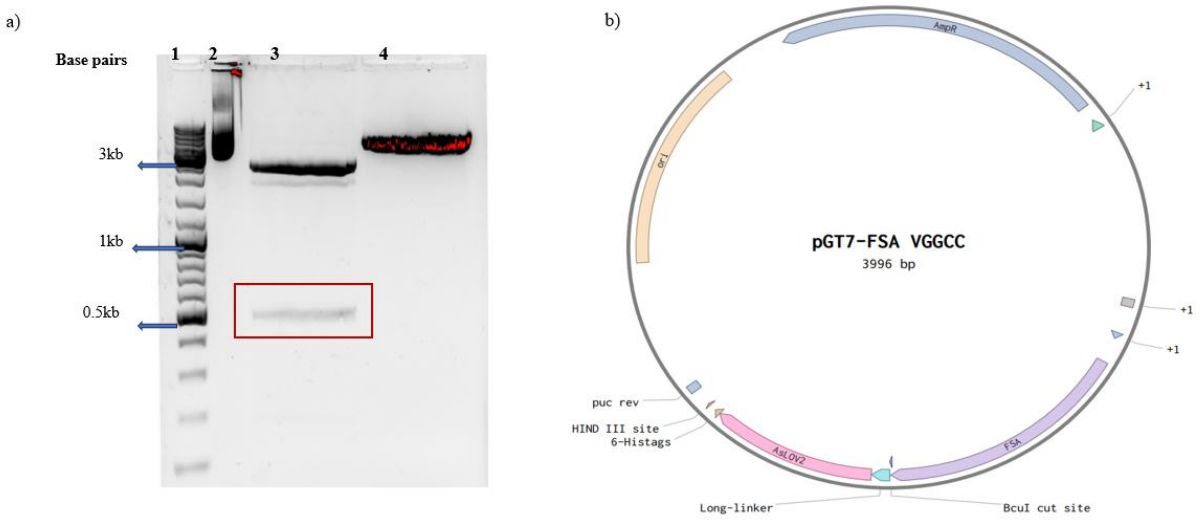

Figure 1. a) Digested plasmid DNA separated by agarose gel electrophoresis during cloning. Lane 1. DNA bp marker, 2. Undigested (supercoiled) plasmid (control), 3. *Spe*I (*Bcu*I)/*Hin*dIII Digested plasmid with AsLOV2 insert (0.5kb band) and 4. Digested vector plasmid continuing native FSA. b) Example plasmid map of FSA-TS(QAAS)4-AsLOV2 cloned construct.

Protein expression and purification

The constructs were transformed into *E. coli* BL21-AI which allows for induction by L-arabinose of the T7 phage RNA polymerase gene. The T7 RNA polymerase would then bind the T7 promoter upstream the gene of interest in the vector. Expressed proteins were purified by immobilized metal ion (Ni(II)) affinity chromatography (IMAC) and size-exclusion chromatography after the necessary lysis steps as described in the manuscript. The molecular weight of a single subunit of the hybrid proteins were approximated to be 25 kDa following SDS-PAGE. The molecular weight of native FSA single subunit has been determined to be ~22kDa via mass spectrometry(Schürmann & Sprenger, 2001). Size exclusion chromatography suggested a complex of molecular weight ~400 kDa according to the protein markers in the column description(Cytiva, n.d.) suggesting decameric oligomers.

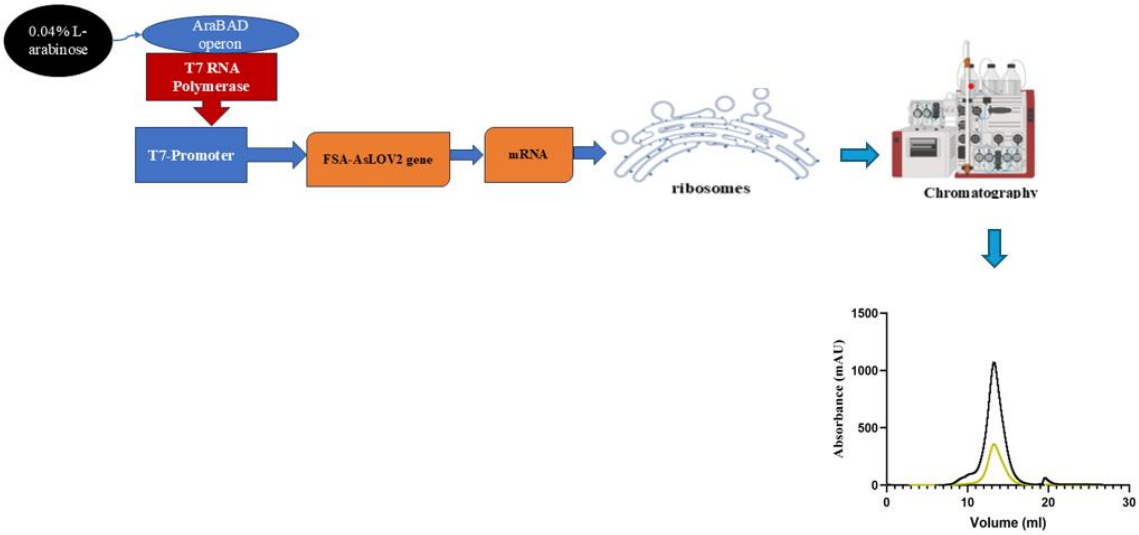

Figure 2. Flow chart of protein expression in BL21-AI cells which contains the arabinose inducible T7 RNA polymerase gene replacing the araB gene of the araBAD operon. Protein expression was induced by adding 0.04 % (w/v) L-arabinose at early lag phase (OD_{600} ~0.4). Purification was done using affinity (nickel immobilised-metal ion chromatography) and size exclusion chromatography.

AsLOV2 folding and FSA activity tests

In order to ascertain that the AsLOV2 domain was properly folded in the fusion protein, a photoactivation cycle test of the FMN chromophore was conducted. The FMN cofactor absorbs light at 450 nm. A diode emitting blue light was used to illuminate the FSA-$(QAAS)_4$-AsLOV2 sample. A time resolved spectroscopy data series depicted the chromophore unfolding in which the FMN forms a covalent bond with a conserved cysteine residue in the protein domain leading to decrease in absorbance at 450 nm(Berntsson et al., 2017; Konold et al., 2016). The chromophore was restored into its ground state by stopping the irradiation and recording the regeneration spectrum in the dark (Paper I). These experiments supported proper folding of the AsLOV2 domain since that is necessary for functional chromophore insertion and photo responsiveness.

The catalytic retro aldol cleavage activity of FSA-$(QAAS)_4$-AsLOV2 protein was confirmed by recording the consumption of NADH in a coupled reaction mixture with glycerol-3-phosphate dehydrogenase, in the presence of fructose-6-phosphate (F6P) and triose phosphate isomerase (TIM). Cleavage of F6P generates equimolar mixtures of glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), equilibrated by TIM, where the DHAP undergoes reduction in the coupled reaction.

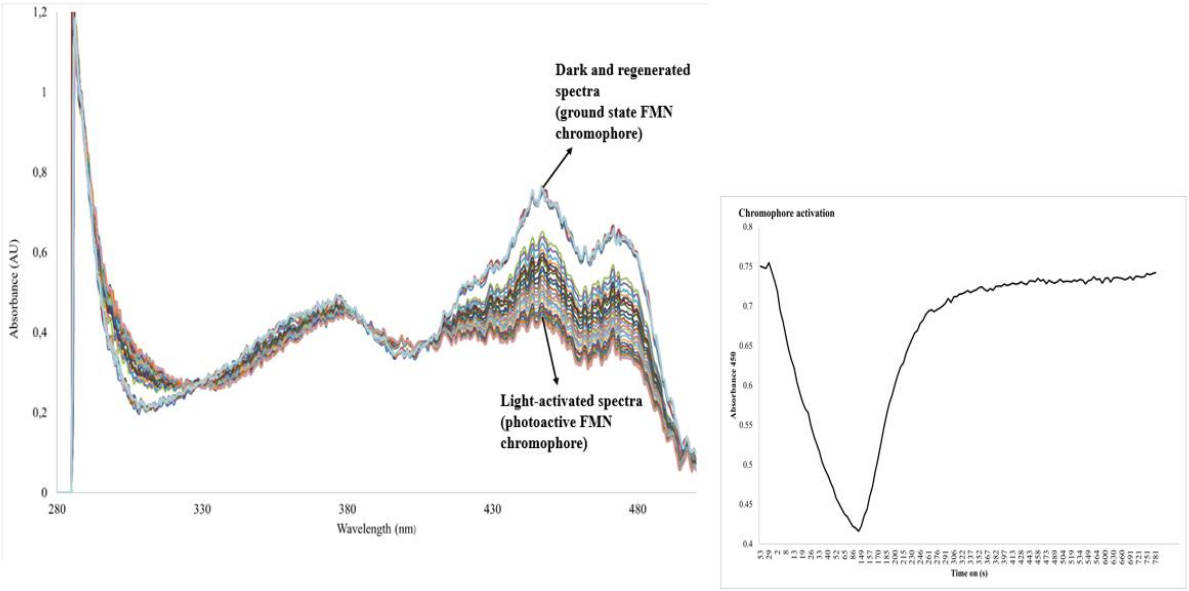

Figure 3. Time series of activation and deactivation of the FMN chromophore in the AsLOV2 domain of FSA-(QAAS)4-AsLOV2. When irradiated with blue the chromophore underwent a conformational change that led to absorbance at 450 nm decreasing. The chromophore photoactivation occur within 200 seconds (inset). Upon switching the blue light irradiation off, the chromophore reverted back to its ground state. The different spectra represent the excitation and regeneration of the chromophore to its ground state as observed.

CryoEM screening

Grid screening suggested small particles with undefined symmetry when compared to previously collected data on native FSA(Hebert et al., 2024). Particles of different fusion proteins were overall smaller than expected, suggesting an abundance of lower oligomeric states than the expected decameric structure (expected cross section ~170 Å). Processed 2D classes were also smaller due to challenges attributed to particle picking since the recorded micrographs were different from the decameric structure of FSA. The processed CryoEM map density using C1 symmetry showed resemblance to the predicted monomeric version of the fusion protein which led to conclusion that the decameric quaternary structure was not retained. A detected signal at the periphery of some 2D classes further suggested that flexibility of linkers could have lead to lack of uniform symmetry in the particles or aggregation of the complexes on the CryoEM sample grids. Attempts to improve the folding included, as previously mentioned, direct fusion of the FSA-AsLOV2 proteins to minimize flexibility in the joined regions at the C- and N-termini of FSA and AsLOV2 structures, respectively. The protein was expressed and directly frozen on grids to omit any aggregation due to storage. Grid screening suggested similar particle size when compared to fusion proteins in thin ice. Squares with thick ice had different particles with different symmetry which suggested different folding of the FSA-AsLOV2 sample or large protein aggregates.

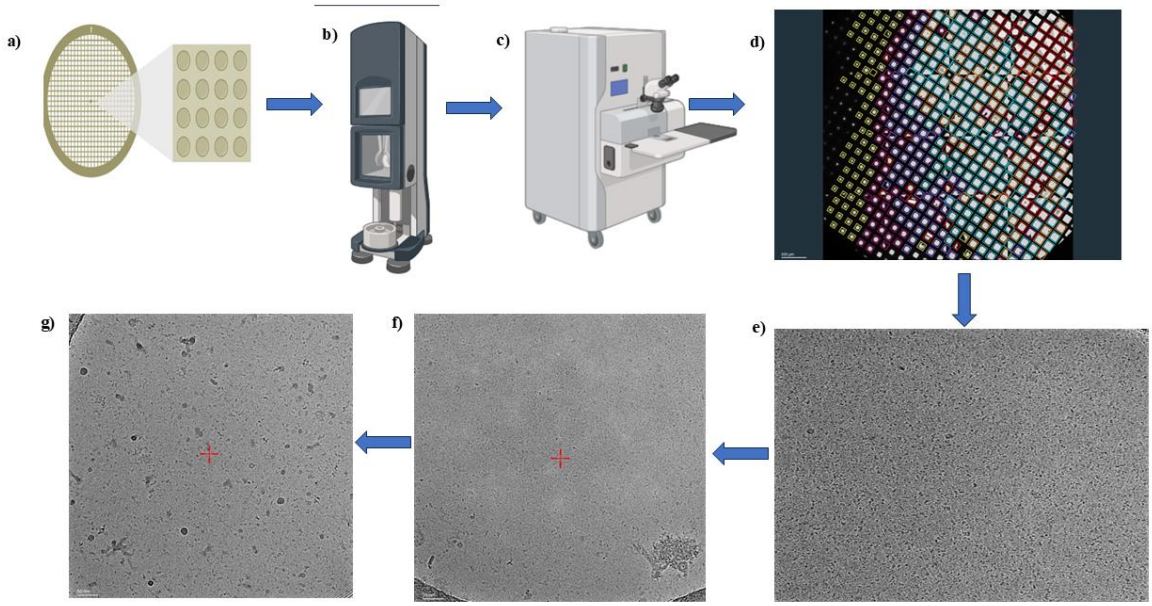

Figure 4. Flow chart of the applied CryoEM process from sample vitrification to data collection. a) sample application on grid, b) Vitrobot for plunge freezing the samples in liquid ethane, c) Glacios microscope, d) image of a grid during screening, e) micrograph illustrating the particle sizes observed for fusion proteins, f) micrograph of particles in thin ice and g) micrograph showing the contrast of particle size in thick ice. (image made in Biorender)

Dynamic light scattering (DLS) and thermal unfolding

DLS was carried out to ascertain the protein size of the fusion proteins following the CryoEM findings. The data suggested a shorter cumulant radius of the fusion proteins ~3 nm as compared to native FSA ~5.5 nm. This difference suggested that the chimeric complexes were smaller, or not of expected oligomeric structures. The FSA-AsLOV2 sample displayed a cumulant radius of ~9 nm which suggested a different protein folding that was not globular as well as recorded protein aggregated (> 40 nm). A limited buffer screen in which FSA-AsLOV2 was stored either in 40 mM bicine (pH 8.4) or 100 mM sodium phosphate (pH 7.4) suggested better stability in the phosphate conditions as judged by DLS. Thermal unfolding data revealed that the fusion proteins unfolded at melting temperatures of approximately 56 °C, where native FSA showed melting at 80 °C. The difference in melting temperatures suggested protein of lower molecular weight (monomers). The cumulant radius of the FSA together with its unfolding data correlated with previously determined mass and thermal stability(Schürmann & Sprenger, 2001). This data suggested that the chimeric proteins were of lesser molecular weight than the expected homo-ologomeric structure. It inferred that experiments that could stabilize the ologomeric state of the expected chimeric proteins were necessary before CryoEM.

PAPER 2.

Summary

This paper demonstrates the use of MHz repetition rates at X-ray free Electron lasers (XFELs) to probe microsecond structural changes in protein scattering. To achieve this, the Jα helix of AsLOV2 domain was used as a model. It is a 20-25 amino acids long helix at the C-terminal of the domain (spans residues 520-546) (Konold et al., 2016; Zayner et al., 2012, 2019b). It is the key signal transduction helix that undergoes structural changes when the FMN chromophore is activated by blue light(Konold et al., 2016). The unfolding of the Jα helix activates downstream kinase activity and is key in engineering light sensitive proteins(Lungu et al., 2012). At the XFEL one can acquire time resolved data by using either "pump probe" mode or reading out multiple probe pulses after a reaction trigger. In this research, time resolved wide angle X-ray scattering (TRWAXS) was used to test and verify the efficiency of acquiring time resolution data from AsLOV2 using the latter scheme. TRWAXS can be used to uncover conformational changes in protein structures in their native state at near atomic scale details upon modelling the recorded curves to a previously modelled/solved structure.

During data acquisition, sample was carried to the X-ray interaction point using a liquid jet through a 3D-printed gas dynamic virtual nozzle. Activation of the AsLOV2 chromophore was by a nanosecond laser at the start of every X-ray series (Figure 1 in Paper 2). Q-range (scattering vector length which determines the length scale that can be probed) of the collected was within 2.1 $Å^{-1}$ to 0.08 $Å^{-1}$. Upon removing data with discrepancies, changes in the data could be detected within q-values up to 1.5 $Å^{-1}$. The data had an exceptionally low noise signal 0.001%, when compared to previous research. This was as a result of the large number of recorded images that were averaged as well as use of high X-ray energy. This low noise level is highly significant in determining structural changes at microsecond level. The Jα unfolding was inferred into a A-B-C model sequential structural changes (Figure 2 in Paper 2). It was deduced that structural conformational change in state C was comparatively larger than in states A and B. State C was thought to be the unfolded state while state A emerged at the first 1.77 μs in correlation with the FMN-cysteinyl adduct formation in previous photoactivation studies(Konold et al., 2016; Pudasaini et al., 2015). State B was inferred to be a novel intermediate formed after the FMN-Cys adduct.

The TRWAXS utilising MHz at XFELs demonstrate the significance of this method in uncovering transient states during a signalling event in small proteins. In this study, the data had low noise levels contributed by the collection of large number of images per pulse rate. This data acquisition scheme and analysis facilitated the discovery of new intermediate states in the Jα unfolding of AsLOV2. TRWAXS thus can be complementary method when studying microsecond/millisecond signalling events in both large and small proteins. Thousands of recorded images using the MHz repetition at the XFELs enhance discovery of new transient states in triggered reactions; which can be crucial in structure-based drug discovery studies.

DISCUSSION

In this thesis I have investigated two methods that can be used to probe and solve structures of small proteins. CryoEM, can provide a viable alternative for acquiring near atomic resolution data of small proteins that may be difficult to study using crystallography or NMR. The size restriction barrier of CryoEM has been challenged with proteins < 200 kDa being successfully imaged and structures obtained to identifiable resolution(Merk et al., 2016). The strategy covered in the first paper, utilizes the ability of linking a small protein domain onto a homo-oligomeric scaffold via an alpha helical extension. In this strategy, rigidity between the two protein domains is important. Therefore, optimizing the peptide linker length and composition is a vital step. The linker should be able to allow independent oligomerization of the chimeric protein. If one succeeds in obtaining chimeric protein size and symmetry should be determined prior to CryoEM imaging. Size determination can be carried out via gel filtration and dynamic light scattering. On the other hand, negative EM staining is a tested method for determining particle symmetry. Once the particle orientation is predetermined, one can proceed to sample vitrification and microscopy. To improve resolution of small protein targets, thin ice layer optimization, use of 300 keV microscope, Volta phase plates technology and gold grids for sample vitrification are some of implementations that can help improve signal-to-noise ratio(M. Wu & Lander, 2020). Future studies should focus on methods of engineering rigid interdomain alpha helical linkers that can be compatible with different protein targets, oligomeric base proteins with preferred symmetry and alpha helical extensions in the C terminal. Other recommendations include methods development for assuring robust oligomerization, retaining structural rigidity and symmetry of constructed chimeric proteins prior to microscopy.

The discussed TRWAXs utilizing MHz XFELs is a significant method for enhancing acquisition of transient states in enzyme catalyzed reactions or folding and unfolding of small proteins modulating a signalling event. The data acquisition strategy employing delivery of thousand of X-ray pulses at high repetition rates enables probing of microsecond transition details during chemical reactions. It can be widely applied in studying domains involved in major signalling pathways and this can provide highly accurate data that can be vital for drug discovery studies.

CONCLUSION

Small proteins that occur as modular domains mediate vital roles in cell physiology by activating or enhancing stability of other proteins (usually larger). Structure acquisition of these proteins is vital in structure-activity relationship research in drug discovery. The discussed methods can be applied to solve structures of small proteins of biological importance that are annotated in different databases but lack structural data. It is vital to combine hardware improvement of old methods as well as new strategies to improve structure resolution. Computational methods such as AlphaFold can also be applied at beginning of CryoEM studies for engineering scaffolding strategies to direct the choice of carrier protein.

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