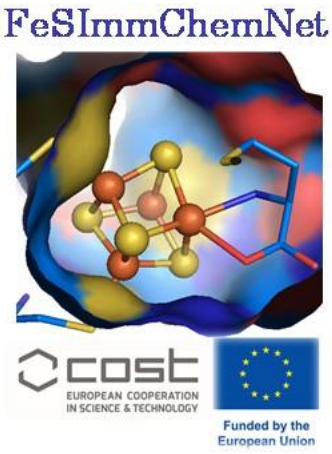

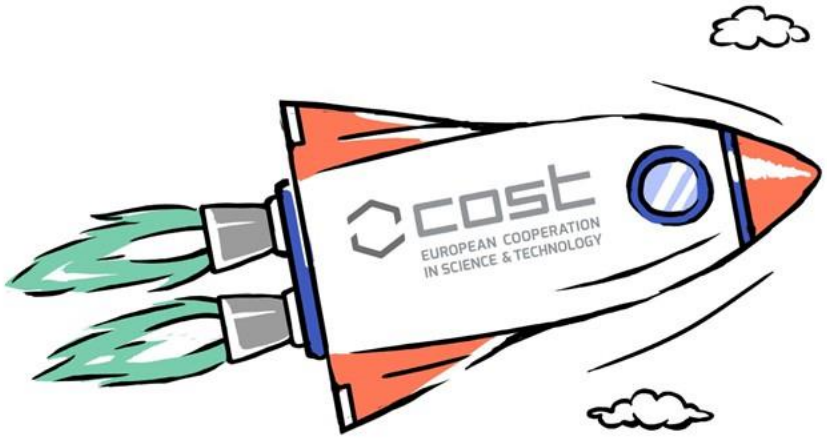

COST Action CA21115

Report date: November 2024

WG2. FeS Clusters in Viral Replication

General description: This report describes the protocols developed and used by Action members to study and work with FeS proteins involved in viral replication

Period: Nov 2022 – Nov 2024

Section 1. Computational methods to study human FeS protein SAND involved in restricting viral replication

Protein-ligand docking is a computational technique used to predict the preferred orientation of a ligand when bound to a protein target, often with the goal of drug discovery. It simulates how a small molecule (ligand) fits into a binding site on a protein. This technique can also be used to insert cofactors in incomplete protein structural models. Within this COST- Action we have mainly employed the AI-based Alphafill tool as well as a flexible docking algorithm implemented in the Autodock Vina program to generate holistic structural models of the human radical S-adenosylmethionine-dependent nucleotide dehydratase (SAND) harboring the radical S-adenosylmethionine (SAM) moiety and the nucleoside CTP ligand. Protein-ligand docking algorithms can be integrated with classical molecular dynamics (MD) simulations to refine structural models, enhancing the accuracy of predicted binding poses by accounting for dynamic interactions and conformational flexibility.

Paper 1

Nghi Thao Hoang, Deborah Grifagni, Meritxell Wu Lu, Yujie Sheng, Theo Situmorang, Pei-Hsin Tai, Astrid Maluta, Mohammed Hakil, Yvain Nicolet, Shahram Kordasti, Peter-Leon Hagedoorn, Maria-Andrea Mroginski, Simone Ciofi-Baffoni, Kourosh H. Ebrahimi . Discovery of the Electron Transfer Partner of the innate immune antiviral radical-SAM enzyme**. Manuscript in preparation**

Section 2. Discovery of FeS enzymes producing antiviral lead molecules inhibiting viral replication

A novel assay was developed by Ebrahimi lab at King's College London. The assay named VITAS (Viral polymerase-Inhibition Toxin-Associated Selection). The VITAS assay is based on the hypothesis that an ANA is formed due to the expression and activity of an enzyme in E. coli. The ANA inhibits viral T7 RNA polymerase (Pol)-mediated expression of a toxin protein allowing cell growth. The VITAS assay fundamentally differs from the commonly used live/dead assays in drug discovery. Traditional assays rely on chemical or biological labelling of cells during/after treatment with known purified lead molecules synthesised either chemically or using a purified enzyme. Moreover, purifying oxygen-sensitive radical-SAM enzymes is not straightforward, limiting the use of in vitro assays. These commonly used assays cannot easily be adopted for protein engineering to rapidly screen the activity of enzyme variants in a large library. In sharp contrast, the VITAS assay eliminates the need to purify enzymes and the NPs. Therefore, it enables the screening of enzymes' activity, facilitating the mining of the repository of natural enzymes and protein engineering to discover new ANAs. The VITAS assay is sensitive to the SANDs' activity, unlike the previously reported fluorescence-based assay showing similar activity for human and microbial SANDs.

Paper

Alharbi, A. F., Kim, H., Chumroo, D., Ji. Y., Hakil, M., Ebrahimi, K. H., VITAS, a sensitive in vivo selection assay to discover enzymes producing antiviral natural products, **CHEMICAL COMMUNICATIONS** 2023,59, 5419-5422

Section 3. Protocol for protein production and purification (according to ref [1])

Genes cloning and protein production

The *R633b* gene was amplified from mimivirus genomic DNA. The PCR product was inserted by In-Fusion HD cloning kit (Takara) into an in-house-modified pETDuet expression vector (Novagen), between the *BamH*I/*Not*I restriction sites, allowing the production of the protein fused with a cleavable N-terminal His_6-SUMO tag. A human rhinovirus 3C (Prescission) protease cleavage site allowed tag removal. The cloning was controlled by sequencing (Eurofins).

The genes encoding the Mg762, mm_612b, and ma671a proteins were amplified from megavirus chiliensis, moumouvirus maliensis, and moumouvirus australiensis genomic DNA respectively. The genes encoding the tupanvirus soda lake QKU35266 and moumouvirus (unannotated gene, Genbank ID: JX962719.1, sequence regions 770054 to 770236) proteins were synthetically made (GenScript). They were then cloned as described for *R633b*.

The vectors were transformed in *E. coli* Rosetta (DE3). Cells were grown in 2YT medium containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol at 37 °C to an A_{600} of ~0.7 when the culture temperature was shifted to 17 °C. Protein production was induced at A_{600} ~1, by the addition of 0.5 mM IPTG. Cells were grown 16 h after induction and harvested by centrifugation at 6000 *g* for 15 min at room temperature.

Protein purification

All purification steps were performed in aerobia, unless otherwise mentioned. For each produced protein the purification was made in two steps with the appropriate resuspension buffer. Bacteria pellets were resuspended in buffer A (50 mM Tris-HCl, 300 mM NaCl, pH 8.5 for R633b and Mg762

proteins, and 50 mM Tris-HCl, 150 mM NaCl, pH 9.0, for AUL79376.1, mm_612b, ma671a, and moumouvirus proteins, containing 0.1 mg/mL DNase, 0.1 mg/mL lysozyme, 0.2% Triton X-100 and antiprotease tablets (Roche Applied Science). Cells were lysed by 6 cycles of 3 min sonication. The lysate was clarified by centrifugation at 13,000 *g* for 45 min at room temperature (RT) and filtrated through a 0.2 µm PES filter (Millipore) before being loaded on Ni-NTA resin (Thermo Scientific) pre-equilibrated with buffer A. The column was washed with 10 column volumes of buffer A and 10 column volumes of buffer A containing 50 mM imidazole. Elution was performed by steps of 5 column volumes at 0.25, 0.5, and 1M imidazole. The protein was eluted during the 0.25M step. All fractions were controlled by SDS-PAGE electrophoresis. All the purification steps were performed at room temperature (RT), as the protein did precipitate under 10°C.
Microdialysis tests were performed using different buffers, which returned that the protein was soluble in CAPS pH 10.5 buffer. Thus, the purified tagged protein (~20 kDa) was dialyzed on a 12.5 kDa cut-off, regenerated cellulose membrane (Spectrum), in 50 mM CAPS pH 10.5 for 16h at RT, and concentrated on a 30 kDa concentrator (GE Healthcare) at RT.

Paper

Villalta, A.; Srour, B.; Lartigue, A.; Clémancey, M.; Byrne, D.; Chaspoul, F.; Loquet, A.; Guigliarelli, B.; Blondin, G.; Abergel, C.; Burlat, B., Evidence for [2Fe-2S](2+) and Linear [3Fe-4S](1+) Clusters in a Unique Family of Glycine/Cysteine-Rich Fe-S Proteins from Megavirinae Giant Viruses. *J. Am. Chem. Soc.* **2023,** *145* (5), 2733-2738.