

Tackling crystallization issues of challenging targets: Three case studies from Novartis Basel

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Introduction

At the interface of biology and chemistry, the Structural Biophysics Department at NIBR Basel delivers structural information on target proteins and their interactions with ligands to guide and support integrated lead finding and optimization.

Our success relies on the availability and quality of crystals. Therefore we focus on the development of techniques to systematically screen and optimize protein crystals in an automated high throughput manner. For challenging targets we often have to face specific issues, which can occur at any step of the process from protein expression, purification to crystal diffraction. For those difficult cases, we use the available knowledge to identify the most suitable approach to obtain well-diffracting protein crystals.

The approaches can be addressed at different steps in the process:

- Cloning: limited proteolysis, entropy reduction, surface mutations
- Expression: solubility tags, co-expression with ligands in various hosts
- Purification: optimization and refolding, methylation, posttranslational modification
- Crystallization: screening methods, (matrix-) seeding, crosslinking, LCP
- Data collection: cryo optimization, in-situ diffraction tests

Here we present 3 techniques that allowed us to successfully determine the three-dimensional structure of challenging protein targets.

Limited proteolysis - Identification of promising protein domains for crystallization

Issue

- Multiple domain protein of large size (>130 kD).
- Constructs designed by in-silico methods do not yield crystals.
- Flexible domains and large loops prevent crystallization.
- No known ligands/ proteins to stabilize the protein conformation.
- Sensitive for protease digestion.

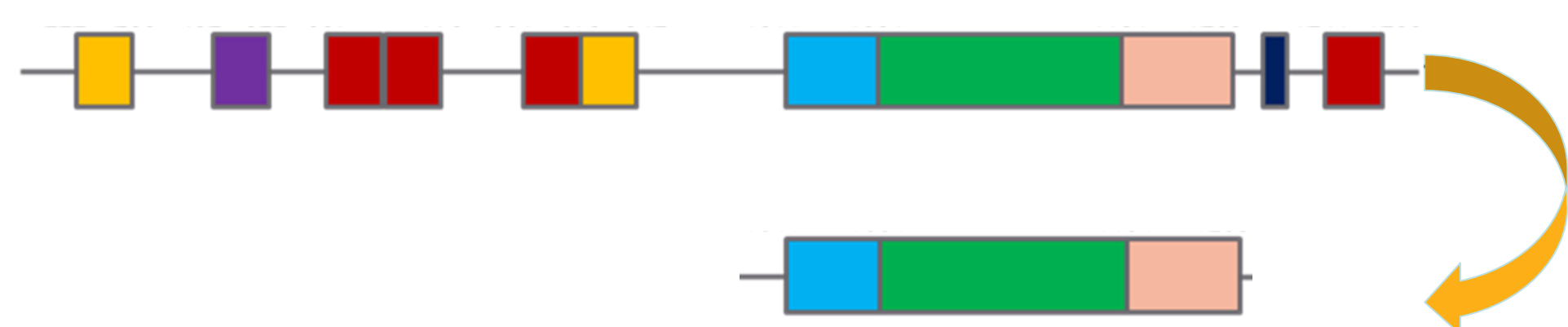


Figure 1: Full length protein and limited proteolysis construct after digestion

Strategy

- Limited proteolysis.
- Analysis by SDS-Page, characterization by mass spectrometry.
- Design of new constructs.
- Small scale protein purification to access protein yields and thermal stability by DSF.
- Crystallization trials.

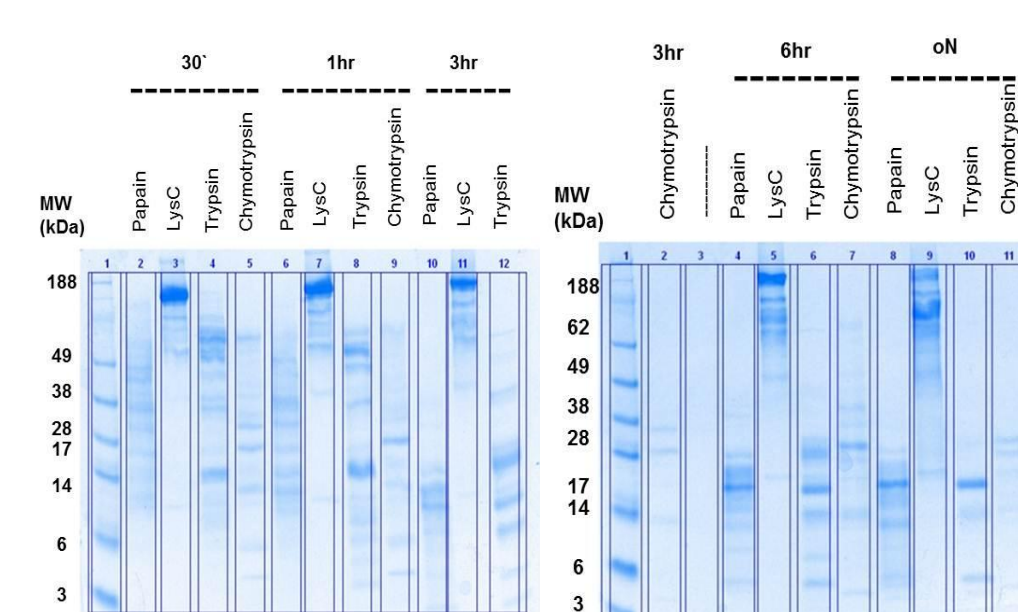


Figure 2/3: SDS-Page analysis of target protein. Comparison of several proteases for indicated times

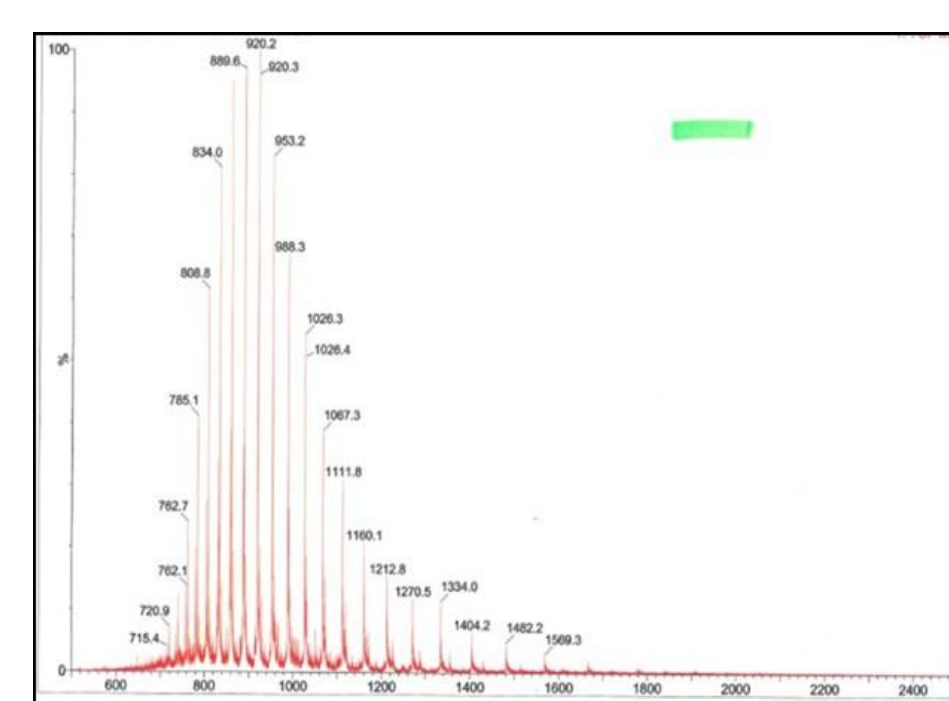


Figure 4: Mass spectrometry analysis for indication of domain boundaries.

Results

- Good quality crystals obtained in a short period.
- Easy implementation and use.
- Rapid method with adaptability for high-throughput analysis.



Figure 5: Protein crystals obtained with new construct designed. Protein diffraction of 2.3 Å

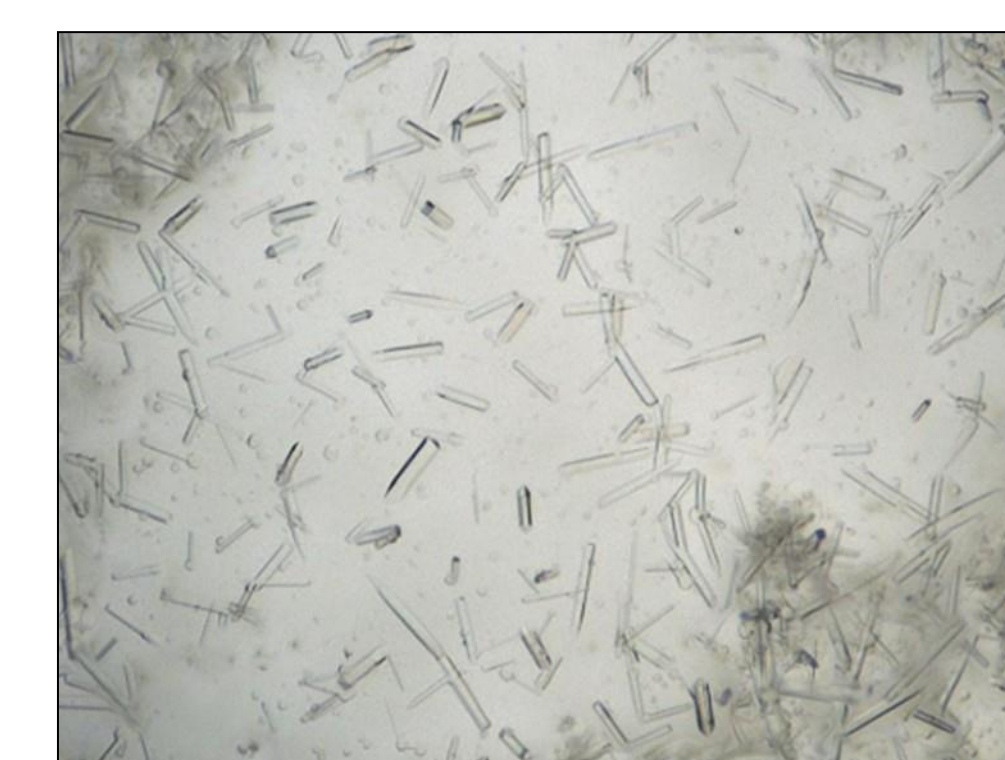
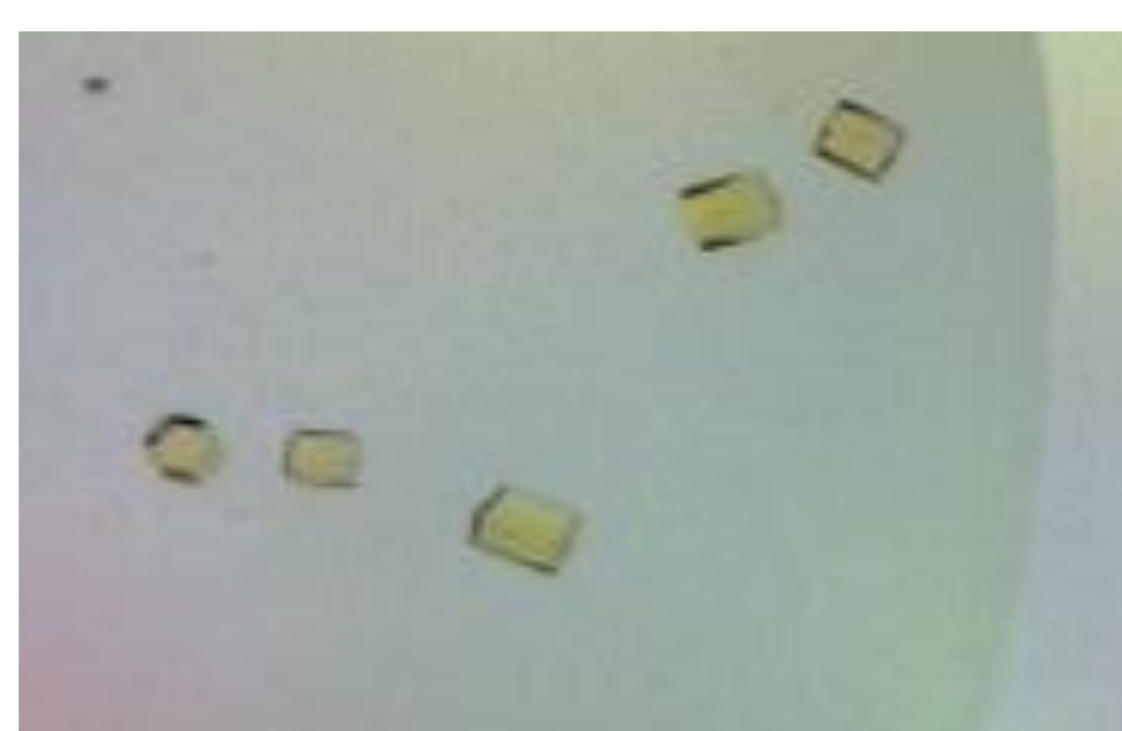


Figure 6: Further crystal optimization. Better crystal fishing and freezing.

SERM* (surface entropy-reduction mutagenesis) - Improving crystal resolution and quality

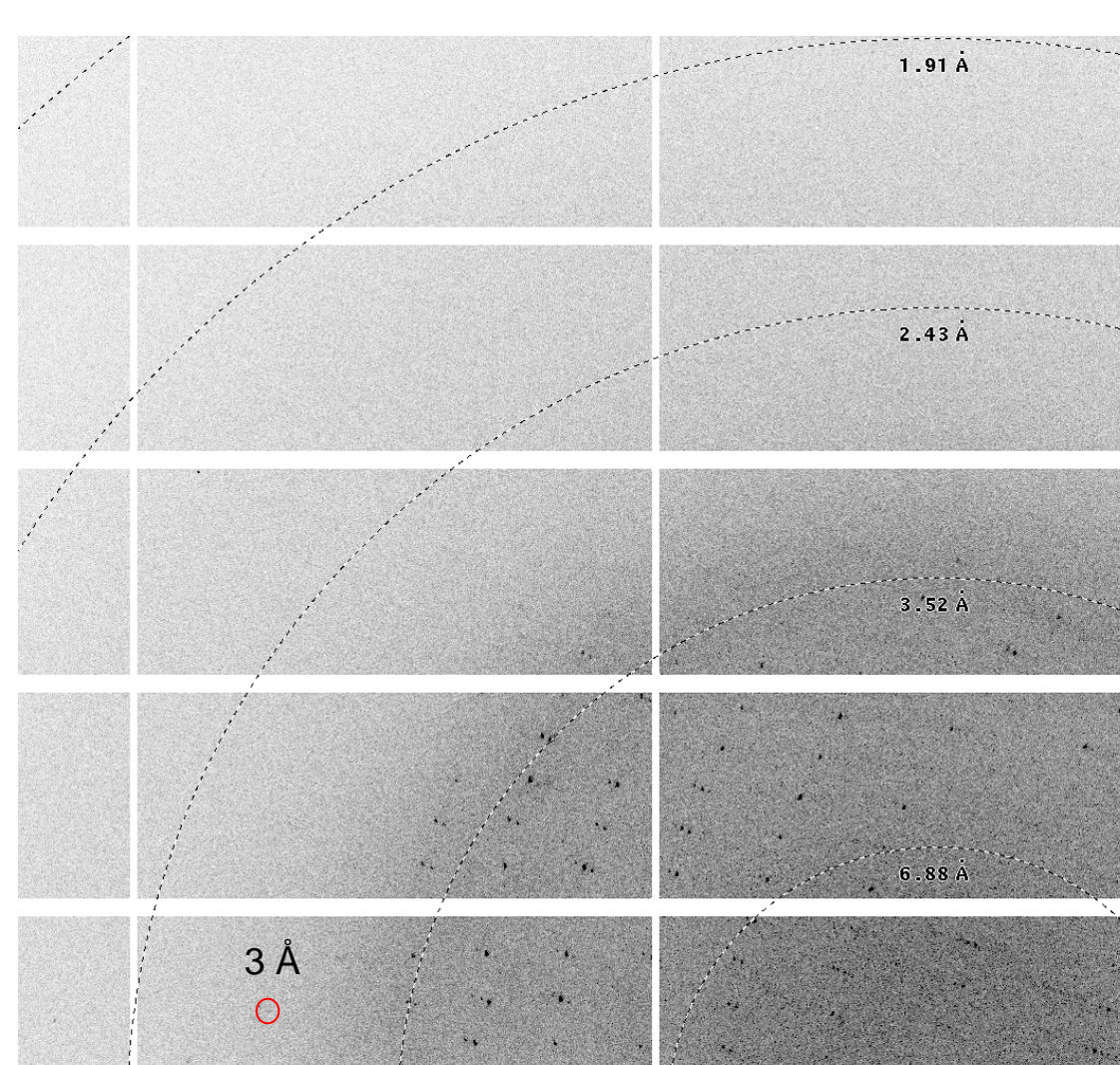
Issue

Kinase co-crystallized with many compounds: plenty of crystallization hits with nice looking crystals.



Poor crystal quality:

- 1 of 40 crystals diffract to about 3 Å.
- Laborious crystal testing necessary to obtain a low resolution structure.
- Structure refinement very time-intensive: low resolution and mostly 3-4 molecules/ ASU.



* Derewenda ZS, Acta Cryst. (2011). D67, 243-248

Goal & Strategy

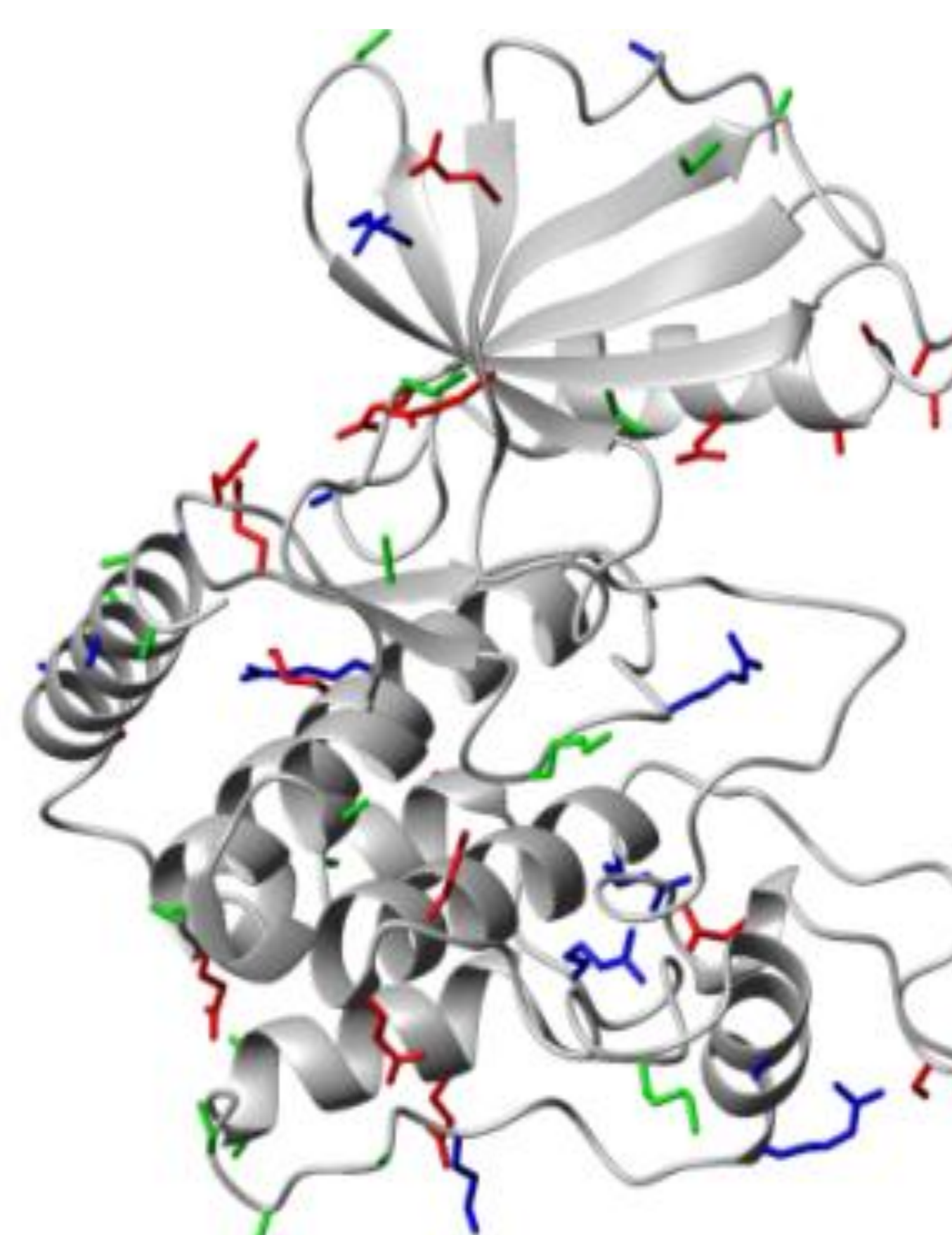
Improve crystal diffraction, make the system more efficient.

Engineered protein surfaces:

8 different mutants designed.

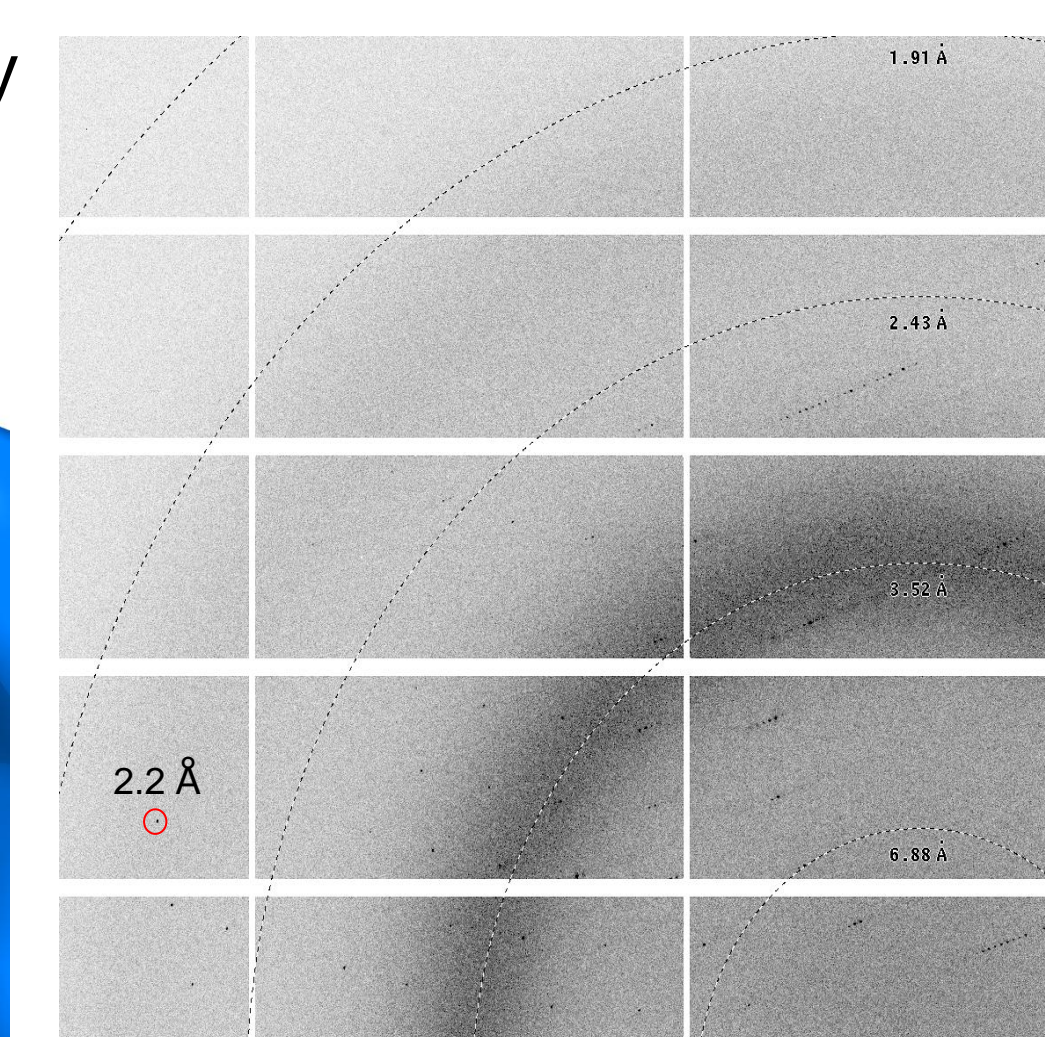
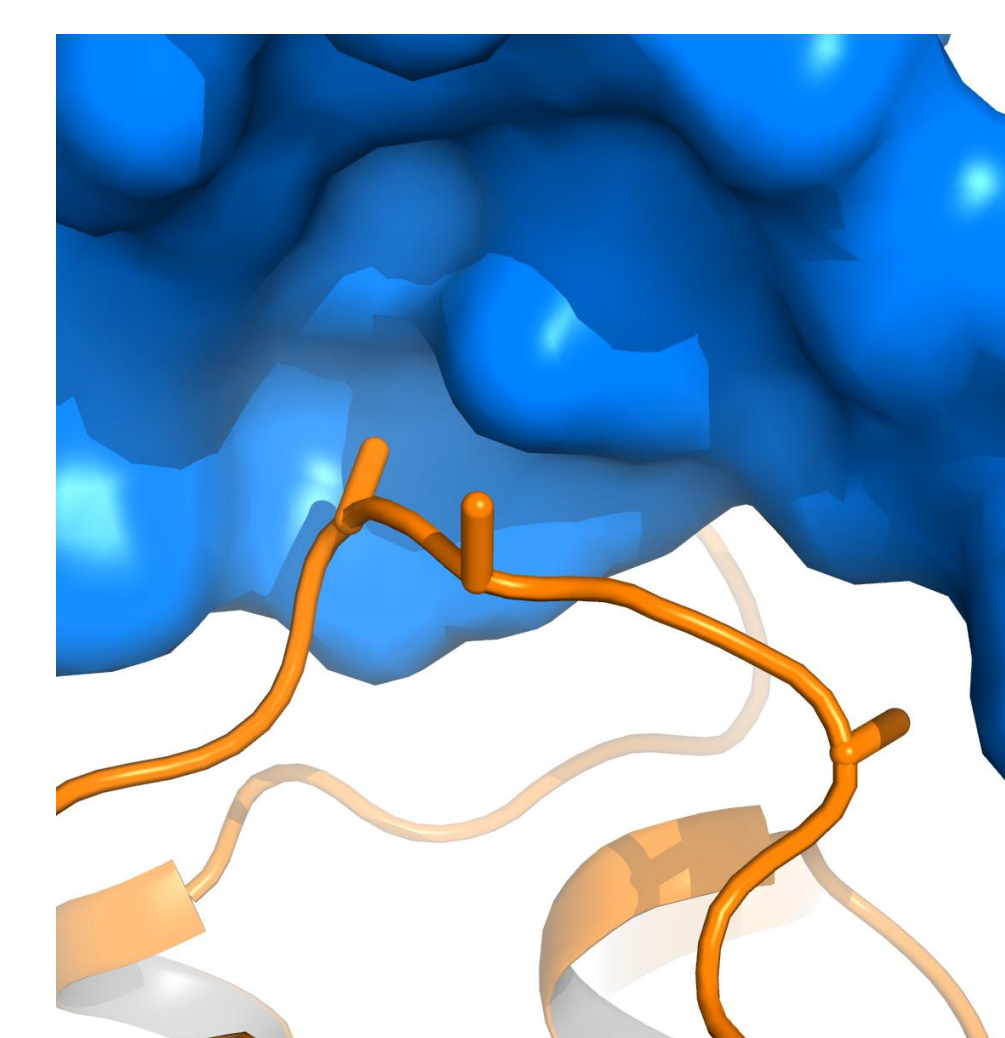
Cluster of 2-3 surface residues with high conformational entropy (K, E, R), replaced by a low entropy residue (A).

Higher propensity to mediate crystal contacts. Enhance protein crystallizability.



Results & Discussion

Mutant X gave significantly better resolution.



Crystal contact mediated by mutated patch of mutant X.

Validation mutant X by testing and reproducing existing structures:

- Massively improved resolution/ quality of diffraction data (3 Å to 2.2 Å on average).
- Improved efficiency in data collection.
- Often less molecules/ ASU – faster structure refinement.

The Histone-Methyltransferases family - obtaining cofactor-free crystallization-grade protein for structural and biophysical studies

Issue

- Obtain co-crystal structures of small molecules bound in the cofactor binding site.
- Compounds initially identified were unable to displace the cofactor: $K_{dSAM} \sim 300nM$ and $K_{dSAH} \sim 100nM$.
- All structures solved contained only SAM.

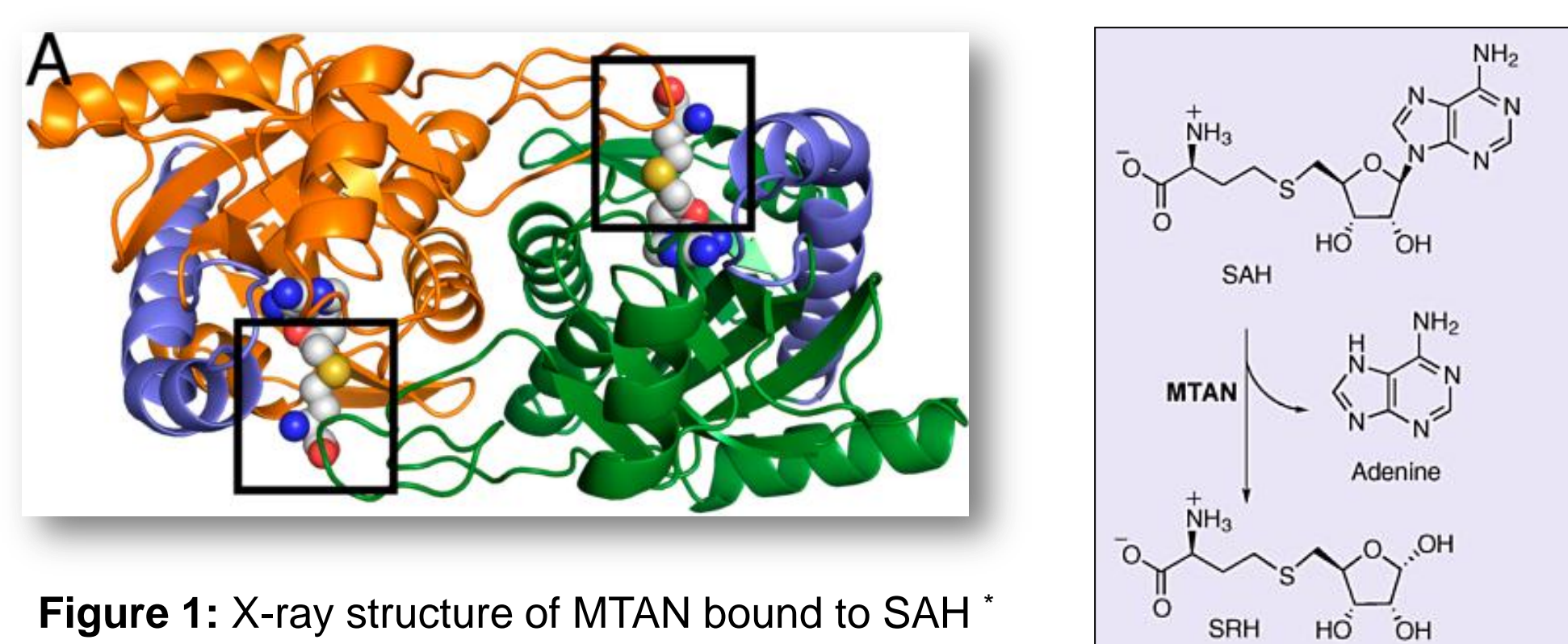


Figure 1: X-ray structure of MTAN bound to SAH

* Ronning, D., Biochemistry (2012) 51, 9763-9772

Strategy

- Family-based approach with different HMT targets.
- New purification protocol established using MTAN to hydrolyze SAH into Adenine and SRH, which prevent re-binding to the HMT.
- Co-crystallization screening performed with the «SAM-free» protein samples.

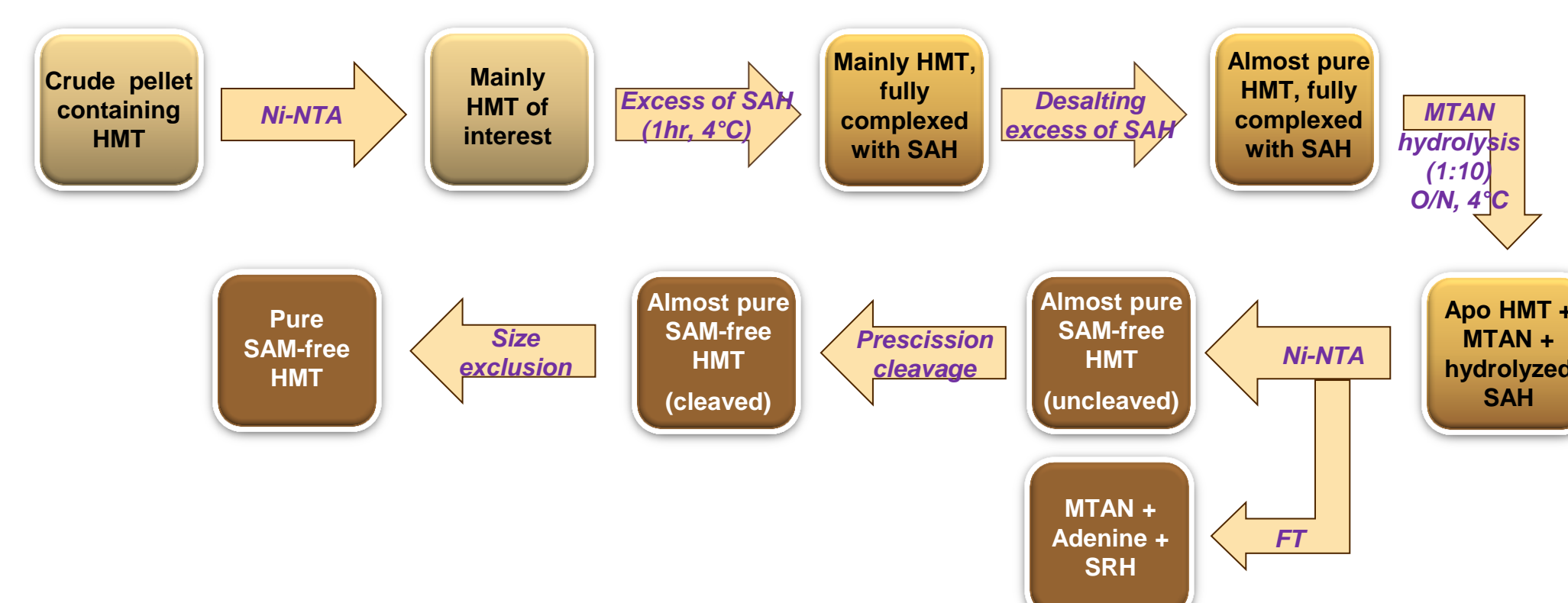
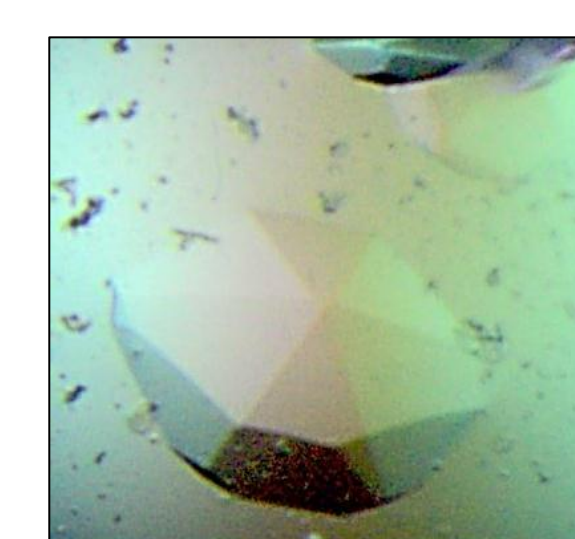


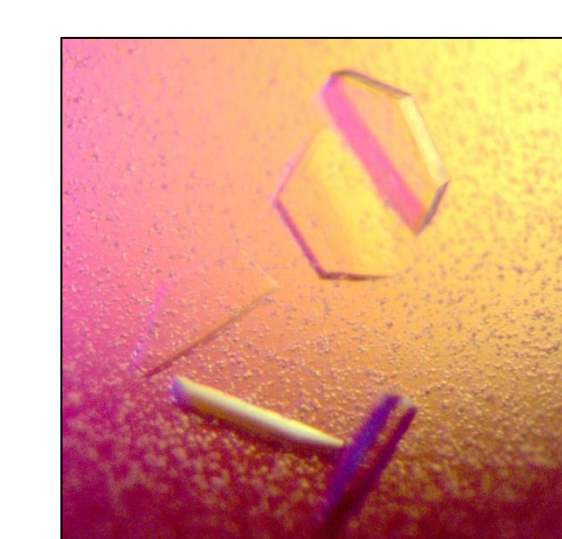
Figure 2: Optimized purification protocol to produce SAM-free protein

Results

- Initial difficulties to solve SMW-inhibitor complex structures, due to SAM blocking the binding site.
- After producing SAM-free protein, co-crystals were obtained within 2 weeks, leading to a high-resolution complex structure.
- Additional complex structures were solved quickly afterwards.
- Overall, several dozens of structures were deposited for this project.



HMT+SAM
SG P6₅
a=b=153.1
c=51.5
Res. 2.3Å



HMT+compound
SG P6₃
a=b=158.6
c=73.4
Res. 2.2Å

Figure 3: SAM-bound vs SAM-free crystal forms