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Title: X-Ray Crystallography to Study the Oligomeric State Transition of the *Thermotoga Maritima* M42 Aminopeptidase TmPep1050

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Y**

If **Yes**, can you record movies/images using your own microscope camera?

Y

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Louis Droogmans**: This protocol facilitates the study of aminopeptidases with different oligomeric states and oligomerization-dependent activities that are balanced between inactive dimers and active dodecamers **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Louis Droogmans**: This conventional technique can be easily performed in any lab and, aside from requiring access to a beamline or X-ray crystallography facility, does not require special devices **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Raphaël Dutoit**: With some adaptation, this method may be applied to any other protein with an activity or function that is dependent on its degree of oligomerization **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.4. **Raphaël Dutoit**: To process the X-ray data, users must have a minimal understanding of crystallography. We invite users to follow trainings, to visit the XDS wiki, and to watch Phenix tutorials **[1]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Introduction of Demonstrator on Camera

1.5. **Louis Droogmans**: Demonstrating the procedure will be Raphaël Dutoit, **[1][2]**.

1.5.1. INTERVIEW: Author saying the above

1.5.2. Named demonstrator(s) looks up from workbench or desk or microscope and acknowledges camera

Protocol

2. Activity Assay

2.1. To perform an activity assay, add 25 microliters of 100-millimolar L-Leucine-p-nitroanilide to 965 microliters of 50-millimolar MOPS (mops), 250-micromolar cobalt 2 chloride, and 10% methanol **[1-TXT]**.

2.1.1. WIDE: Talent adding reagent(s) to reaction, with millimolar L-Leucine-p-nitroanilide, MOPS, CoCl2, and methanol containers visible in frame **TEXT: MOPS: 3-(N-morpholino)propanesulfonic acid**

2.2. Preincubate the reaction mix in a 75-degree Celsius dry bath **[1]** before diluting the enzyme to a 1-micromolar final concentration with additional 50-millimolar MOPS **[2]**.

2.2.1. Talent placing reaction at 75 °C in dry bath

2.2.2. Talent adding MOPS to reaction, with MOPS container visible in frame

2.3. Next, add 10 microliters of 1-micromolar enzyme to the reaction mix **[1]** and vortex the resulting solution **[2]** before returning the reaction to the 75-degree Celsius dry bath for no more than 1 hour **[3]**.

2.3.1. Talent adding **the enzyme** to mix
2.3.2. Talent vortexing mix
2.3.3. Talent placing reaction to dry bath

2.4. When the solution has turned yellow, stop the reaction with 1 milliliter of 20% acetic acid **[1]** and vortex the mixture **[2]** before allowing it to cool to room temperature **[3]**.

2.4.1. Shot of yellow reaction, then acid being added, with acid container visible in frame
2.4.2. Talent vortexing solution
2.4.3. Talent placing solution at room temperature

2.5. Then transfer an aliquot of the reaction mix to a spectrophotometer cell **[1]** and read the absorbance at 410 nanometers against an incubated reaction mix without enzyme as a negative control **[2]**.

2.5.1. Talent adding mix to cell

2.5.2. Talent loading cell onto spectrophotometer

3. Apoenzyme Preparation

3.1. For apoenzyme preparation, add 10 microliters of 1,10-phenanthroline stock solution to 890 microliters of 50-millimolar MOPS, 0.5-molar ammonium sulfate, and 100 microliters of purified TmPep1050 (T-M-pep-ten-fifty) **[1]**.

3.1.1. WIDE: Talent adding reagent(s) to reaction, with phenanthroline, MOPS, $(NH_4)_2SO_4$, and aminopeptidase containers visible in frame

3.2. Check the activity loss using the activity assay as demonstrated without the addition of cobalt 2 chloride **[1]**.

3.2.1. Talent pick up CoCl2 and moving it out of frame/away from containers, with millimolar L-Leucine-p-nitroanilide, MOPS, CoCl2, and methanol containers visible in frame

3.3. After the assay, transfer the sample into a dialysis tube **[1]** and dialyze the sample against 200 milliliters of 50-millimolar MOPS and 0.5-molar ammonium sulfate at 4 degrees Celsius **[2]**.

3.3.1. Talent adding sample to tube
3.3.2. Talent adding reagent(s) to tube, with MOPS and $(NH_4)_2SO_4$ containers visible in frame

3.4. Exchange the dialysate with fresh buffer three times during the 48-hour dialysis **[1]** and collect sample from the dialysis tube **[2]**.

3.4.1. Talent exchanging buffer
3.4.2. Talent collecting sample

3.5. Using ultrafiltration units with a 30-kilodalton cutoff, concentrate the sample back to 100 microliters **[1]** and check the concentration on nano-volume spectrophotometer at 280 nanometers **[2]**.

3.5.1. Talent loading solution into ultrafiltration units
3.5.2. Talent adding sample to spectrophotometer

4. Dimer Preparation

4.1. To prepare the dimers, dilute the apoenzyme to a 1-micromolar concentration in 50-millimolar MOPS and 0.5-molar ammonium sulfate **[1]** and incubate the reaction for 2

hours in a 75-degree Celsius dry bath **[2]**.

4.1.1. WIDE: Talent adding reagents to reaction, with MOPS and $(NH_4)_2SO_4$ containers visible in frame
4.1.2. Talent placing reaction into dry bath

4.2. Then concentrate the enzyme sample to an at least 50-microliter concentration **[1]** and check the molecular weight by size exclusion chromatography **[2-TXT]**.

4.2.1. WIDE: Talent loading ultrafiltration units in a centrifuge. NOTE: Same as 3.5.1
4.2.2. Talent adding sample to column **TEXT: Elution peak must shift from about 82 to about 95 milliliters**

5. Microseeding

5.1. To improve the shape, size, and crystallinity of the protein crystals, add the appropriate volume of optimized crystallization solution **[1-TXT]** to a drop of crystals to bring the crystal droplet volume to 10 microliters **[2-TXT]**.

5.1.1. WIDE: Talent aspirating solution from well **TEXT: See text for crystallization solution optimization details**
5.1.2. Talent adding solution to crystals **TEXT: See text for crystal preparation details**

5.2. Transfer the droplet to a 1.5-milliliter microtube on ice **[1]** and add 90 more microliters of crystallization solution to the seeded well **[2]**.

5.2.1. Talent transferring droplet to microtube
5.2.2. Talent adding solution to microtube

5.3. For each seed dilution, add 500 microliters of crystallization reagent per well to the appropriate number of wells in a crystallization plate **[1]** and mix 2 microliters of protein sample with 2 microliters of crystallization reagent and 0.2 microliters of seeds in one crystallization support per well **[2]**.

5.3.1. Talent adding reagent to well(s), with reagent container visible in frame
5.3.2. Talent mixing materials in support, with sample, reagent, and seed containers visible in frame

5.4. Then fix the supports onto each well of crystallization reagent **[1]**.

5.4.1. Support being fixed onto well

6. X-Ray Diffraction

6.1. For crystal picking, fill a bath with liquid nitrogen **[1]** and plunge any vials or basket used for sample handling into the nitrogen **[2]**.

6.1.1. WIDE: Talent filling bath
6.1.2. Talent plunging vial and/or basket

6.2. **Raphaël Dutoit**: Handling liquid nitrogen is hazardous and can cause severe frostbites. Be sure to wear proper individual protections like cryogenic gloves and protective eye googles **[1]**.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

6.3. Set up sample picking loops of different sizes according to the crystal size **[1-TXT]** and use a binocular to select a drop containing crystals **[2]**.

6.3.1. Talent selecting loop(s) **TEXT: *e.g.*, 100-, 150-, 200-micrometer loops**
6.3.2. Talent putting on binocular

6.4. The use a loop to gently select an isolated crystal from one well **[1]** and immediately plunge the loop in liquid nitrogen **[2]** before placing the loop in a suitable vial **[3]**.

6.4.1. ECU: Crystal being selected OR Talent selecting crystal *Videographer: Important step*
6.4.2. Talent plunging loop *Videographer: Important step*
6.4.3. Talent placing loop into vial *Videographer: Important step*

7. Indexation

7.1. To measure diffraction spot intensities, create a folder from which XDS (X-D-S) will be run **[1-TXT]** and locate the path to the images **[2]**.

7.1.1. WIDE: Talent creating folder, with monitor visible in frame **TEXT: https://strucbio.biologie.uni-konstanz.de/xdswiki/index.php/Main_Page**
7.1.2. SCREEN: screenshot_1: 00:00-00:07

7.2. To run XDSME (X-D-S-me), enter the command in a terminal window **[1-TXT]**.

7.2.1. SCREEN: screenshot_1: 00:08-00:18 **TEXT: https://github.com/legrandp/xdsme**

7.3. After XDS has ended the job, check the CORRECT.LP file and note the probability of the space group determination, data completeness, the highest resolution, crystal mosaicity, and data quality **[1]**.

7.3.1. SCREEN: screenshot_1: 00:19-00:24

7.4. After checking the XDS_pointless.log to obtain the likelihood of space groups **[1]**, use the commands to rerun XDS-ME with different space group solutions proposed by XDS in separate folder to avoid overwriting the previous process **[2]**.

7.4.1. SCREEN: screenshot_1: 00:24-00:52 *Video Editor: please speed up*
7.4.2. SCREEN: screenshot_2: 00:00-00:10

7.5. After selecting the best solution based on the data statistics, enter the commands to run the XSCALE (X-scale) and XDSCONV (X-D-S-conv) **[1]**.

7.5.1. SCREEN: screenshot_2: 00:17-00:44 *Video Editor: please speed up* **TEXT:** Input **xscale.py XDS_ASCII.HKL** to run XSCALE; input **xdsconv.py XSCALE.HKL ccp4**

8. Molecular Replacement

8.1. To determine the phase without an anomalous scattering atom, use 4P6Y (four-P-six-Y) coordinates to prepare the starting model for molecular replacement **[1]**.

8.1.1. WIDE: Talent using coordinates, with monitor visible in frame

8.2. From the pdb (P-D-B) file, use the Phenix PDB file editor to extract the A monomer and to truncate its aminoacids in alanine **[1]**.

8.2.1. SCREEN: screenshot_3: 00:00-00:12

8.3. Run X-triage with the reflection file generated by XDSCONV and the sequence as inputs **[1]**.

8.3.1. SCREEN: screenshot_4: 00:00-00:12

8.4. Check the log file from X-triage. Note the completeness, the number of subunits in the asymmetric unit, the anisotropy, the presence of ice rings, and the twinning occurrence **[1]**.

8.4.1. SCREEN: screenshot_5: 00:00-00:10

8.5. To run Phaser-MR (fazer-M-R) in Phenix for molecular replacement, select the reflection

file, the sequence, and the starting 4P6Y model truncated in poly alanine, and click **Run [1]**.

8.5.1. SCREEN: screenshot_6: 00:00-00:29 *Video Editor: please speed up*

8.6. Upon completion, check if a model has been found and check the score of the molecular replacement. A translation factor Z-score of at least 8 indicates that the solution is definitively correct **[1]**.

8.6.1. SCREEN: screenshot_6: 00:30-00:34

9. Model Building

9.1. After determining the phase by molecular replacement, select **Run Autobuild [1]** and click **Run**. All of the required files will be automatically added **[2]**.

9.1.1. WIDE: Talent selecting Run Autobuild
9.1.2. SCREEN: screenshot_7: 00:14-00:18

9.2. Upon completion, check the model in Coot and build and refine the model manually according the electron density map in Coot **[1-TXT]**.

9.2.1. SCREEN: screenshot_8: 00:00-00:29 *Video Editor: please speed up* **TEXT: Caution: Do not overinterpret electronic density**

9.3. Using this model, the sequence, and the diffraction data as inputs, refine the manually curated model in Phenix, referring to Phenix help to select the right strategy **[1]**.

9.3.1. SCREEN: screenshot_9: 00:00-00:25 *Video Editor: please speed up*

9.4. After refinement, check the results. Refinement-free and refinement-work must decrease, Molprobity indicators must be respected, and outliers with low real-space correlation must be limited **[1-TXT**

9.4.1. SCREEN: screenshot_9: 00:26-00:49 *Video Editor: please speed up* **TEXT: Repeat model building and refinement until best model generated**

9.5. When the best refined model has been generated, run Molprobity on the server **[1-TXT]** and check any outliers identified by the program **[2-TXT]**.

9.5.1. SCREEN: screenshot_10: 00:00-00:49 *Video Editor: please speed up* **TEXT: http://molprobity.biochem.duke.edu/**
9.5.2. SCREEN: screenshot_10: 00:49-01:05 **TEXT: Repeat model building, refinement,**

and Molprobity until best model generated

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
6.4.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
9.2. The manual refinement in Coot is the most critical part in model building. During step, the user may introduce bias by overinterpreting the electronic density. The automatic refinement will always try to fit user's input, so caution is advised. The most important tip is to not overinterpret the data. Simply, we have to build the model only on what we can see in the electron density.

Results

10. Results: Representative *Thermotoga Maritima* M42 Aminopeptidase TmPep1050 Oligometric State Transition

10.1. Size exclusion chromatography can be used to determine the apparent molecular weight of the purified protein **[1]**.

10.1.1. LAB MEDIA: Figure 3B

10.2. The crystallization condition of the peptide **[1]** can be optimized by varying the pH **[2]** versus the PEG (peg) concentration around the condition of the dimer **[3-TXT]**.

10.2.1. LAB MEDIA: Figure 4A
10.2.2. LAB MEDIA: Figure 4A *Video Editor: please emphasize pH triangle*
10.2.3. LAB MEDIA: Figure 4A *Video Editor: please emphasize PEG triangle* **TEXT: PEG: polyethylene glycol**

10.3. For example, the best TmPep1050-H60A H307A (H-sixty-A H-three-oh-seven-A) crystals **[1]** were obtained in 0.1-molar sodium citrate with a pH of 5.2 and 20% PEG3350 (thirty-three-fifty) concentration after one cycle of microseeding to improve the monocrystallinity **[2]**.

10.3.1. LAB MEDIA: Figure 4C
10.3.2. LAB MEDIA: Figure 4D

10.4. In this analysis, data indexation showed that the space group of the TmPep1050-H60A H307A crystal was $C222_1$ (C-two-two-two-one) **[1]**, but XDS proposed another solution, the *m*P (M-P) space group **[2]**.

10.4.1. LAB MEDIA: Figure 5
10.4.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize mP Data row*

10.5. As illustrated by the X-triage analysis, the data set is twinned **[1]**, making the C2221 space group unlikely and setting up the data set to be processed in P21 (P-two-one) **[2]**.

10.5.1. LAB MEDIA: Authors: What figure and what part of the figure should be highlighted to illustrate these data? NOTE: Authors are supposed to upload a screenshot from the X-triage for this. If it is not uploaded, wither prompt them or remove 10.5 all together

10.6. The structure of TmPep1050-H60A H307A could be completed after several cycles of automated and manual refinement **[1]**, confirming the oligomeric state with an interface surface of 1710-square angstroms between both monomers and a free energy of interface formation of minus 16.2 kilocalorie/mole **[2]**.

10.6.1. LAB MEDIA: Figure 6A
10.6.2. LAB MEDIA: Figure 6A *Video Editor: please emphasize dimerization domain of structure*

10.7. Here a close-up of the TmPep1050-H60A H307A active site **[1]** compared to the active site of the TmPep1050 dimer **[2]** and dodecamer can be observed **[3]**.

10.7.1. LAB MEDIA: Figure 6B *Video Editor: please emphasize red structures*
10.7.2. LAB MEDIA: Figure 6B *Video Editor: please emphasize blue structures*
10.7.3. LAB MEDIA: Figure 6B *Video Editor: please emphasize white structures*

Conclusion

11. Conclusion Interview Statements

11.1. **Raphaël Dutoit**: When building a model, take care to build only what you see in the electron density to avoid overinterpretation the data and to take the time to polish your model **[1]**.

11.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (9.1., 9.2.)

11.2. **Raphaël Dutoit**: The molecular weight of the dimer and dodecamer can also be determined by native mass spectrometry and the method can be useful for detecting transition oligomers **[1]**.

11.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera