Week 40 - Overview

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Weekly summary:

During Week 40, we purified the ci2350-MBP produced last week, along with ci2350-MBP double mutant (R215A and V263I) produced. Testosterone reduction assays were done on day 58.

Main results of the week:

We evaluated CI2350–MBP preparations using an NADH-based spectrophotometric assay to investigate testosterone-associated cofactor consumption. Over 30 minutes, reactions with and without testosterone showed similar decreases in absorbance at 340 nm, while the no-substrate control showed a smaller decrease. Expressing the measurements relative to their initial absorbance confirmed that the enzyme-containing reactions followed closely overlapping trends.

These results identified background NADH consumption as a major limitation of the assay and did not provide clear evidence of testosterone-dependent CI2350–MBP activity. Further work should focus on improving protein purification, resolving background cofactor consumption and directly measuring steroid conversion.

Day 57: [September 30, 2026], Wednesday

Responsibles:
Participants: Rytis, Giulia, Ruiqi in the afternoon

Daily overview of experiments

Lysis and purification of samples from previous week cultivation

Activity	***Protocol File***	***Comments***
Sonication of ci_MBP, ci double mutants		
Purification of ci_MBP, ci double mutants		
Prep of Binding buffer		

Results and interpretation

Sonication

Four samples were used for cell washing: 2 of ci_MBP (sequence corrected) (0.8 mM IPTG, 17°C, cultivated on 24.09), 2 of ci_double mutants (0.8 mM IPTG, DATE). Cell pellets were thawed on ice, washed with 10 mL of cell wash buffer (500 mM NaCl, 20 mM sodium phosphate) per tube, and resuspended. The samples were centrifuged at 4700 x g for 10 minutes at 4°C. The resulting pellets were weighed, and a lysis buffer (500 mM NaCl, 20 mM sodium phosphate, 30 mM imidazole; same as binding buffer) was added at a ratio of 5 mL of buffer per 0.4 g of pellet weight. Double mutants were pooled prior to sonication, a single tube of the wt ci_MBP was lysed, the other cell wash was refrozen at -80°C due to time constraints.

Samples were resuspended in the lysis buffer and sonicated. The sonicator was operated at 30% amplitude for 6 cycles for ci_double mutant, 8 cycles for ci_MBP, with each cycle consisting of 2 minutes of active time using a 3-second on/off pulse interval.

The lysate was subsequently clarified via centrifugation at 20,000 x g for 1.5 hours at 4°C.
NOTE: the lysate sample (for total protein content) for SDS and Bradford was NOT taken.

Prep of binding buffer

Binding buffer was prepared following the usual concentrations: 500 mM NaCl buffer, 20 mM Sodium phosphate buffer, 30 mM imidazole.

Purification of ci_mbp (corrected sequence) and double mutant via Column Binding

The column was equilibrated with a 10 mL binding buffer. The sample flow rate was set to 1.5 (2.2 as the actual setting) mL/min throughout the run. All clarified lysate was processed via column (flowthrough). Then, the column was washed with 10 mL of binding buffer (wash), and the target protein was eluted using 5 mL of elution buffer (500 mM NaCl, 20 mM sodium phosphate, 500 mM imidazole). This procedure was done for both samples.

The elutants were added to Viva Spin columns* (30 kDa cutoff), +4 mL of Phosphate buffer (100 mM) was added to each, then spun down (4000 g, 30 min, +4C) (Swinging bucket rotor).
*The scale was towards the outer rim and membranes were facing to the sides, instead of the opposite (that would be the proper way)

There was much more flowthrough in ci-MBP than in ci-MBP_DM, thus 1 mL fraction of it was collected to be tested in SDS-PAGE.
Viva spin was repeated again, same procedure, but with tube direction corrected.
~2 mL of ci-MBP, and ~3.5 mL of ci-MBP_DM were collected.

Day 58: [October 03, 2026], Saturday

Responsibles:
Participants: Hamsi, Eleonora

Daily overview of experiments

Lysis and purification of samples from previous week cultivation

Activity	***Protocol File***	***Comments***
Testosterone Assay	Spectrophotometric …	

Results and interpretation

Testosterone Assay

A 40 mM testosterone stock was prepared by dissolving 0.0052 g of testosterone (MW 288.42 g/mol) in 450 µL of absolute ethanol. A [40] mM progesterone stock was prepared by dissolving [0.0115] g of progesterone (MW 314.46 g/mol) in [914] µL of absolute ethanol. A 5 mM NADH stock was prepared by dissolving 0.0119 g of NADH disodium salt (MW 709.5 g/mol) in 3.35 mL of 100 mM potassium phosphate buffer, the same buffer used for the assays.
Insert experiment discussion and interpretation

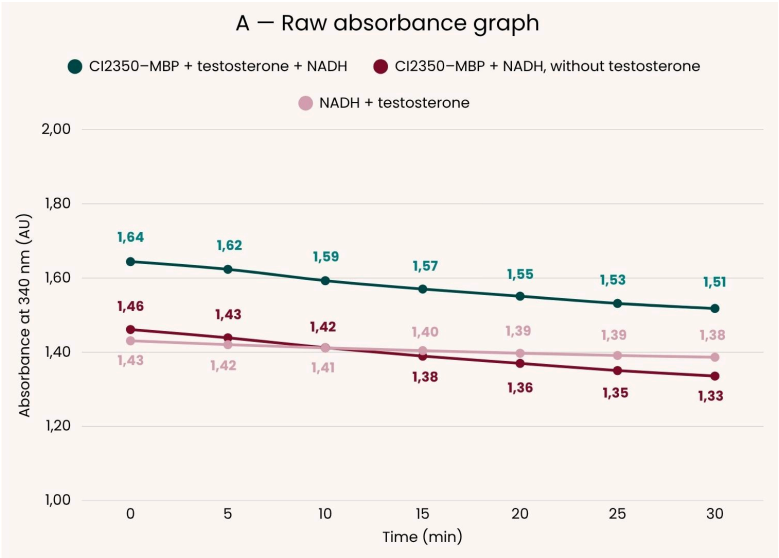
Testosterone and progesterone reduction assays

Reactions were set up in 4 mL cuvettes in triplicate, in a total volume of 4000 µL, and absorbance was followed at 340 nm . Controls lacking substrate , enzyme were included to correct for substrate background, enzyme background respectively.

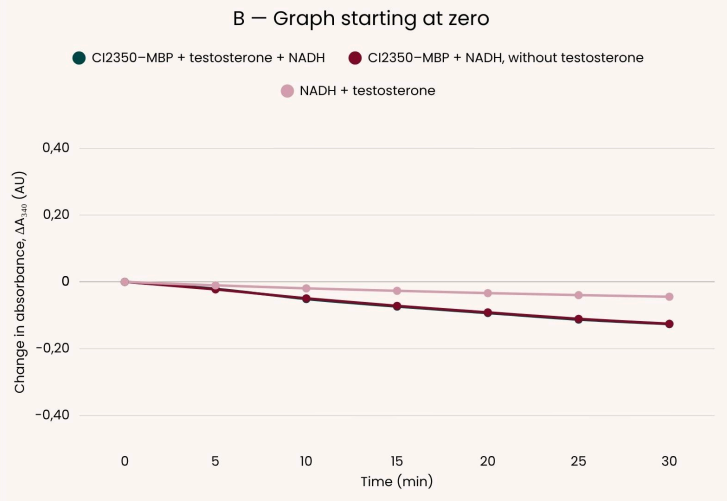
Sample	**Buffer (µL)**	**NADH (µL)**	**Enzyme (µL)**	**Substrate (µL)**

Blank (buffer only)	4000			
Negative control (no enzyme)	3799	200		1 T/P
Negative control (no substrate)	3600	200	200	
Sample + testosterone (triplicate)	[3599]	200	200	1 T
Sample + progesterone (triplicate)	[3599]	200	200	1 P

Data Analysis



Absorbance at 340 nm decreased over 30 minutes in both the CI2350–MBP reaction containing testosterone and the corresponding reaction without testosterone. Based on the rounded values displayed in the results figure, absorbance decreased from approximately 1.64 to 1.51 AU in the testosterone-containing reaction and from 1.46 to 1.33 AU in the no-substrate control. Both conditions therefore showed an overall decrease of approximately 0.13 AU. The no-enzyme control containing NADH and testosterone showed a smaller decrease, from approximately 1.43 to 1.38 AU.



To compare reactions with different starting absorbances, the measurements were also expressed as the change from each condition's initial value: $\Delta A_{340}(t) = A_{340}(t) - A_{340}(0)$. The resulting traces closely overlapped for the enzyme-containing reactions with and without testosterone. This baseline adjustment removes starting offsets

but does not subtract background cofactor consumption.

The similar absorbance decreases indicate that adding testosterone did not produce a clearly distinguishable increase in NADH consumption under the tested conditions. The larger decrease in enzyme-containing reactions relative to the no-enzyme control is consistent with background cofactor consumption associated with the protein preparation, although these measurements do not identify its cause or establish whether CI2350 itself contributed.

This experiment did not provide clear evidence of CI2350–MBP-dependent testosterone conversion. It also does not establish that the enzyme was inactive, because substrate-associated activity could not be resolved from the background signal.

Progesterone assay results: lost during saving process.