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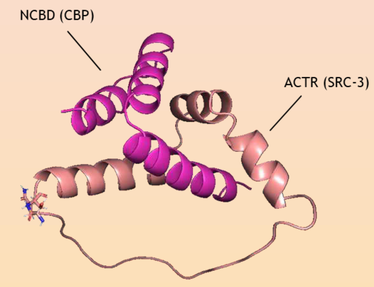
Chemoenzymatic synthesis of protein pseudo-[2]rotaxane



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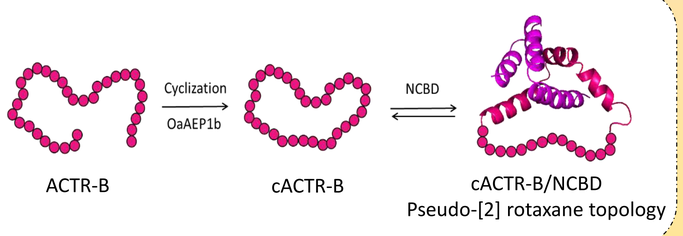
Introduction :

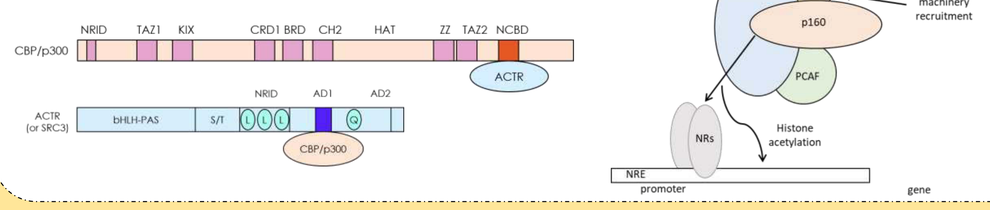
Intrinsically disordered proteins (IDPs) display enhanced conformational flexibility and structural heterogeneity. They are able to recognize diverse molecular targets and engage in multivalent interactions[1]. For instance, transcriptional co-activators ACTR (p160) and p300/CBP contain IDP regions which are involved in the formation of multiprotein complexes with nuclear receptors regulating the transcription of many genes. These proteins are overexpressed in breast and ovarian cancers[2,3].

Goal of the project :

Target and disrupt protein-protein interactions between ACTR and NCBD. To do this, we want to increase the stability of the ACTR/NCBD complex by linking the two domains through non-covalent interactions enhanced by non-classical topology (pseudo-[2]rotaxane protein complex) .

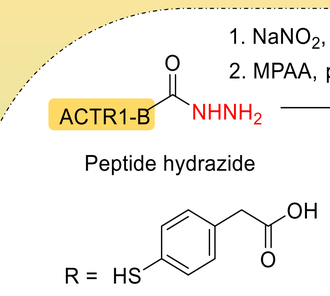


Complex :

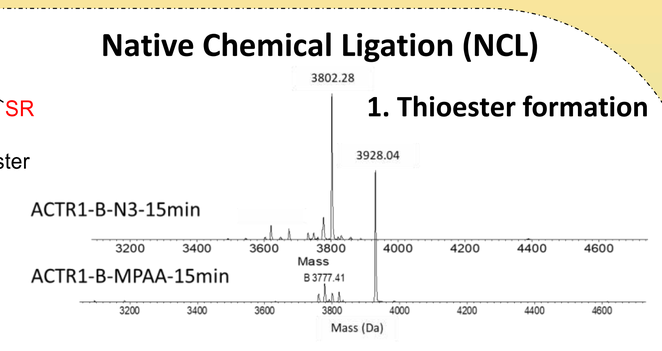


Chemoenzymatic synthesis

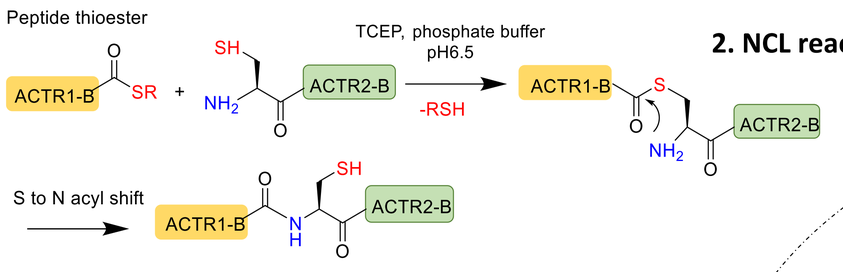
Native Chemical Ligation (NCL)

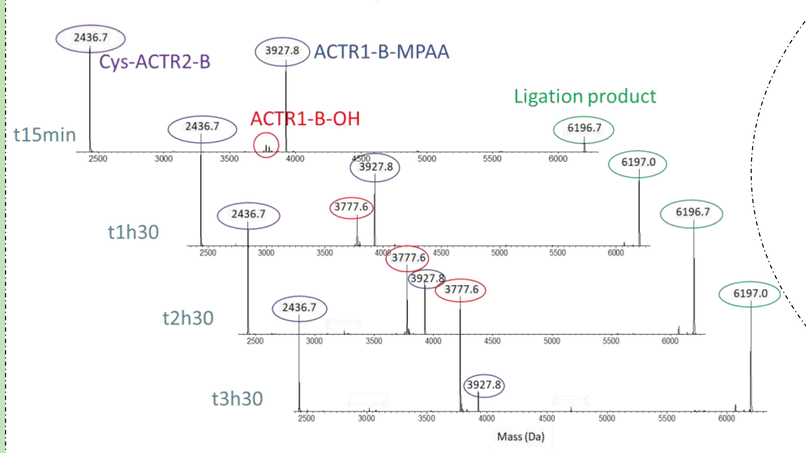


1. Thioester formation

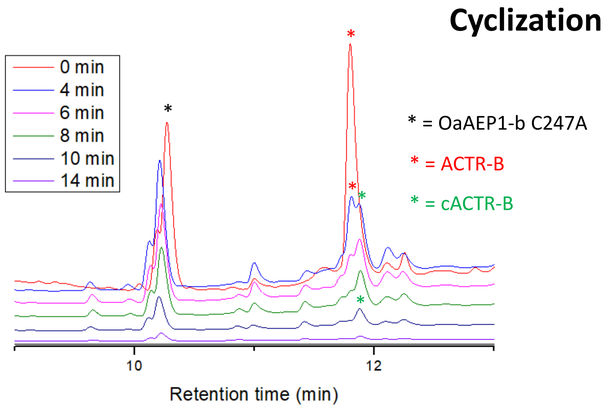


2. NCL reaction: Amide bond formation



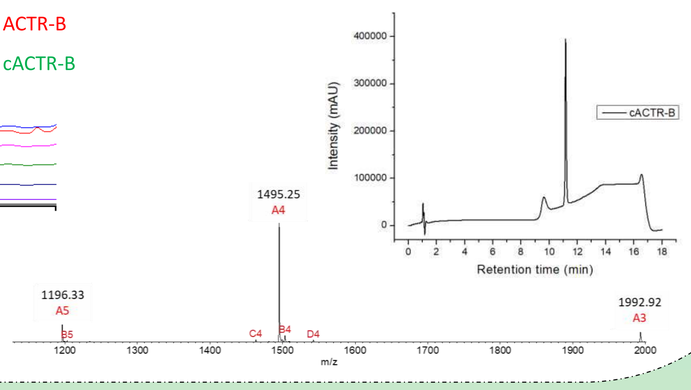


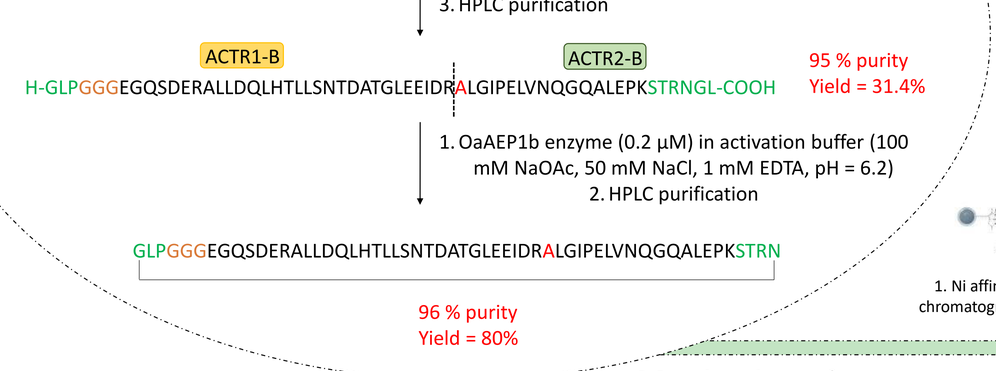
Cyclization



Cyclization reaction monitored by analytical HPLC

LC-MS and analytical HPLC analysis of purified cACTR-B (MW = 5978.55 g/mol)





Enzyme asparaginyl endopeptidase

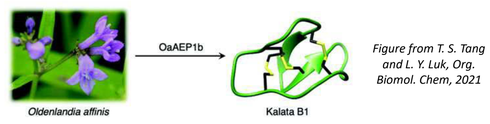
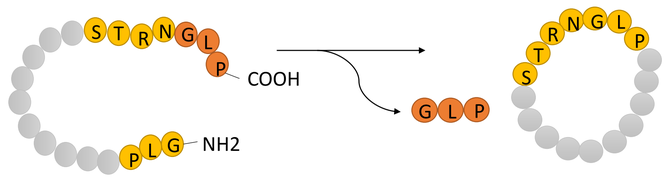
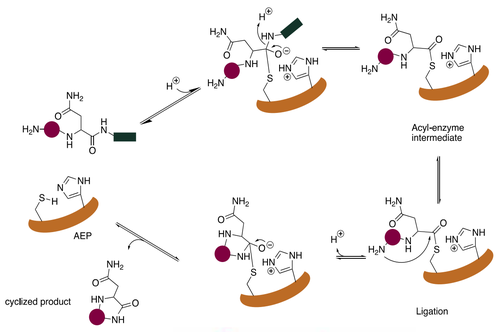


Figure from T. S. Tang and L. Y. Luk, Org. Biomol. Chem, 2021

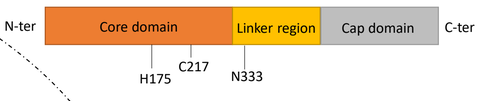
Motif of recognition for cyclization by OaAEP1b

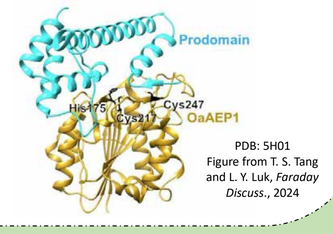


Proposed mechanism of AEPs catalysis



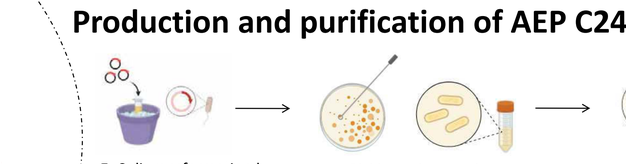
AEP activation and improvement of the catalytic efficiency with AEP C247A mutant





PDB: 5H01
Figure from T. S. Tang and L. Y. Luk, *Faraday Discuss.*, 2024

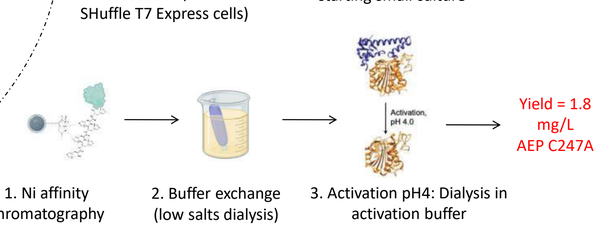
Production and purification of AEP C247A mutant



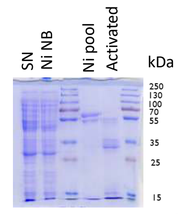
E. Coli transformation by heat shock (*E. coli* SHuffle T7 Express cells)

Picking colony and starting small culture

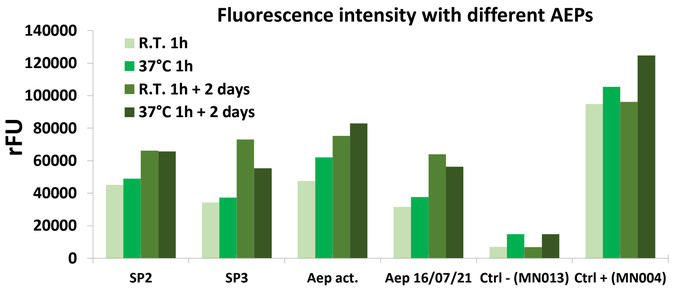
At OD ~ 0.8, IPTG induction overnight at 18°C

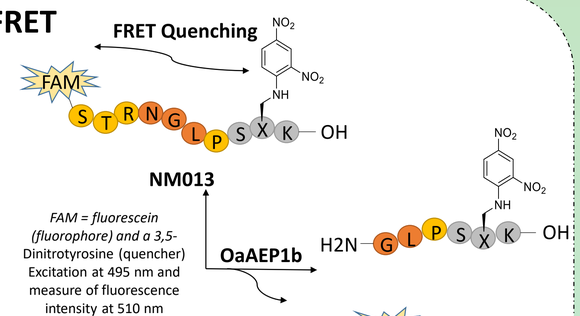


Yield = 1.8 mg/L AEP C247A



Monitoring of enzyme activation by FRET





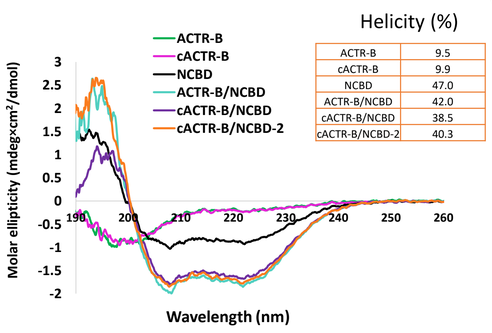
FAM = fluorescein (fluorophore) and a 3,5-Dinitrotyrosine (quencher) Excitation at 495 nm and measure of fluorescence intensity at 510 nm

	SP2	SP3	Aep Act.	Aep (16/07/21)	NM013 (substrat)	Ac-YVAD-CMK (inhibitor)	Ctrl + : MN004 (desired product ref.)
Concentrations (µM)	1.02	0.6	24.9	0.6	0.5	15	0.5

Activity buffer:
- 100 mM NaOAc, pH4
- 500 mM NaCl
- 5% glycerol

Biophysical characterization and cell penetration studies

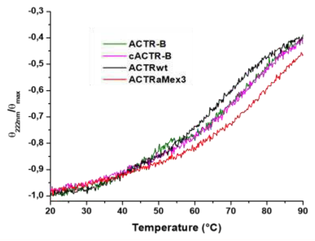
Circular Dichroism (CD)



Helicity (%)

ACTR-B	9.5
cACTR-B	9.9
NCBD	47.0
ACTR-B/NCBD	42.0
cACTR-B/NCBD	38.5
cACTR-B/NCBD-2	40.3

Thermal Denaturation (TD)

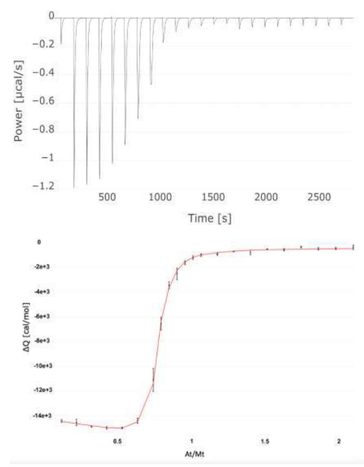


Variant	Thermal denaturation
ACTR-B/NCBD	86°C
cACTR-B/NCBD	79°C
ACTR/NCBD W.T.*	67°C
ACTRαMex3*	110°C

* Data from Alexis Jouin, PhD student in the team of Biosystem chemistry

Isothermal Titration Calorimetry (ITC) of cACTR-B

Series of 23 injections of cACTR-B solution into NCBD solution

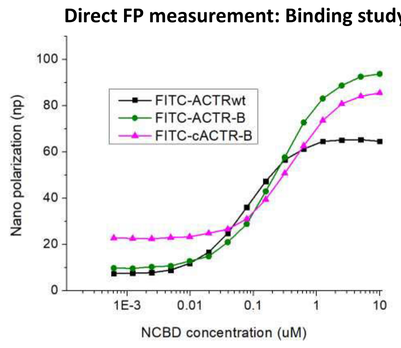


Variant	K_d (nM)	ΔH (cal.mol^{-1})	ΔG (kcal.mol^{-1})	-TΔS (kcal.mol^{-1})
cACTR-B	170.0	-16344	-9.4	6.9
ACTRwt	206.0	-12700	-9.3	3.4

→ Better binding for cACTR-B than W.T.
→ Decreasing of enthalpy and increasing of entropy → More interaction that stabilize the complex and destabilization because less freedom of conformation degree (enthalpy-entropy compensation)

Fluorescence polarization measurement (FP measurement)

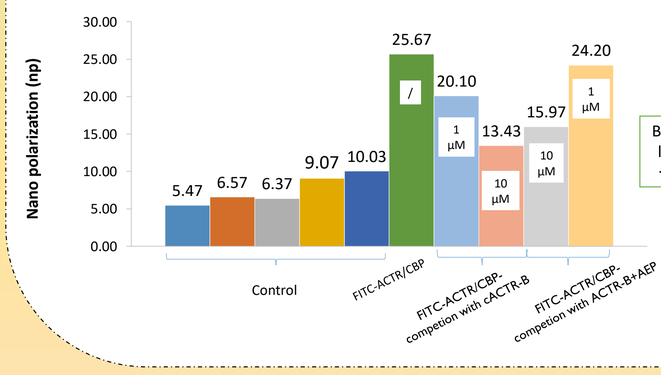
Direct FP measurement: Binding study of cACTR-B/NCBD complex (N=3)



Variant	K_d (nM)
ACTR-B	167.7
cACTR-B	102.1
ACTRwt	57.7

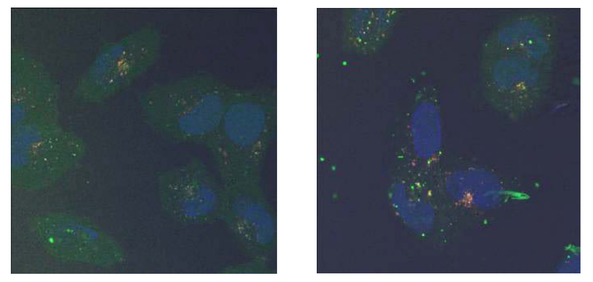
FITC is not in the same place for ACTRwt than ACTR-B and cACTR-B

Competition binding experiment: Targeting NCBD domain in full length 265 kDa CBP (N=3)



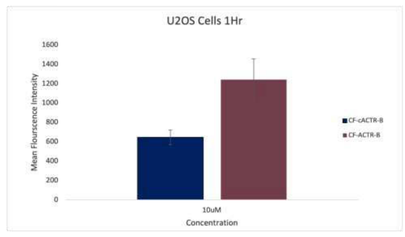
Binding of cACTR-B to full lenght CBP is confirmed
→ Binding mechanism ?

Cell penetration studied by confocal microscopy



Cell uptake of ACTR-B and cACTR-B (10 µM peptide, 1h treatment in PBS, U2OS cell line)

Quantitative determination of cell uptake by flow cytometry



Mean fluorescence intensity of CF-cACTR-B (10 µM) and CF-ACTR-B (10 µM) (p-value = 0.014) (1 h treatment at 37°C in PBS)

Conclusion and perspectives

ACTR-B could be chemically synthesized and cyclized enzymatically via the ligase asparaginyl endopeptidase. cACTR-B/NCBD pseudo-[2]rotaxane complex was formed. CD analysis shown an helical structure similar for ACTR-B/NCBD and cACTR-B/NCBD complex. We proved that complex cACTR-B/NCBD and ACTR-B/NCBD have an higher thermal denaturation than ACTRwt/NCBD. It was found that cACTR-B is a better binding with NCBD compare to ACTRwt. Fluorescence polarization measurement allowed us to see a binding between cACTR-B and NCBD, but also full length CBP. For the future it would be interesting to determine the binding mechanism between this cyclic cACTR-B and NCBD (and CBP) and get a cristal structure of the complex. The next step will be to study the biological impact of cACTR-B into cells.

References

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