TEV蛋白酶热稳定性突变体的理性设计

Rational design of TEV protease mutants with enhanced thermostability

G05D　刘逸珩

2021.11.23

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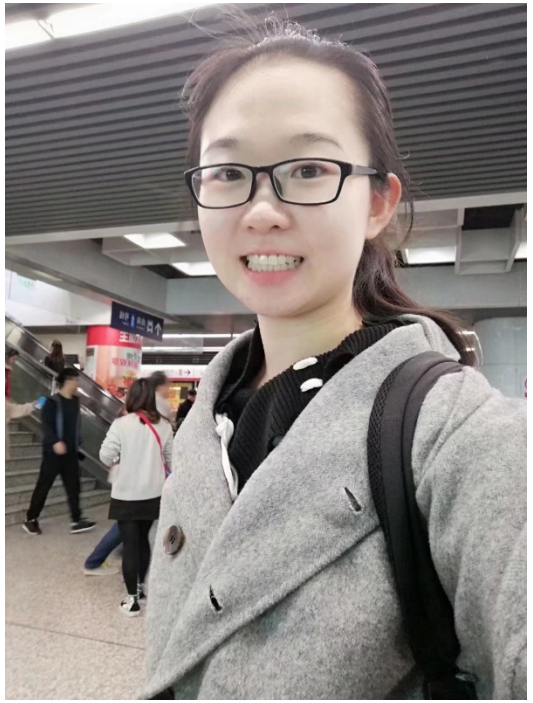

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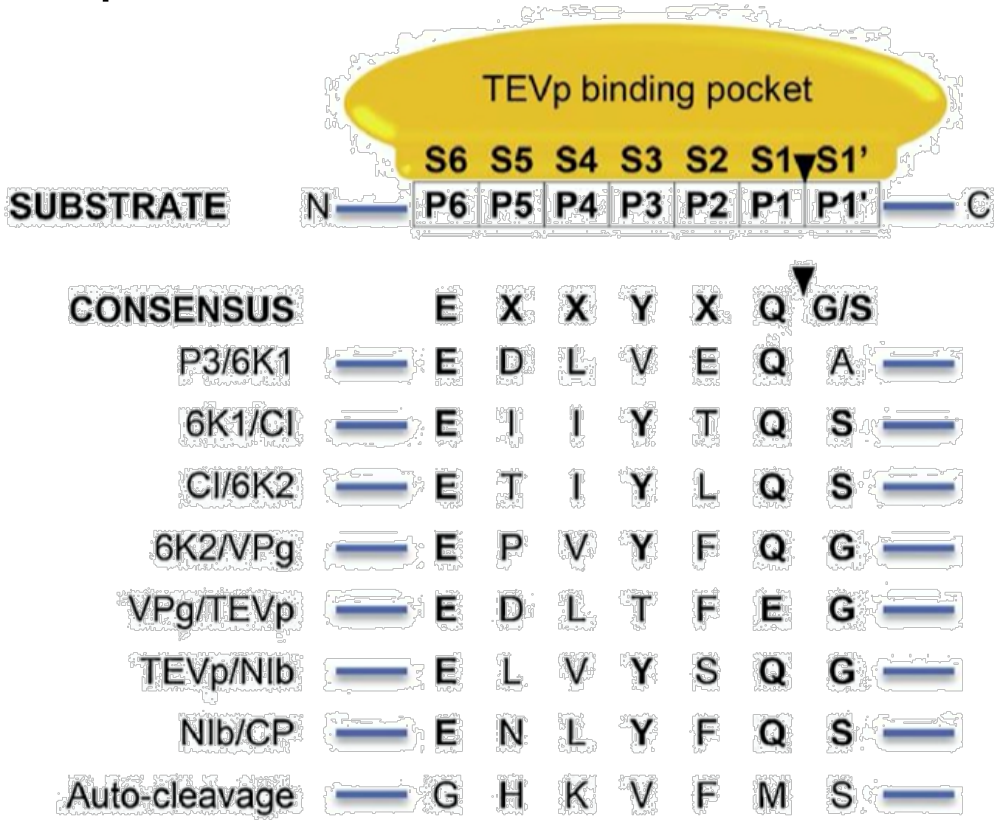
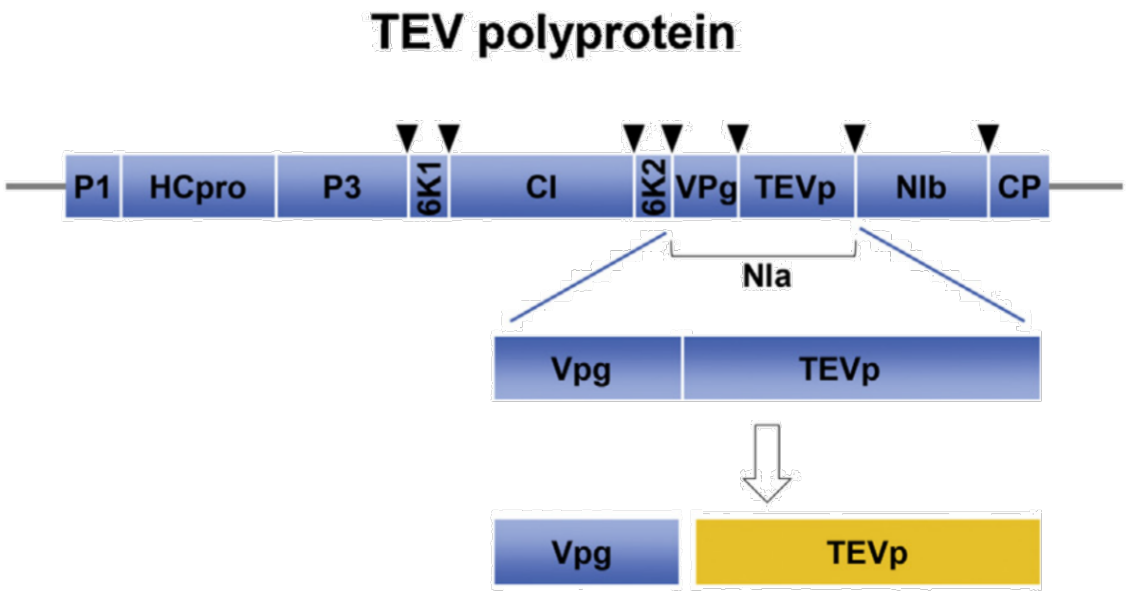
G05B	张萃雯	范六民	逆境小体形成的分子机制
G05C	李展	李磊	miRNA和铜稳态
G05D	刘逸珩	王忆平	生物固氮、合成生物学

背景——TEV蛋白酶（TEVp）

- TEVp来源于烟草蚀纹病毒TEV，其基因组全长为9500bp，编码一个3054氨基酸的多肽，在感染细胞后经过三种病毒蛋白酶加工成10个成熟产物。
- TEVp的最适温度为30℃，当温度达到或高于37℃时，其酶活性会急剧下降。

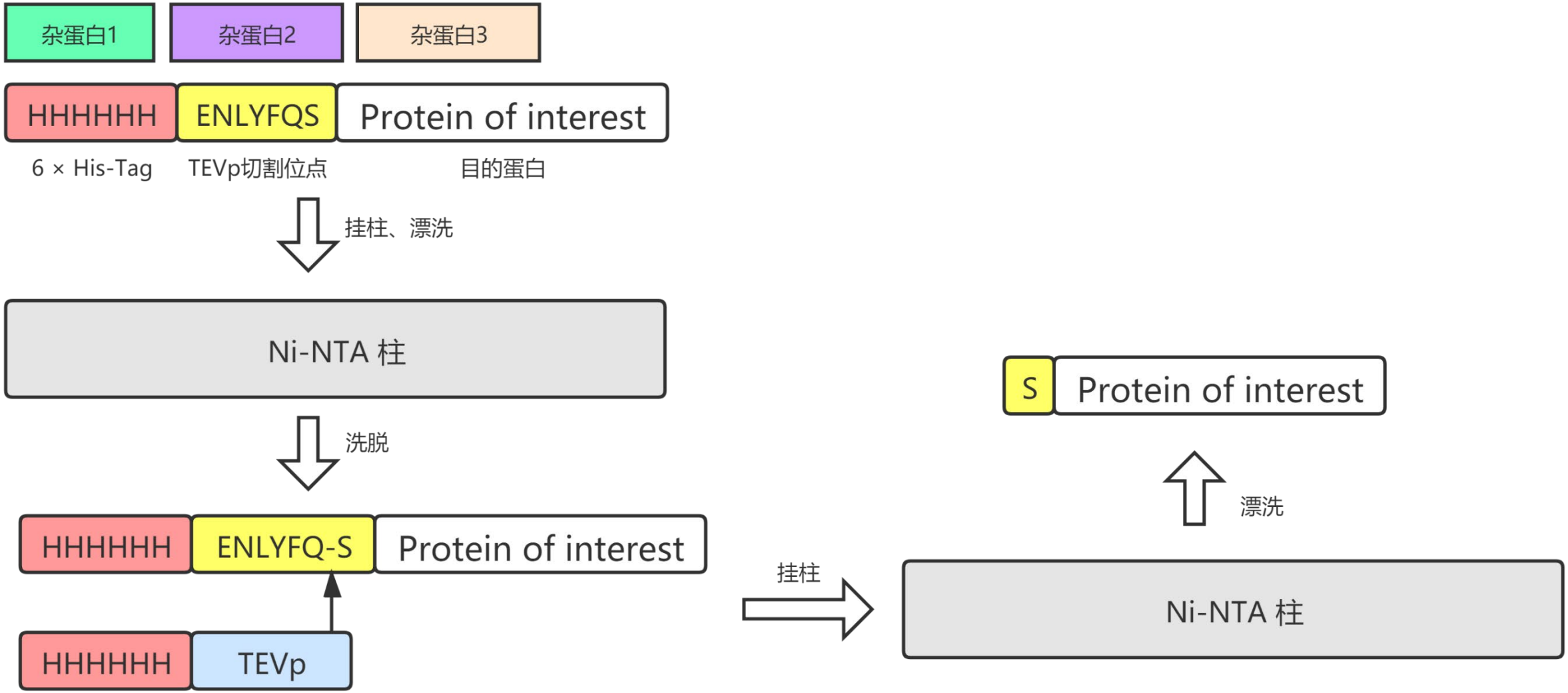




F. Cesaratto, O. R. Burrone, G. Petris, Tobacco Etch Virus protease: A shortcut across biotechnologies. *J. Biotechnol.* **231**, 239-249 (2016).

背景——应用：蛋白质纯化

- 蛋白质纯化过程常需要一些亲和标签，如GST-Tag、His-Tag等。



背景——应用：定化学计量比多组分表达系统

PNAS

Polyprotein strategy for stoichiometric assembly of nitrogen fixation components for synthetic biology

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Edited by Caroline S. Harwood, University of Washington, Seattle, WA, and approved July 6, 2018 (received for review March 22, 2018)

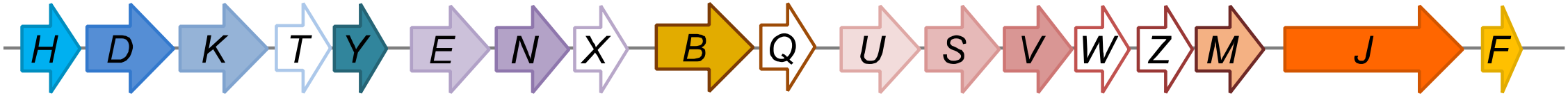
Polyprotein ABC N_A' M-X-X-X-X-E-N-L-Y-F-Q-S-M-X-X-X-X-E-N-L-Y-F-Q-S-M-X-X-X-X C_C'

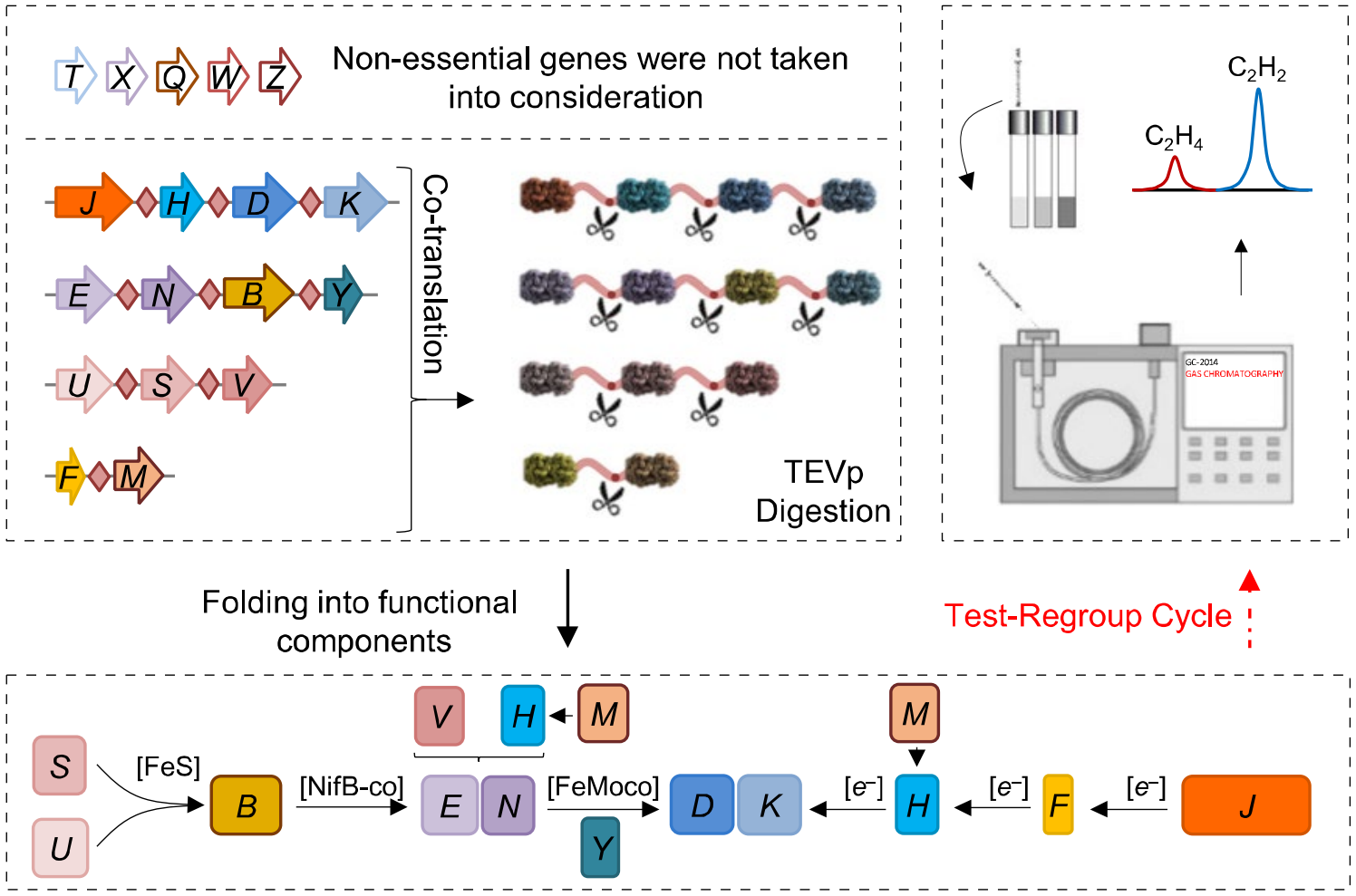
TEV protease (TEVp) Digestion

Protein A	Protein B	Protein C
N_A' M-X-X-X-X-E-N-L-Y-F-Q C_A'	N_B' S-M-X-X-X-X-E-N-L-Y-F-Q C_B'	N_C' S-M-X-X-X-X C_C'
C′ Tail	N′ cap + C′ Tail	N′ cap

J. Yang *et al.*, Polyprotein strategy for stoichiometric assembly of nitrogen fixation components for synthetic biology. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E8509-E8517 (2018).

背景——应用：定化学计量比多组分表达系统





- 将*Klebsiella oxytoca*原本的18个基因融合为5个巨型基因，且能够维持原本表达量的化学计量比值。

J. Yang *et al.*, Polyprotein strategy for stoichiometric assembly of nitrogen fixation components for synthetic biology. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E8509-E8517 (2018).

背景——脯氨酸效应：现象

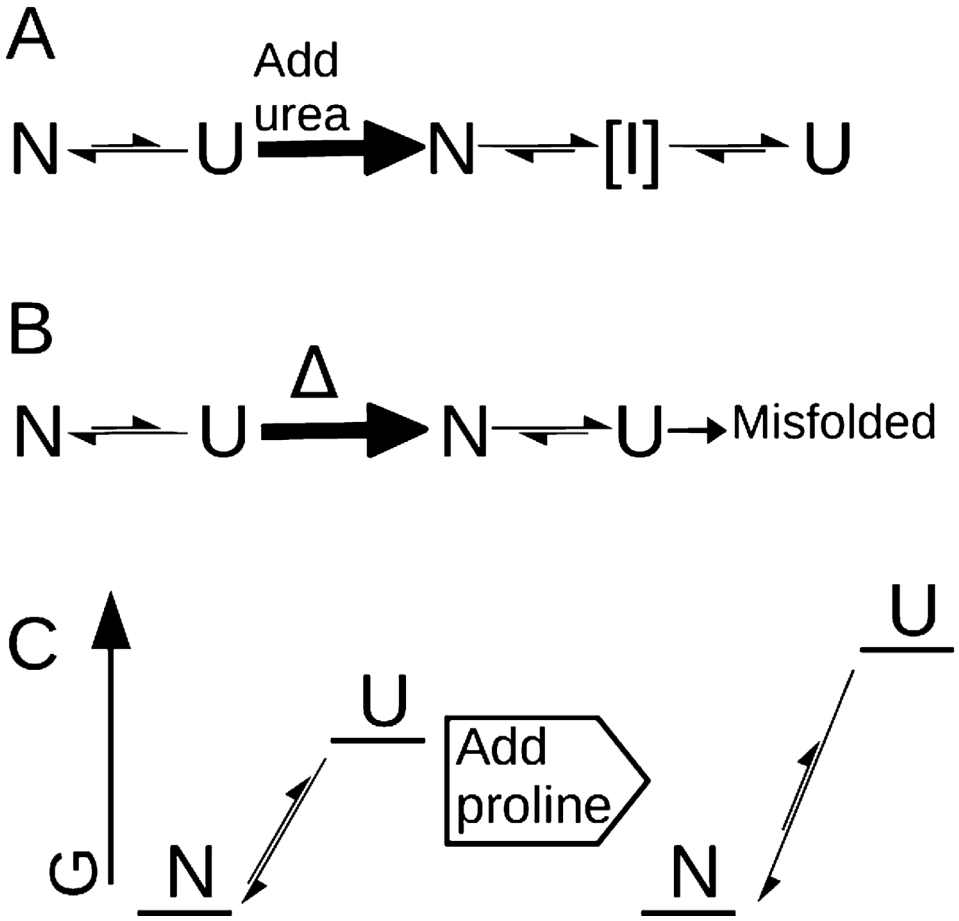
- 脯氨酸在寡聚-1,6葡萄糖苷酶中的占比与来源菌株的偏好温度呈现显著的正相关关系。

来源菌株	生境特征	Proline %
Bacillus cereus ATCC 7064	嗜温菌	2.37
Bacillus coagulans ATCC 7050	兼性嗜温菌	4.63
Bacillus sp. KP 1071	嗜热菌	5.67
Bacillus thermoglucosidasius KP 1006	专性嗜热菌	6.79
Bacillus flavocaldarius KP 1288	极端嗜热菌	8.52

Y. Suzuki, K. Oishi, H. Nakano, T. Nagayama, A strong correlation between the increase in number of proline residues and the rise in thermostability of five Bacillus oligo-1,6-glucosidases. *Appl. Microbiol. Biotechnol.* **26**, 546-551 (1987).

背景——脯氨酸效应：原理

- 脯氨酸具有较强的刚性，在合适位点引入脯氨酸突变能够稳定蛋白结构，是一种常用的蛋白质工程策略。
- 脯氨酸可限制蛋白质未折叠形式的柔性，将未折叠形式的熵优势降低~1.2 kcal/mol。
- 脯氨酸突变在β-转角、无规卷曲以及α-螺旋和β-折叠的N端等位点容忍性通常较好。



J. Huang, B. J. Jones, R. J. Kazlauskas, Stabilization of an alpha/beta-Hydrolase by Introducing Proline Residues: Salicylic Acid Binding Protein 2 from Tobacco. *Biochemistry* **54**, 4330-4341 (2015).

R. S. Prajapati *et al.*, Thermodynamic effects of proline introduction on protein stability. *Proteins* **66**, 480-491 (2007).

背景——脯氨酸效应：应用

- 通过序列比对以及分子动力学模拟，确定潜在的突变位点后，通过定点引入脯氨酸突变的方式将烟草的SABP蛋白的$T_{1/2}^{15min}$提升了25.7℃。

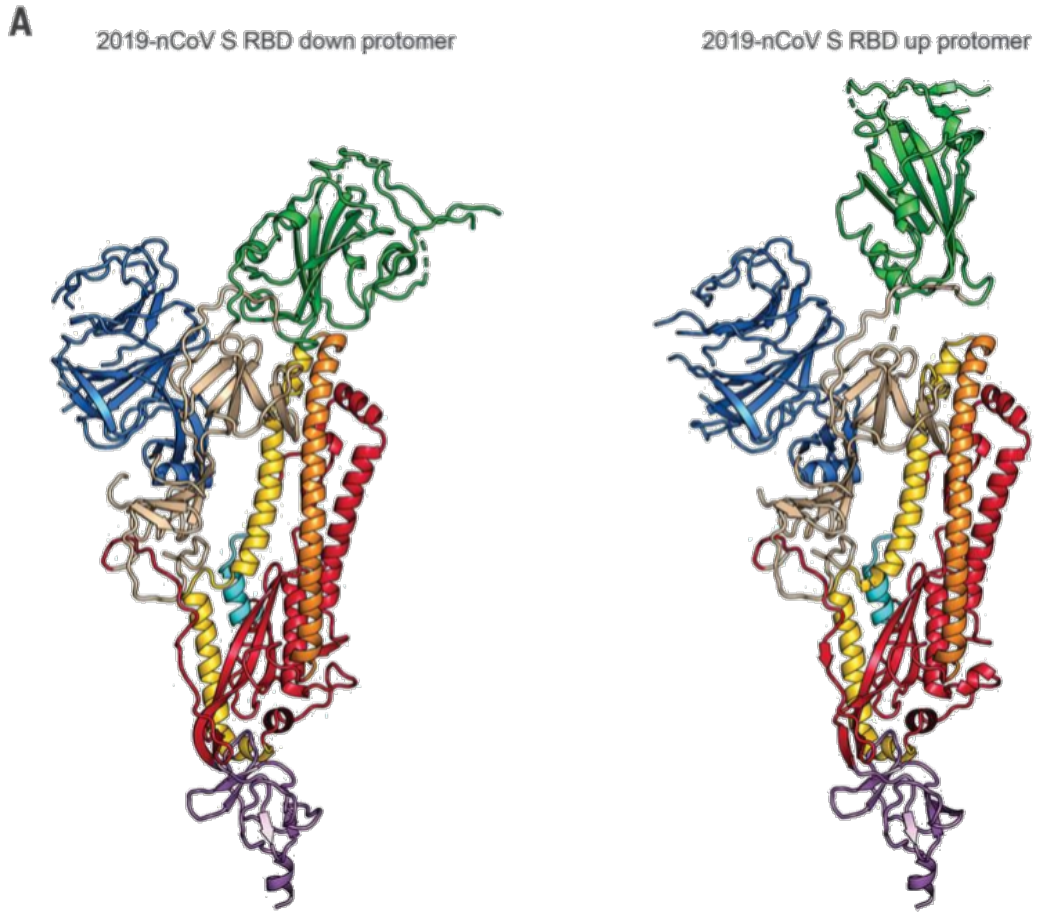
Table 2. Steady-State Kinetic Constants[a] and Stability of SABP2 Variants with Added Proline Residues

SABP2	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (mM^{-1} s^{-1})	$\Delta C_{1/2}^{urea}$[b] (M)	$T_{1/2}^{60°C}$[c] (min)	$T_{1/2}^{15min}$[d] (°C)	$\Delta T_{1/2}^{15min}$ (°C)
WT	1.82 ± 0.02	2.24 ± 0.05	0.81		3.50 ± 0.03	49.2 ± 0.5	
L46P cat	2.44 ± 0.12	3.10 ± 0.23	0.79	+0.2	10.2 ± 1.3	55.6 ± 0.4	+6.4
S70P cat	1.88 ± 0.08	3.10 ± 0.28	0.61	+0.1	7.1 ± 0.2	54.6 ± 0.4	+5.4
S115P cap	0.89 ± 0.04	1.85 ± 0.23	0.48	−0.3	12 ± 1.7	51.0 ± 0.9	+1.8
S141P cap	1.56 ± 0.05	1.60 ± 0.11	0.97	+0.0	9.4 ± 0.3	54.1 ± 0.8	+4.9
K190P cat	1.30 ± 0.07	3.21 ± 0.35	0.41	−1.5	<2	42.2 ± 0.7	−7.0
E215P cat	2.68 ± 0.20	3.49 ± 0.38	0.77	+0.9	5.3 ± 0.1	53.4 ± 0.5	+4.2
V227P cat	2.51 ± 0.21	4.42 ± 0.45	0.79	−0.4	<2	46.9 ± 0.7	−2.3
L46P−S70P cat−cat	2.18 ± 0.06	2.44 ± 0.15	0.89	+0.1	22.5 ± 1.7	74.9 ± 1.7	+25.7
L46P−S141P cat−cap	2.29 ± 0.16	3.03 ± 0.30	0.76	−0.1	11.7 ± 0.1	55.8 ± 0.4	+6.6
S70P−S141P cat−cap	3.42 ± 0.07	3.77 ± 0.15	0.91	−0.2	16.0 ± 0.5	60.5 ± 0.4	+11.3
L46P−S70P−E215P cat−cat−cat	2.34 ± 0.13	6.00 ± 0.56	0.39	+0.9	17.5 ± 0.5	57.2 ± 0.4	+8.0
L46P−S70P−S141P cat−cat−cap	2.05 ± 0.10	2.74 ± 0.10	0.75	+0.0	14.4 ± 0.2	59.6 ± 0.8	+10.4

J. Huang, B. J. Jones, R. J. Kazlauskas, Stabilization of an alpha/beta-Hydrolase by Introducing Proline Residues: Salicylic Acid Binding Protein 2 from Tobacco. *Biochemistry* **54**, 4330-4341 (2015).

背景——脯氨酸效应：应用

- 通过引入2个脯氨酸，将抗原S蛋白稳定在活性构象，从而激发更高效更持久的免疫反应。（在辉瑞及Moderna 的疫苗生产中已应用）

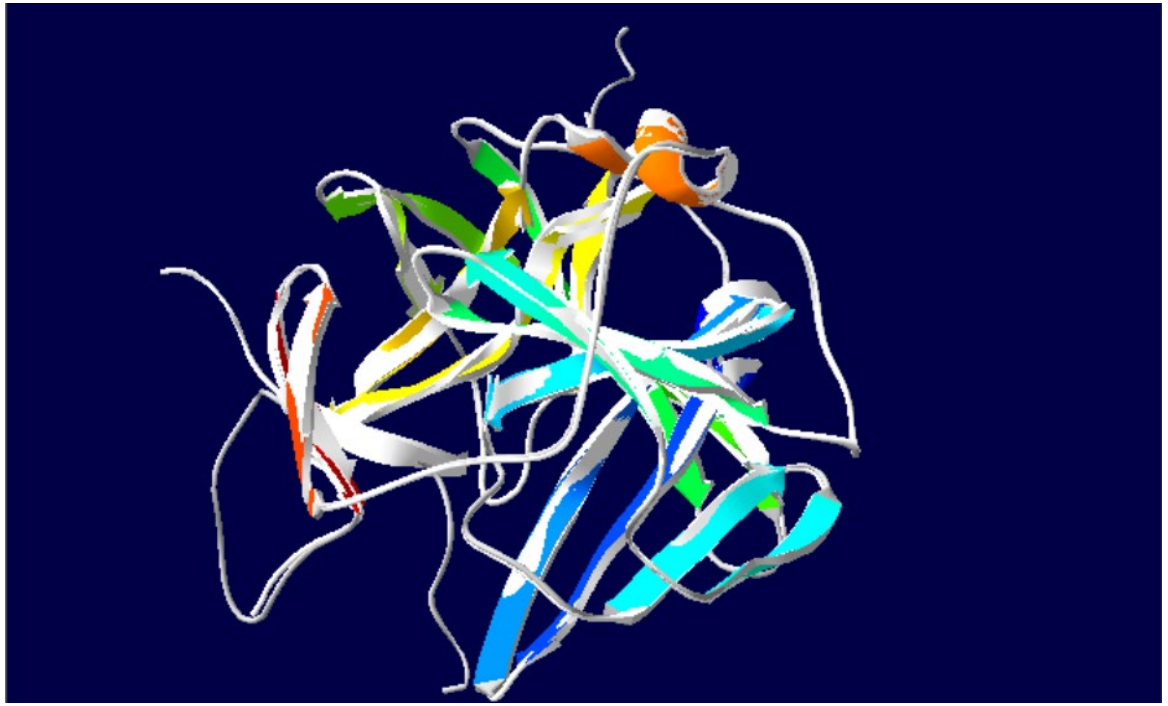


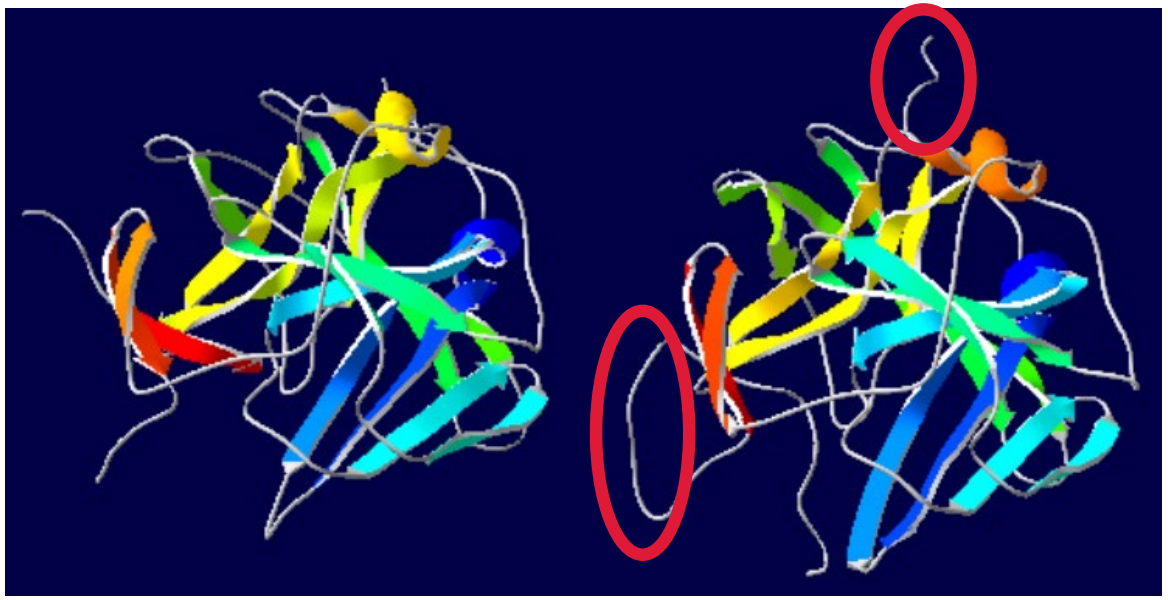
两名共一：Daniel Wrapp、王年爽

D. Wrapp *et al.*, Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* **367**, 1260-1263 (2020).

使用完整序列预测结构

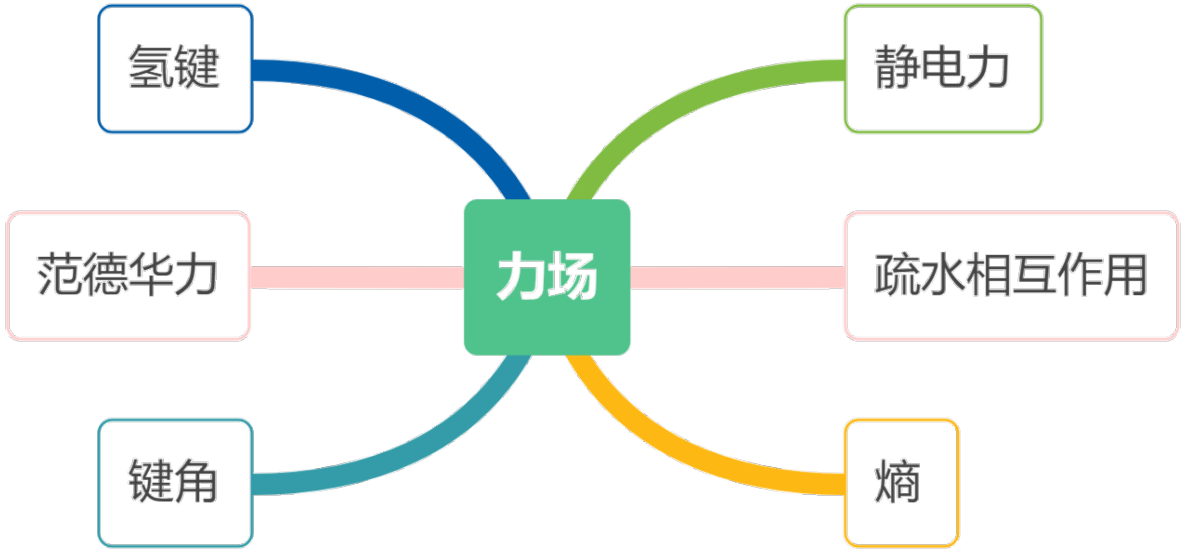
- 发现RCSB PDB中TEVp的结构存在部分片段的缺失(1Q31)
- 使用I-TASSER Sever预测完整的序列结构，补全缺失部分
- 用Swiss-PdbViewer将预测结果与1Q31做拟合





分子动力学模拟

- 利用力场能量迭代求解蛋白质分子和溶剂分子中原子的牛顿运动方程，记录构象在模拟中发生的改变。根据热力学定律，系统往往会趋向于其自由能较低的状态，也就是稳定的状态。



- 可用来解决的问题：

1.蛋白质的折叠问题，获得稳定构象

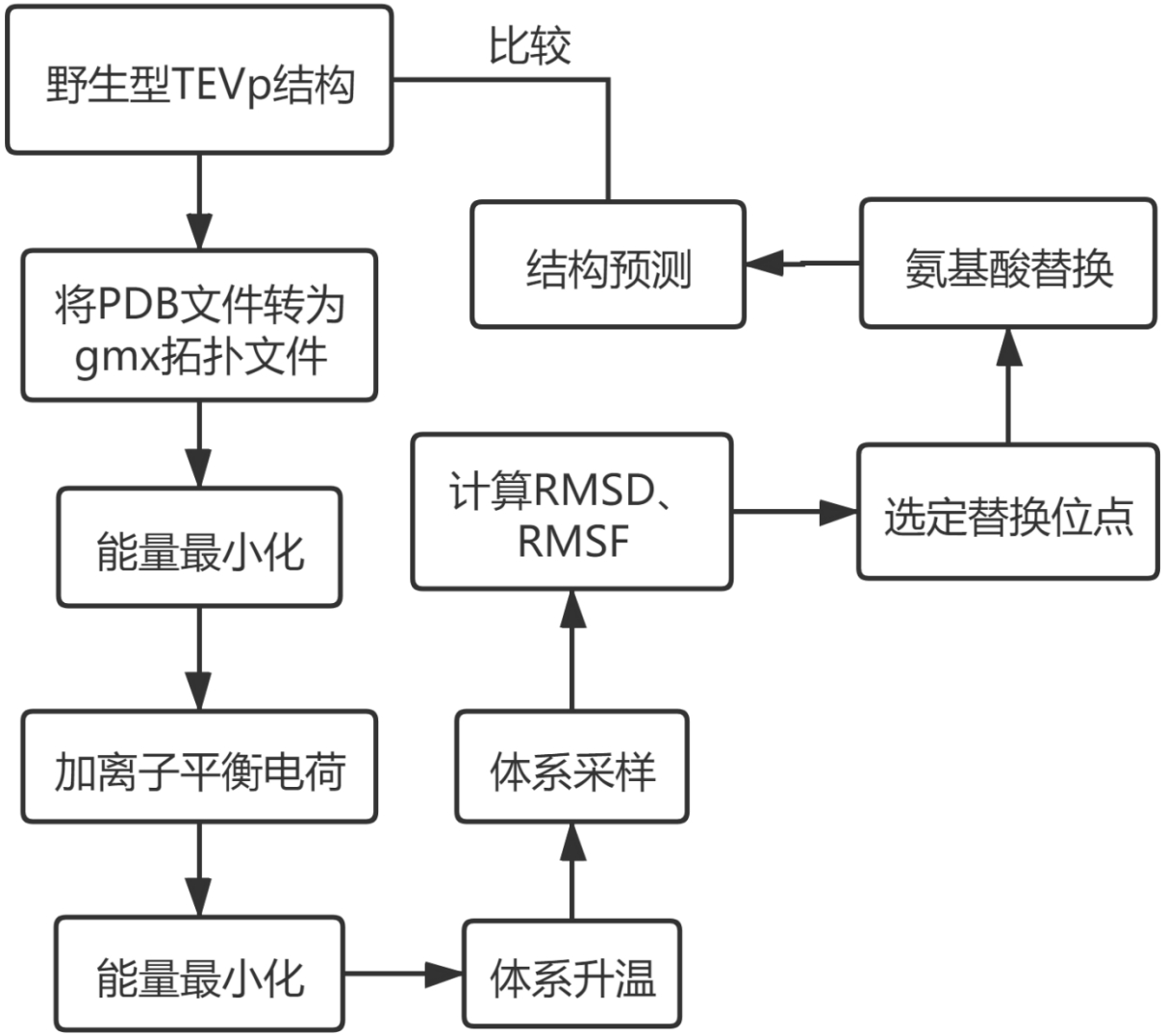
2.配体与蛋白质的对接

3.蛋白质的稳定性问题

……

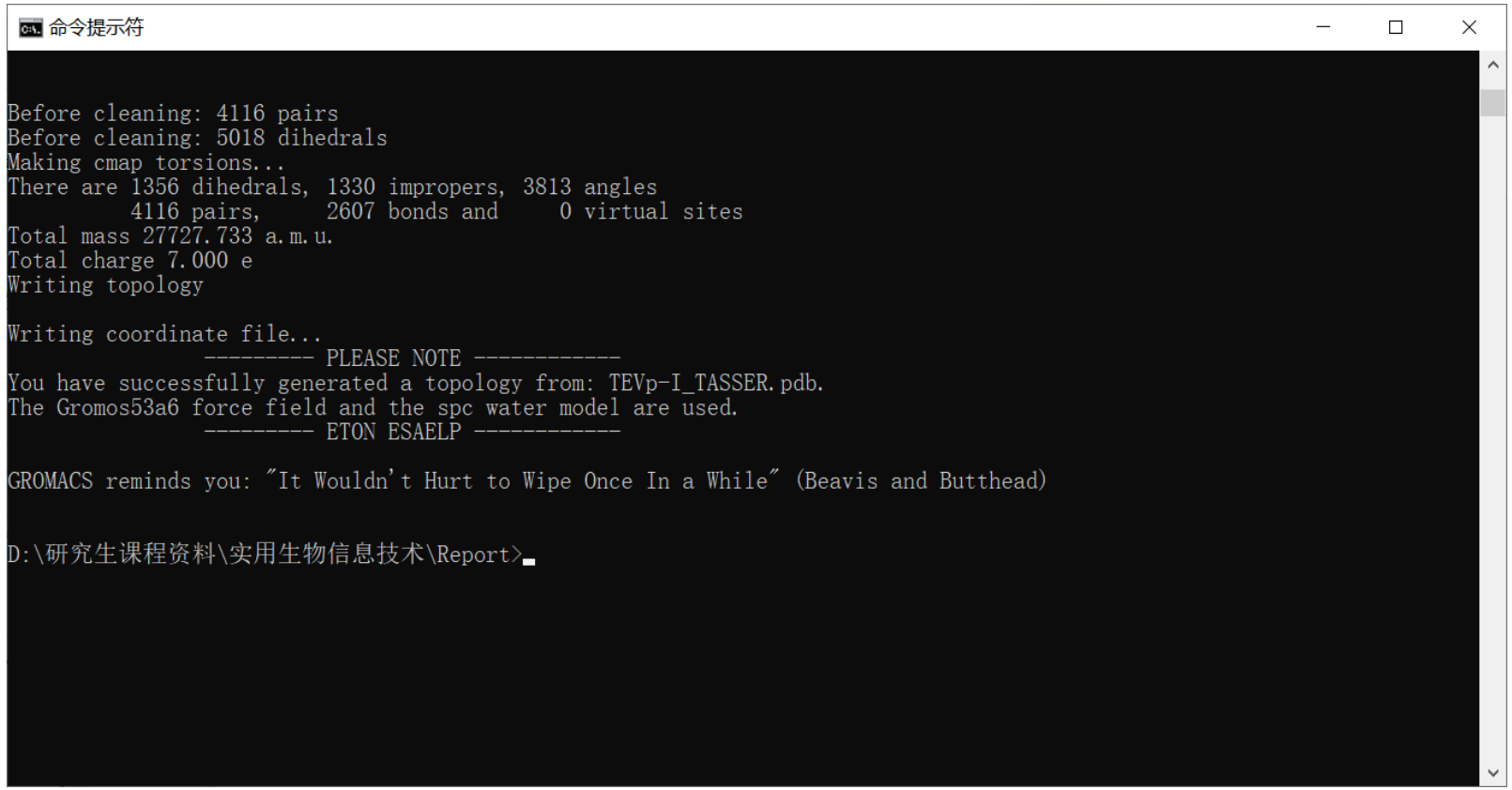
软件：Gromacs 2020.3 - MODIFIED

流程图


野生型TEVp结构
比较
将PDB文件转为gmx拓扑文件
结构预测
氨基酸替换
能量最小化
计算RMSD、RMSF
选定替换位点
加离子平衡电荷
体系采样
能量最小化
体系升温

根据PDB坐标生成拓扑文件

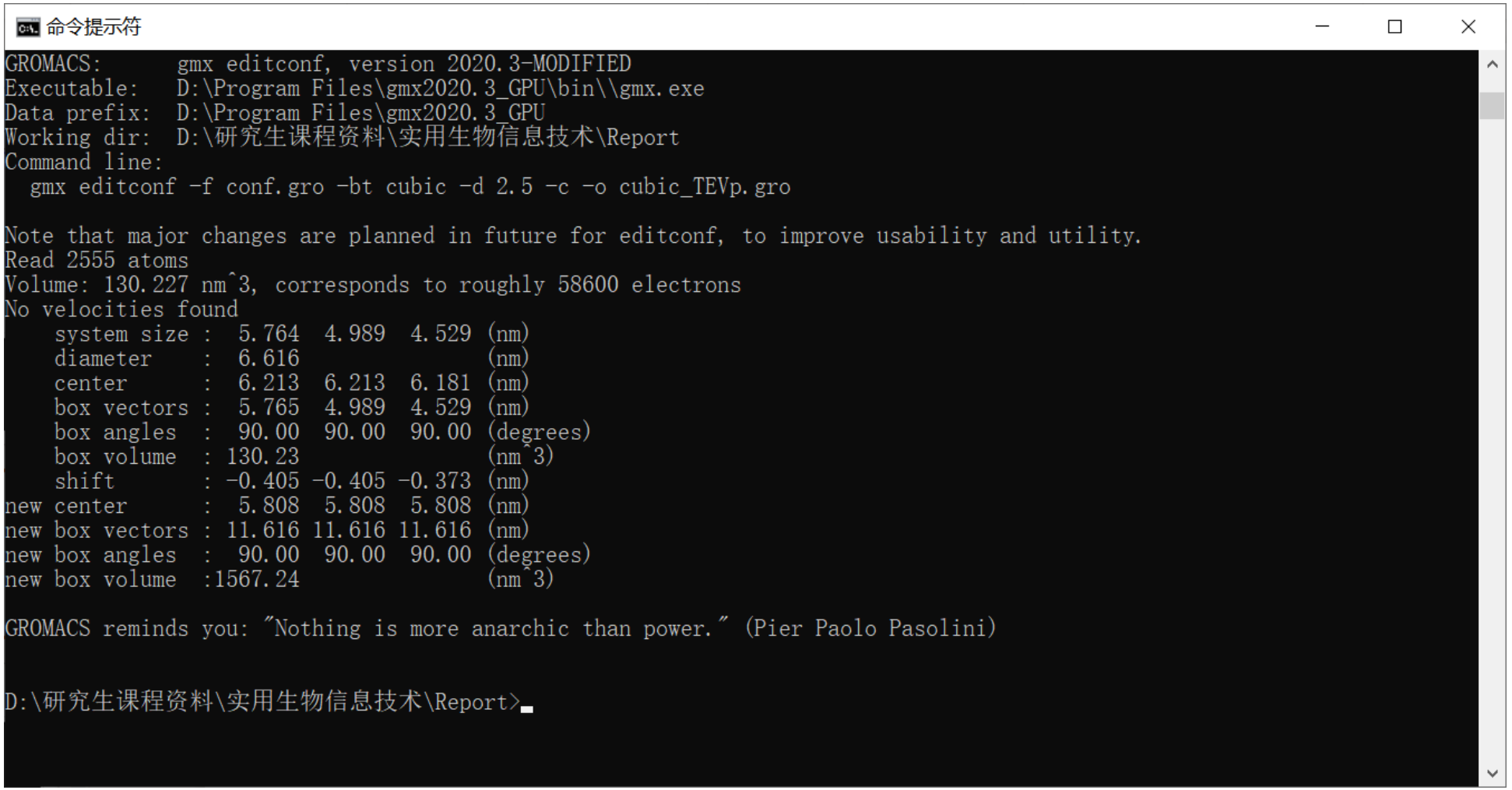
- 命令：gmx pdb2gmx -f TEVp-I_TASSER.pdb -o conf.gro -p topol.top -ignh
- 力场：GROMOS96 53a6
- 溶剂模型：SPC



M. R. Smaoui, J. Waldispuhl, Computational re-engineering of Amylin sequence with reduced amyloidogenic potential. *BMC Struct Biol* **15**, 7 (2015).

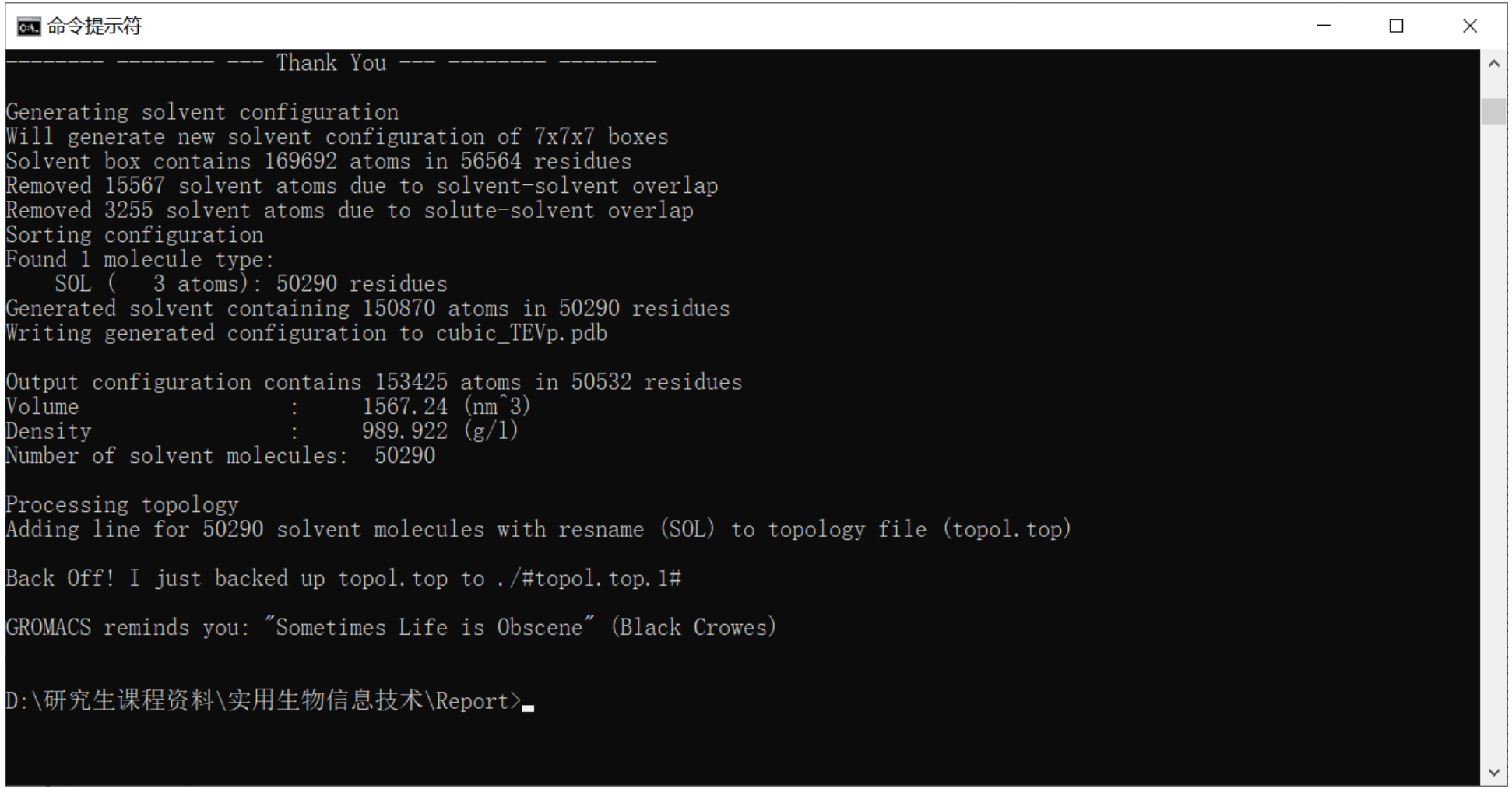
加立方体模拟盒子

- 命令：gmx editconf -f conf.gro -bt cubic -d 2.5 -c -o cubic_TEVp.gro



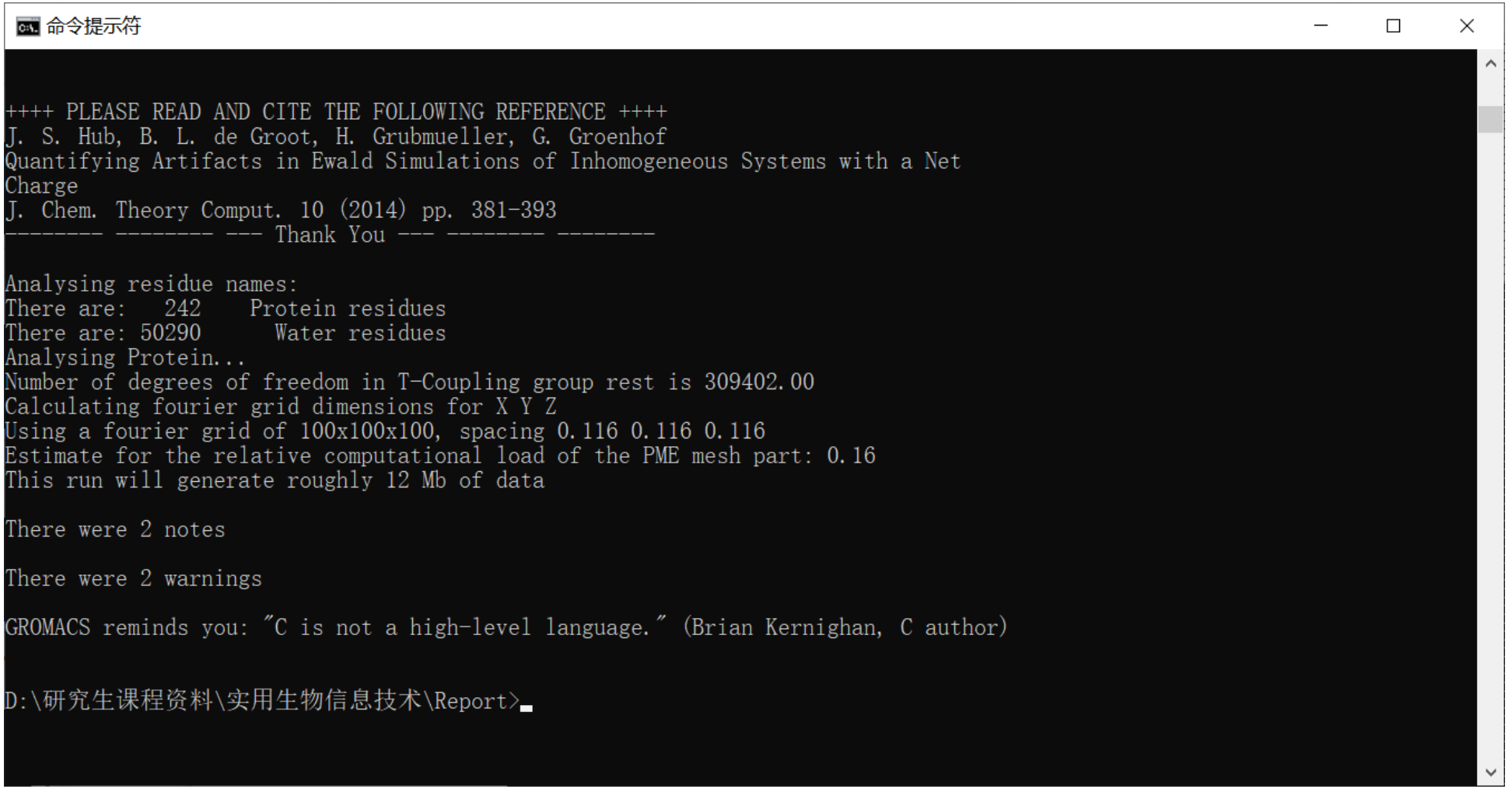
加溶剂

- 命令：gmx solvate -cp cubic_TEVp.gro -o cubic_TEVp.pdb -p topol.top



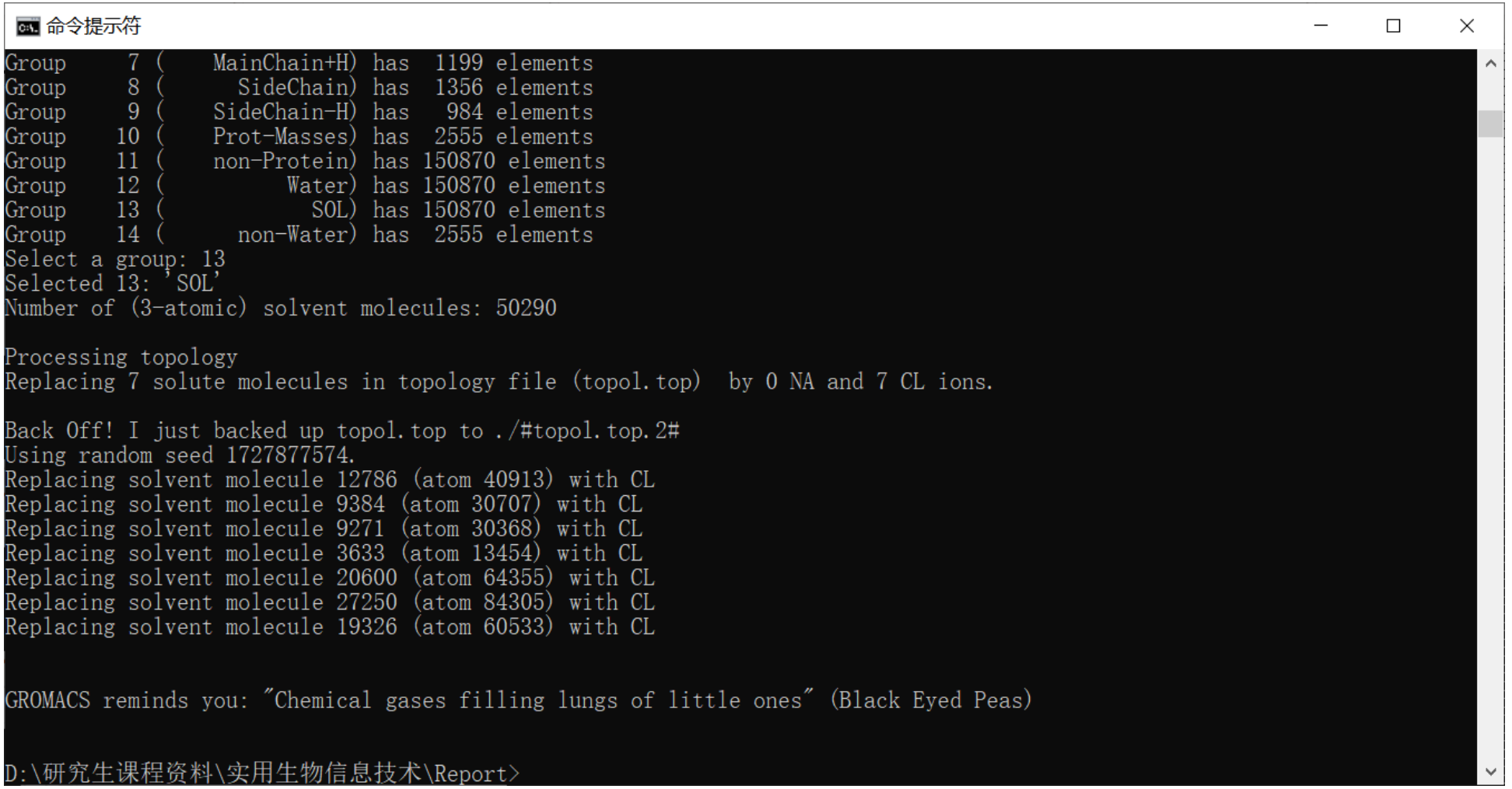
能量最小化

- 命令：gmx grompp -f em.mdp -c cubic_TEVp.pdb -p topol.top -o em_TEVp



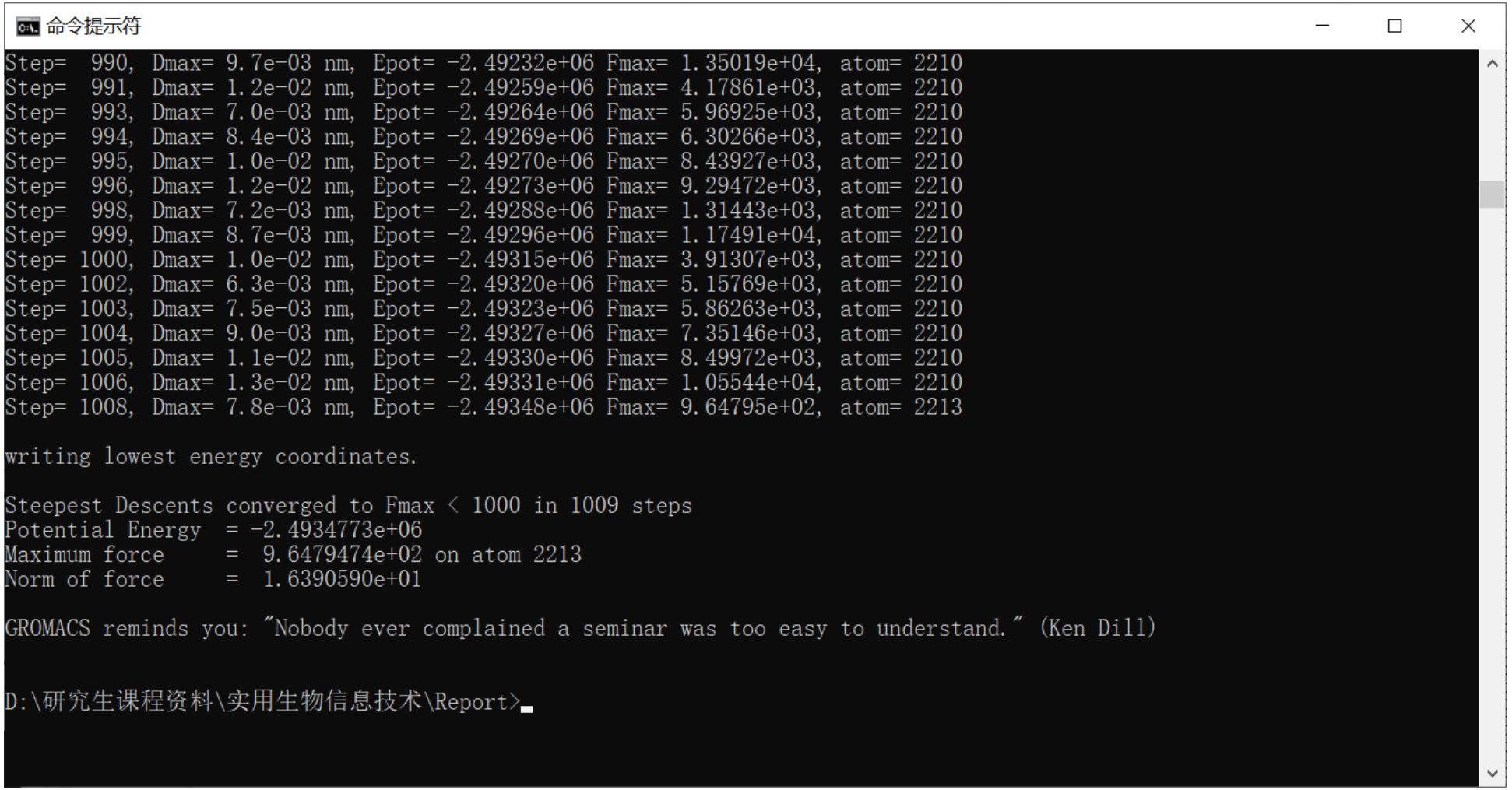
加7个Cl^-用于平衡电荷

- 命令：gmx genion -s em_TEVp.tpr -o ION.gro -p topol.top -nn 7 -nname CL



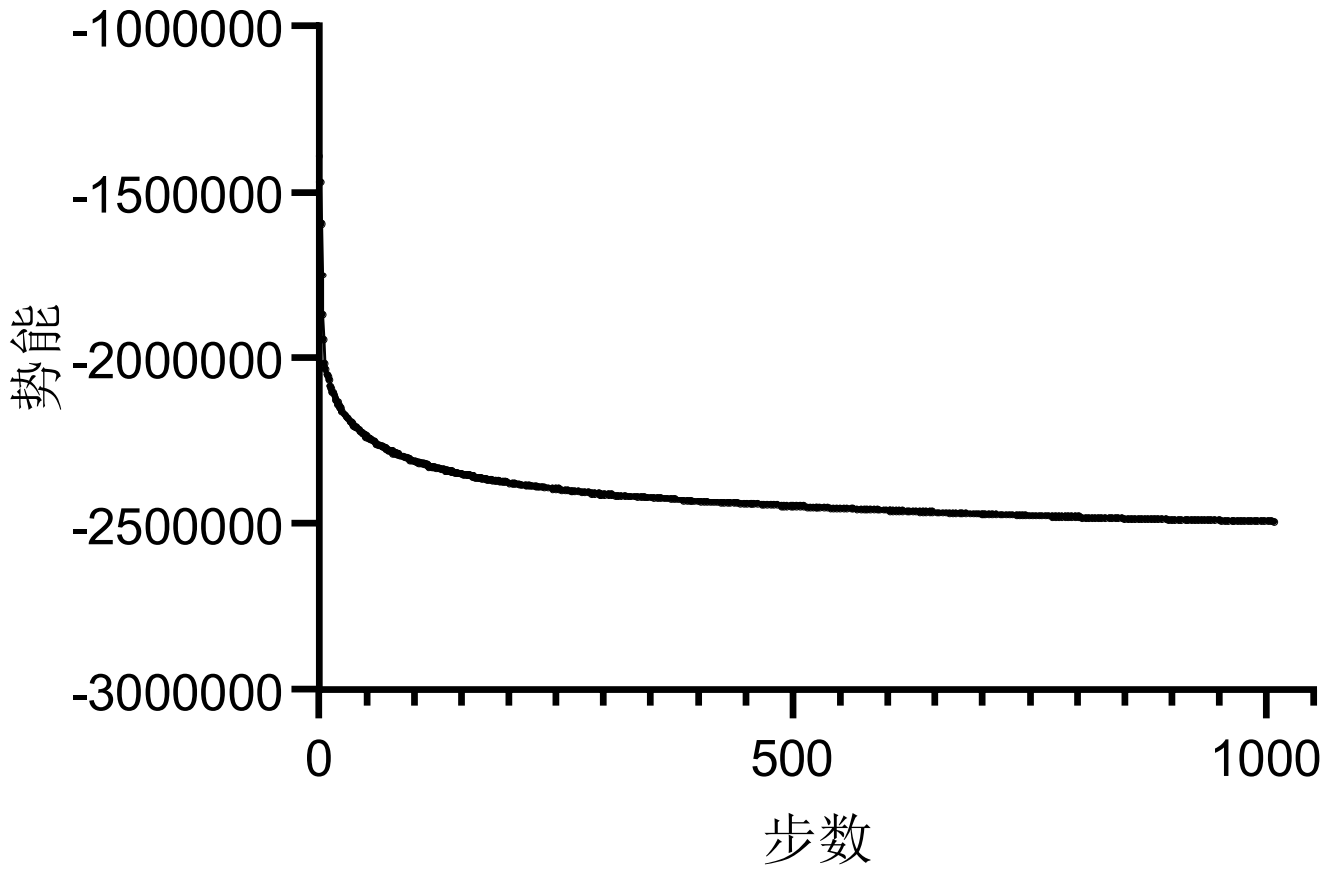
再次能量最小化，并记录轨迹

- 命令：gmx grompp -f em.mdp -c ION.gro -p topol.top -o em_TEVp.tpr
- gmx mdrun -s em_TEVp.tpr -deffnm cold –v



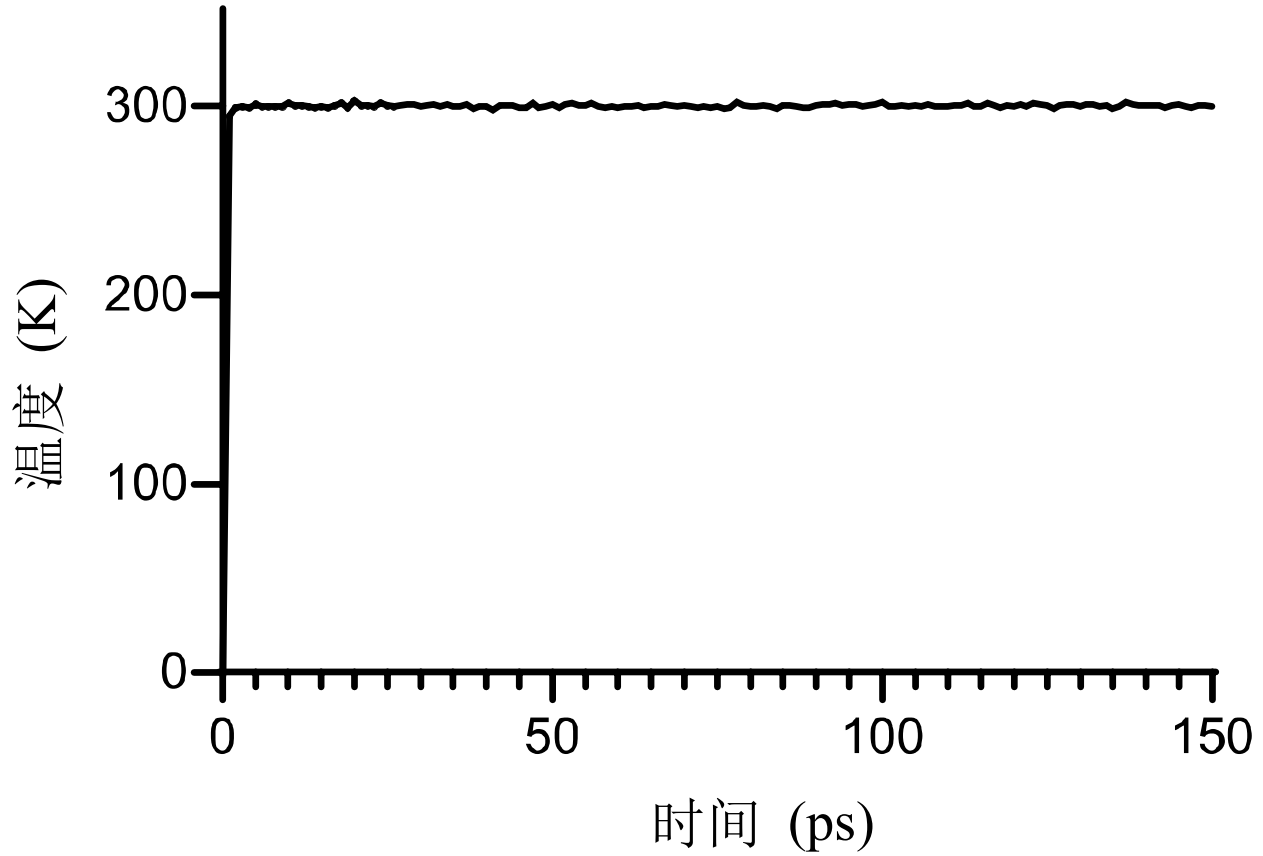
查看能量降低情况

- 命令：gmx energy -f cold.edr -o cold.xvg
- 选择Potential（势能）



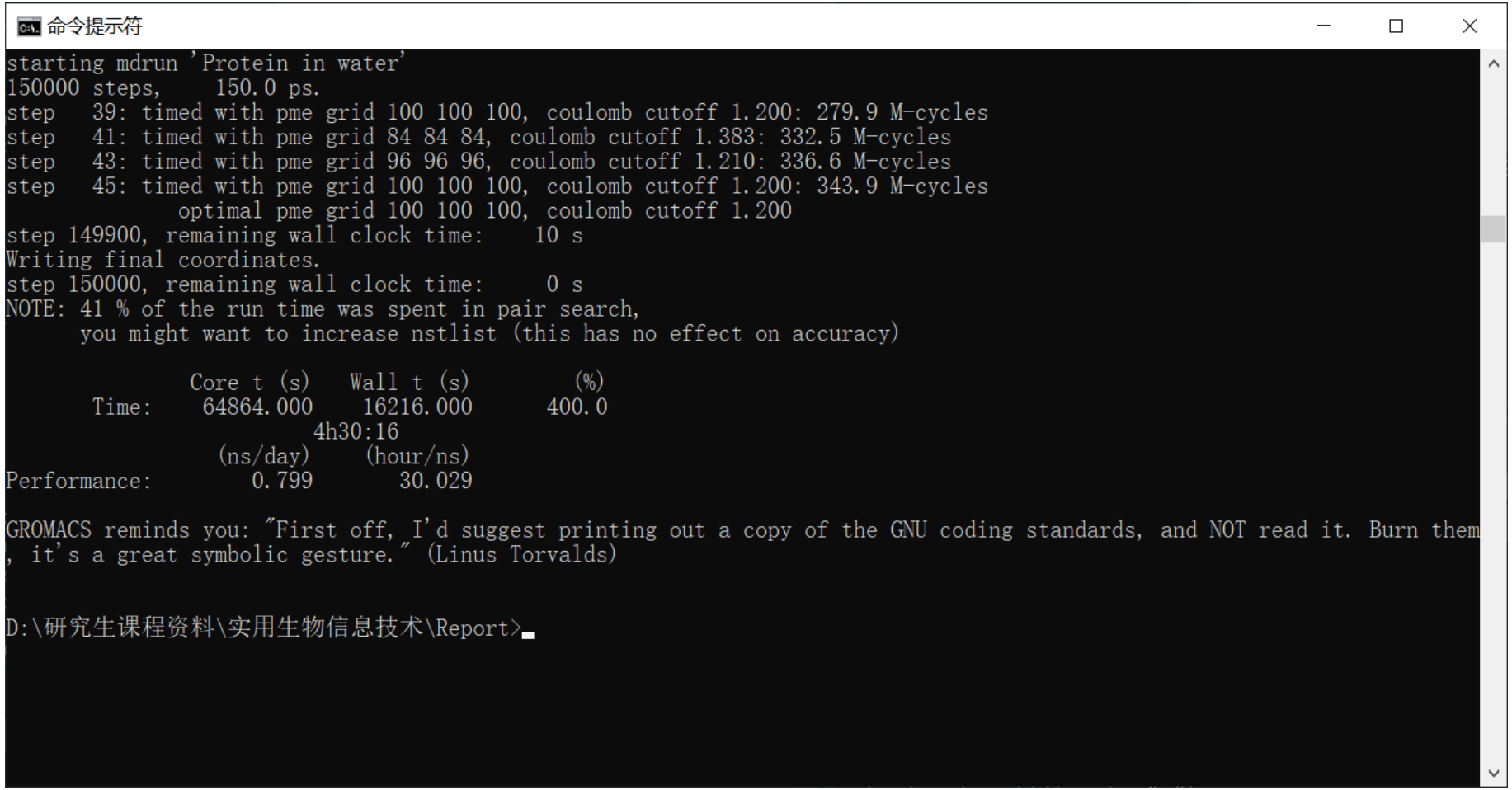
体系升温，查看升温情况

- 命令：gmx grompp -f upgrade.mdp -c cold.gro -p topol.top -o em_TEVp_hot.tpr
- gmx mdrun -s em_TEVp_hot.tpr -v -deffnm hot
- gmx energy -f hot.edr -o hot.xvg



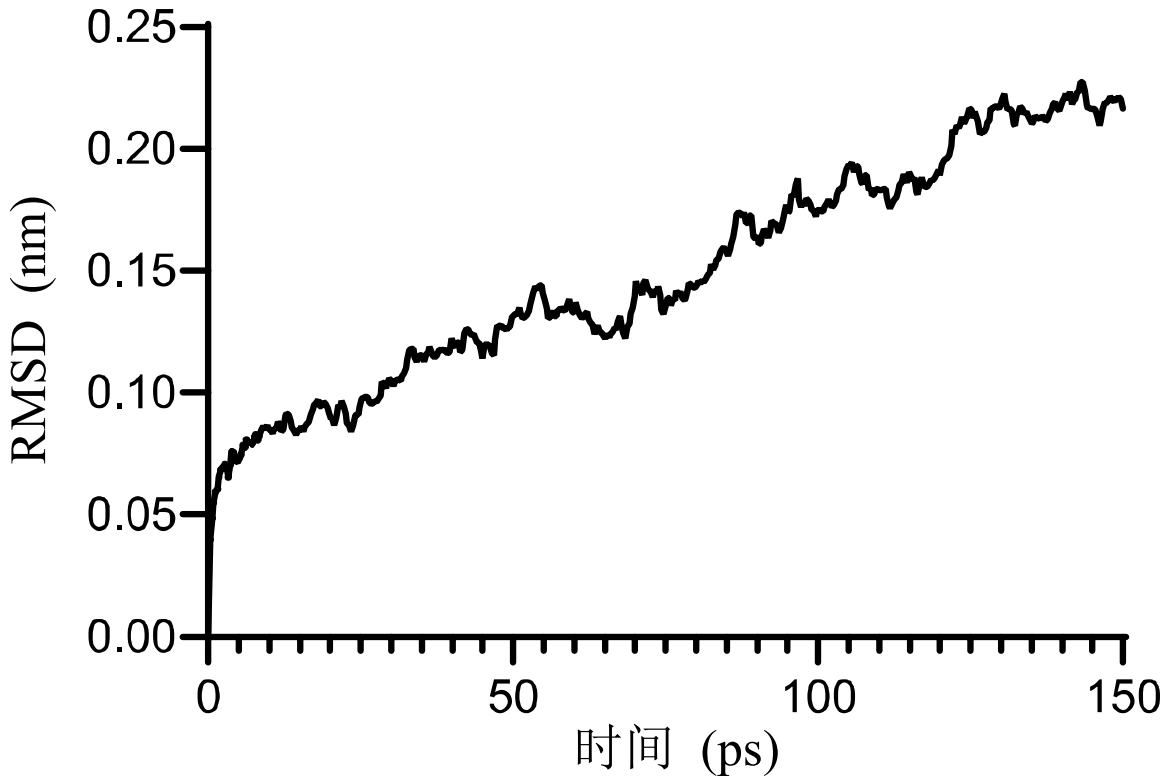
体系升温过程采样

- 命令：gmx grompp -f sample.mdp -c hot.gro -p topol.top -o em_TEVp_hot_sample.tpr
- gmx mdrun -s em_TEVp_hot_sample.tpr -v -deffnm hot_sample



计算RMSD

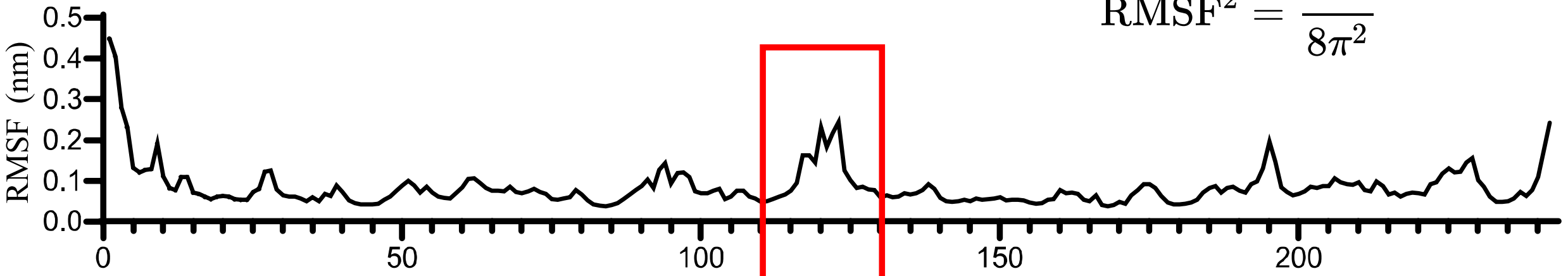
- 命令：gmx rms -s em_TEVp_hot_sample.tpr -f hot_sample.trr -o rmsd.xvg
- 发现150 ps时暂未达到热力学稳态，应继续模拟至RMSD值较稳定

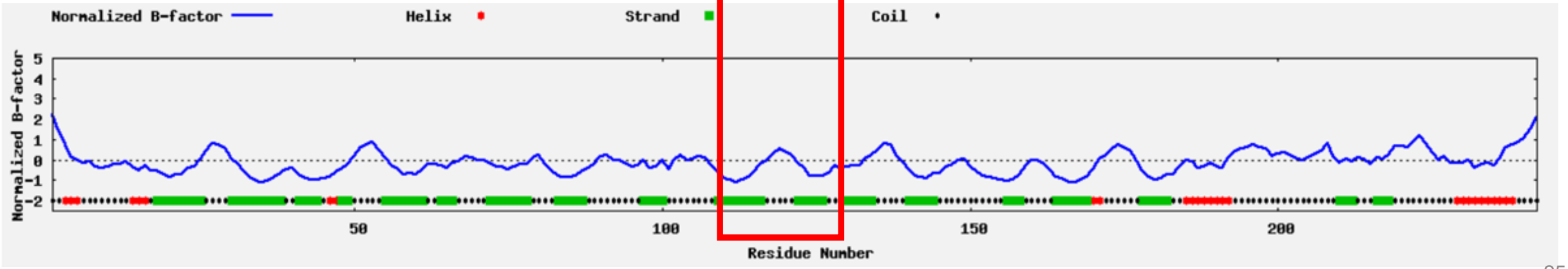


计算RMSF

- 命令：gmx rmsf -s em_TEVp_hot_sample.tpr -f hot_sample.trr -o rmsf.xvg -res
- 可见，各氨基酸残基RMSF值相对大小与I-TASSER Server预测的B-factor基本一致

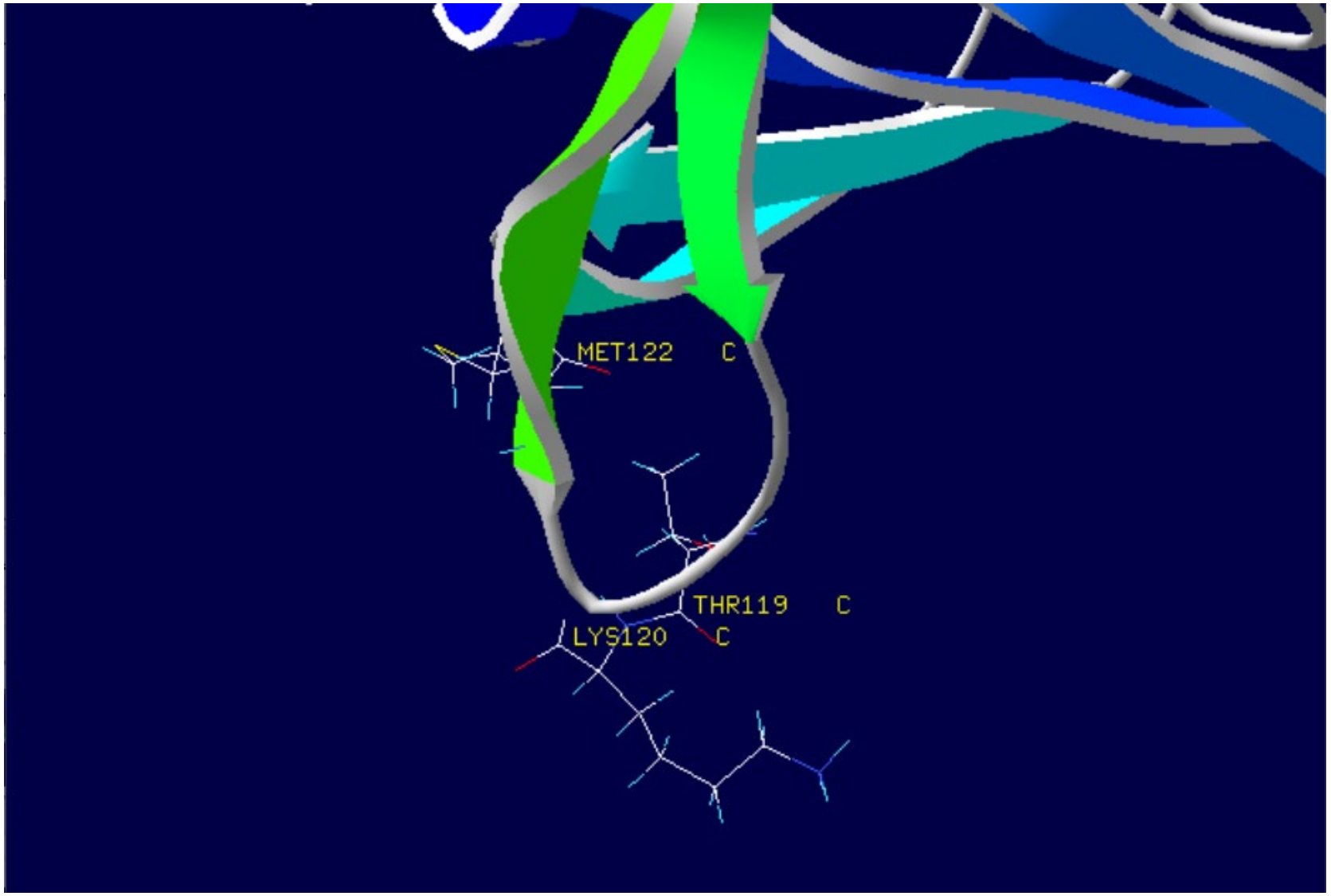
$$\mathrm{RMSF}^2 = \frac{3\mathrm{B}}{8\pi^2}$$





确定候选突变位点

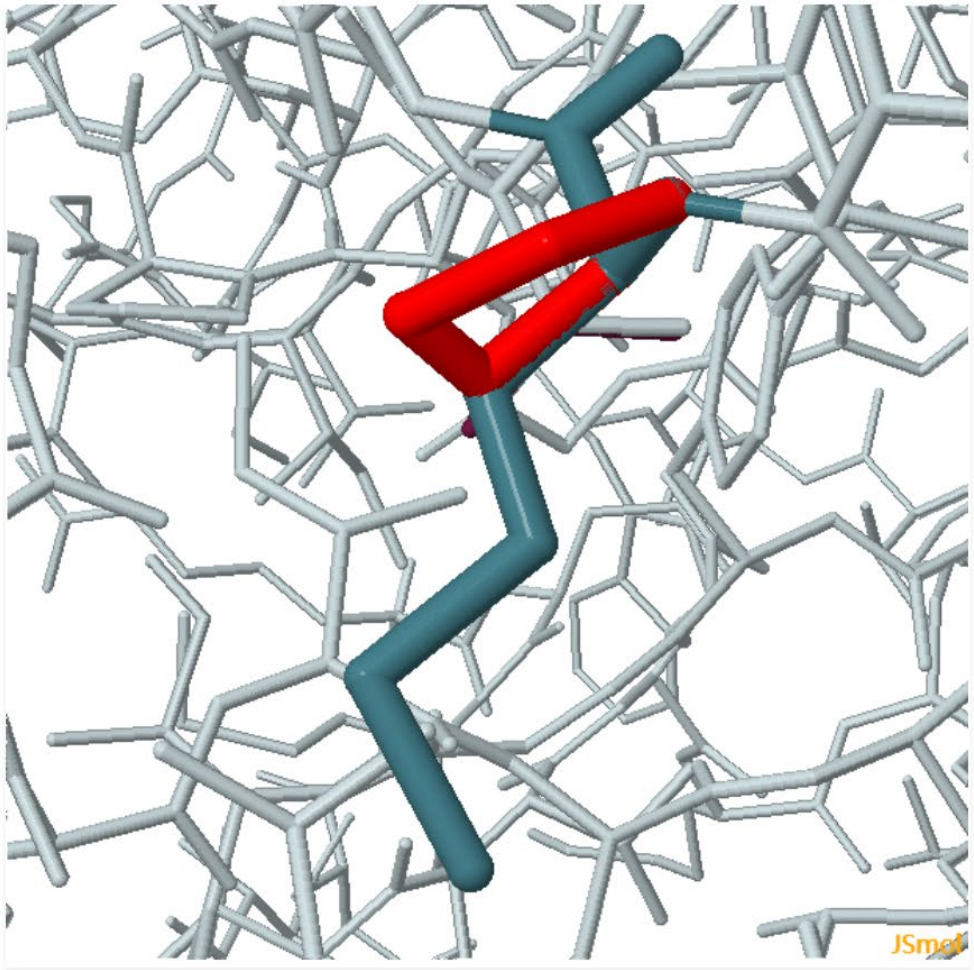
- 选择RMSF值较高的区域，要求尽量不在底物结合位点或者活性中心位置
- 选定T119P、K120P、M122P
- 位于β-转角位置



M122P

MISSENSE 3D

- MISSENSE3D预测该突变会造成结构的改变，因此排除



tevp-i_tasser (A)

Residue ID: 122

Variant: MET > PRO

Structural damage detected

Disallowed phi/psi

Display control | Export image

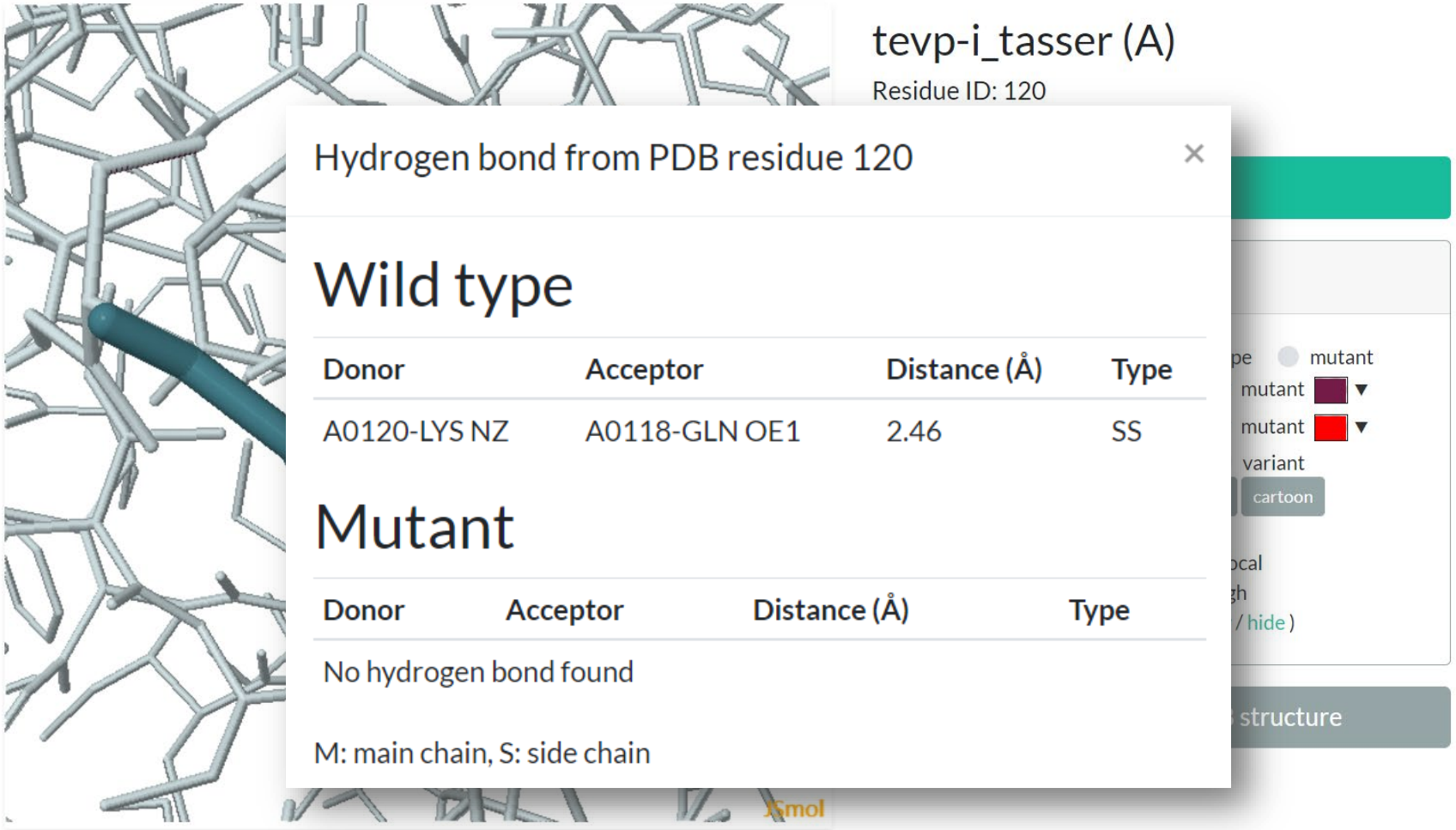
Model	both	wild type	mutant
Chain colour	wild type	mutant	
Residue colour	wild type	mutant	
Centre	protein	variant	
Show as	spacefill	wire	cartoon
Surface	on	off	
Label	none	local	
Image quality	low	high	

(For advanced user: console info show / hide)

Download mutant PDB structure

K120P

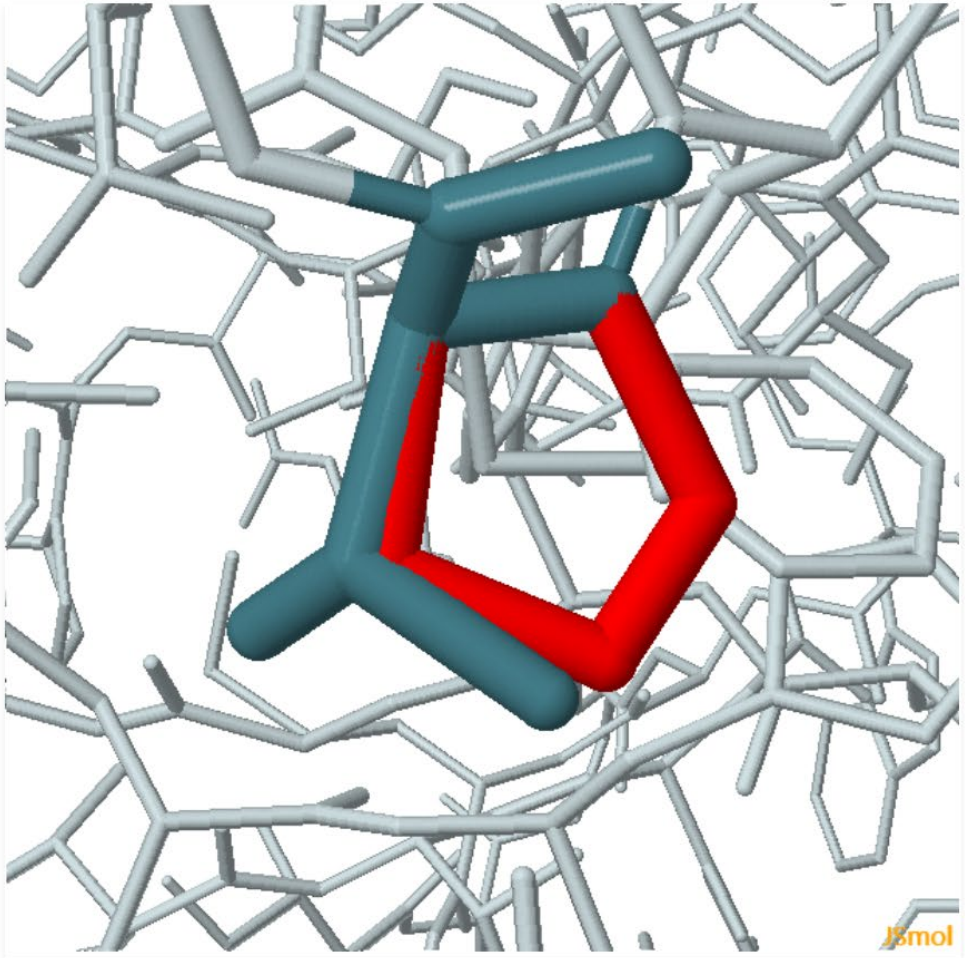
- MISSENSE3D预测该突变不造成结构的改变，但会导致突变后该分子在突变位点减少一个氢键，不利于提高热稳定性，因此排除



T119P

MISSENSE 3D

- 无结构的改变，无氢键数目的改变，因此初步定为较理想的突变位点



tevp-i_tasser (A)

Residue ID: 119

Variant: THR > PRO

No structural damage detected

Display control | Export image

Model: both | wild type | mutant

Chain colour: wild type ▼ mutant ▼

Residue colour: wild type ▼ mutant ▼

Centre: protein | variant

Show as: spacefill | wire | cartoon

Surface: on | off

Label: none | local

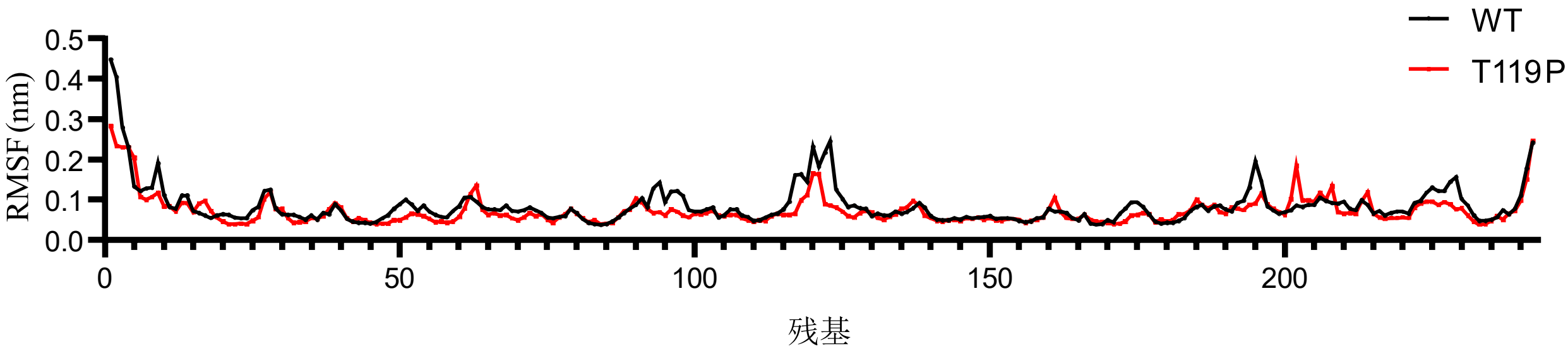
Image quality: low | high

(For advanced user: console info show / hide)

Download mutant PDB structure

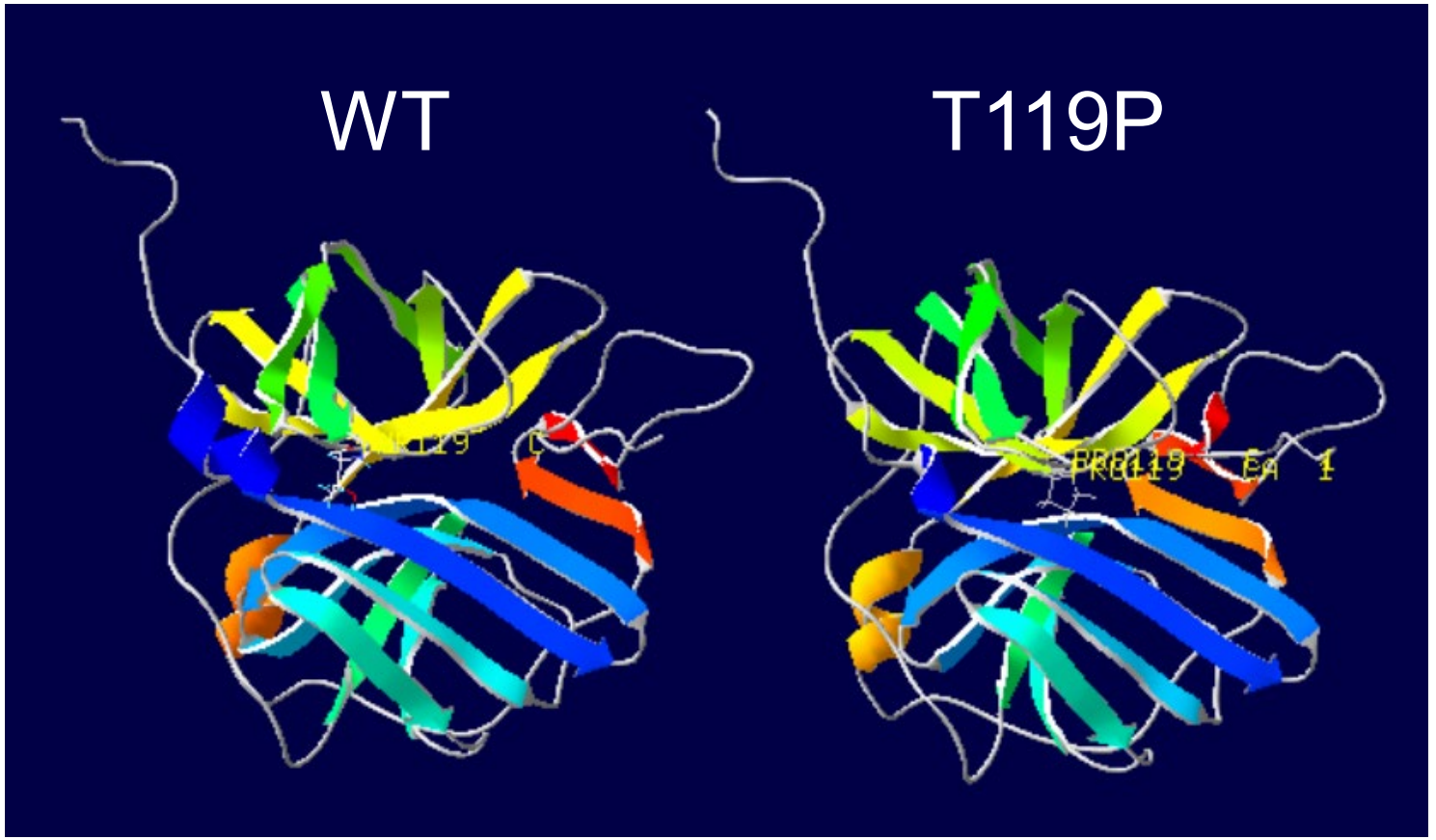
分子动力学模拟T119P对结构的稳定效果

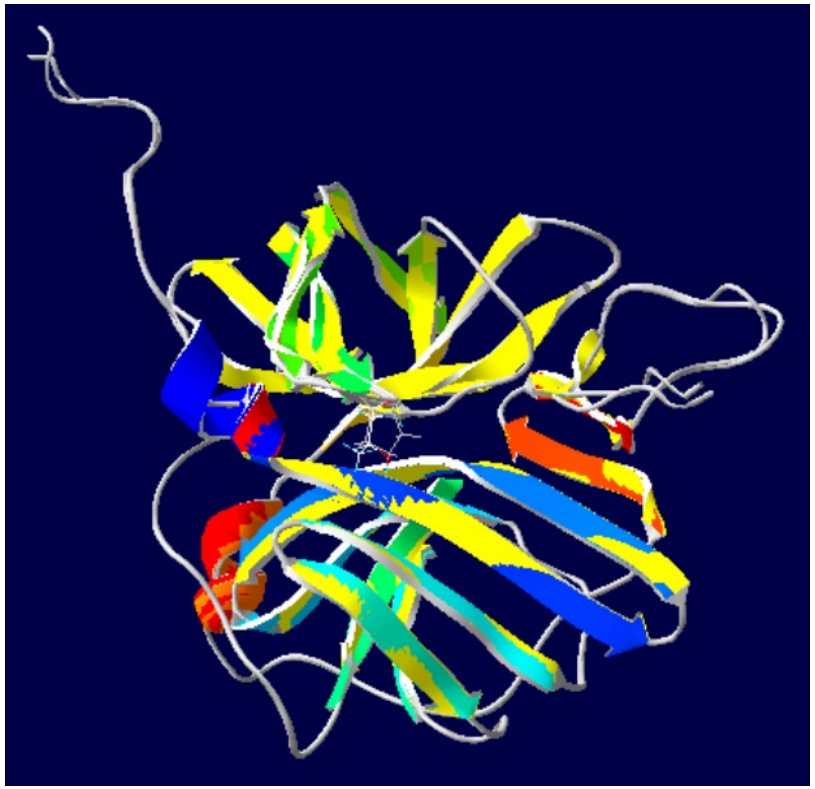
- 使用I-TASSER Server对突变后的蛋白结构做预测，用于分子动力学模拟
- 在同样的参数设置下，模拟结果显示，T119P的分子在120附近的柔性较WT减弱，整体刚性增强，稳定性增强。



WT与T119P的结构比对

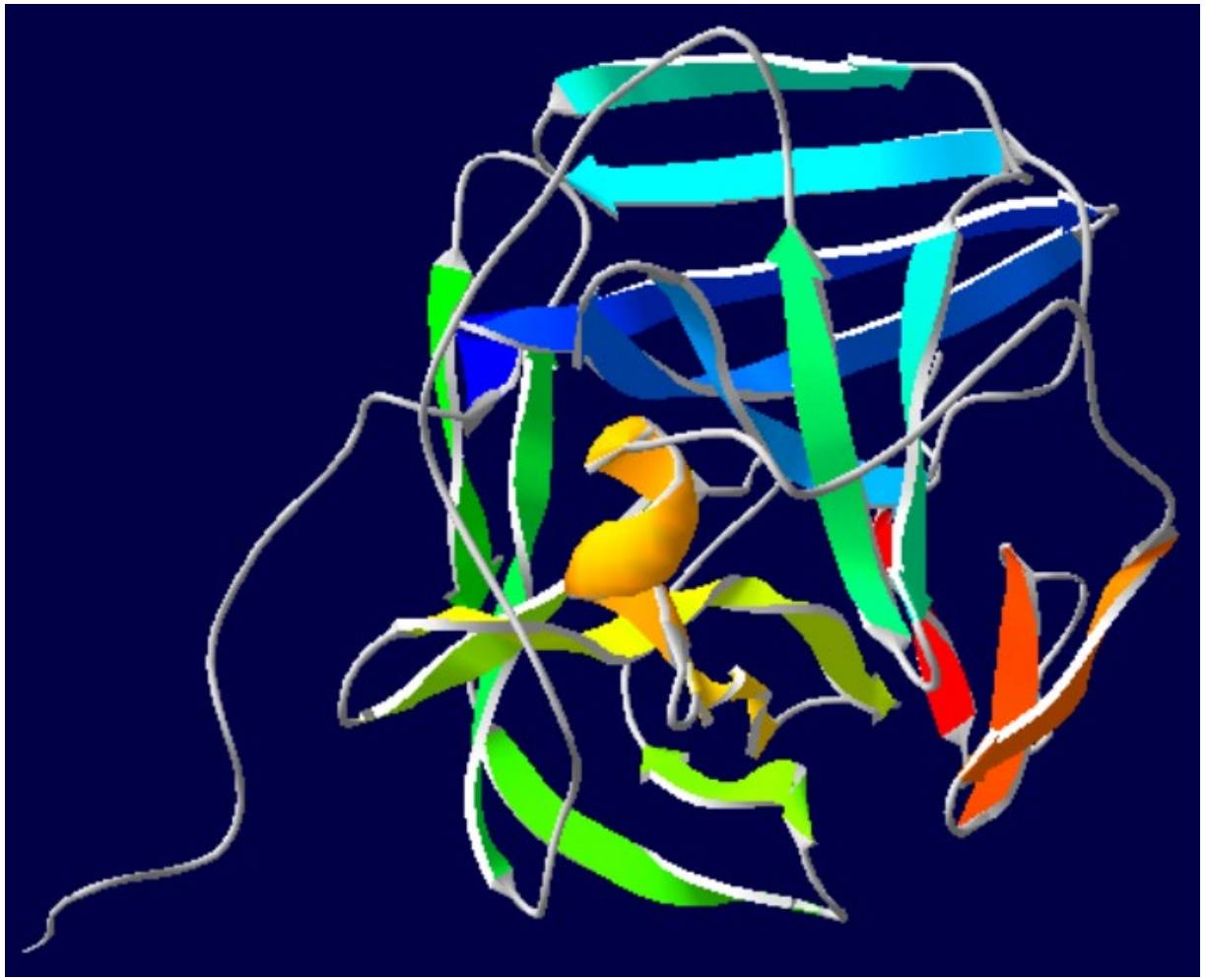
- 预测T119P的结构，发现仍然能够与WT有较高的拟合度。





后续工作

- 延长模拟时间，获得更为准确的模拟结果；
- 对其他潜在的柔性较高的位点进行同样的突变模拟；
- 计算突变位点对整体结构热力学稳定性的贡献；
- 实验验证热稳定性（包括最适温度、变性温度等）是否有提升；
- 实验验证突变后蛋白质的活性、溶解性等其他生化参数是否发生改变。



谢谢！

G05

TEV蛋白酶热稳定性突变体的理性设计
Rational design of TEV protease mutants with enhanced thermostability