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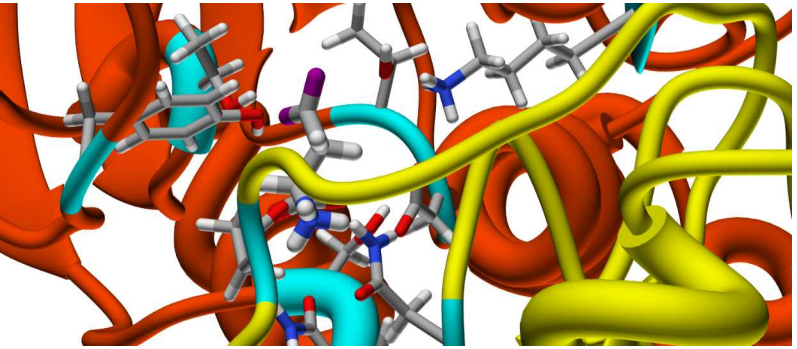

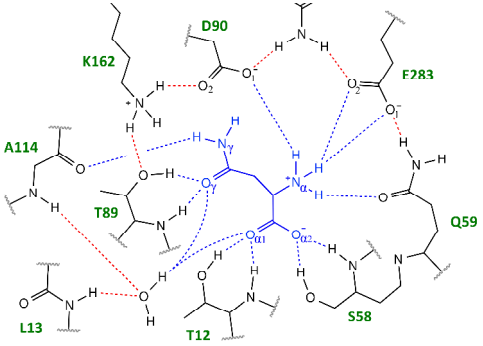


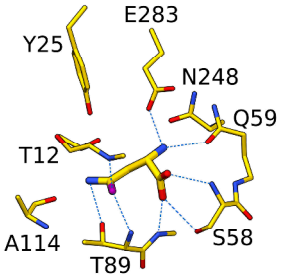


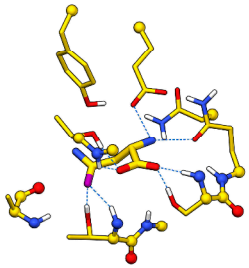

# Active role of the substrate during catalysis by the therapeutic enzyme L-asparaginase II

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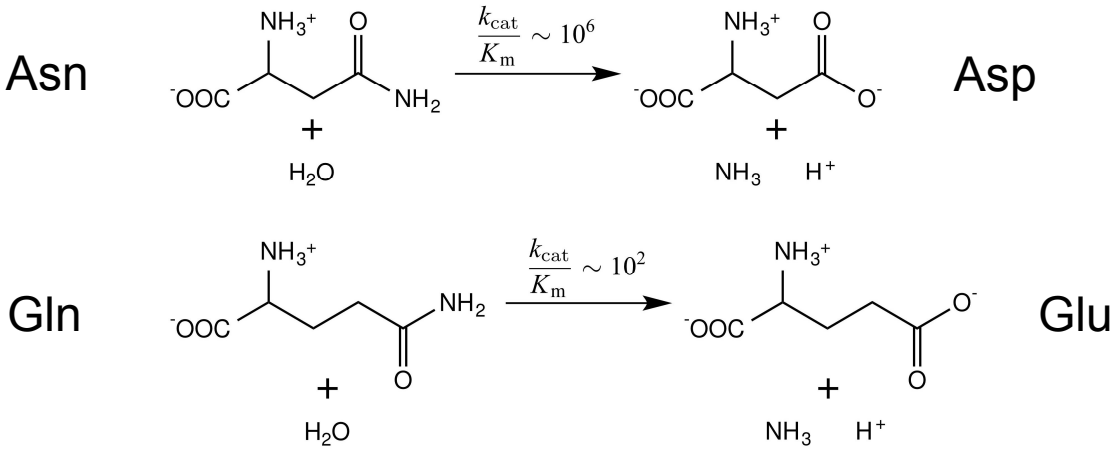
# L-Asparaginase: Starving cancer cells

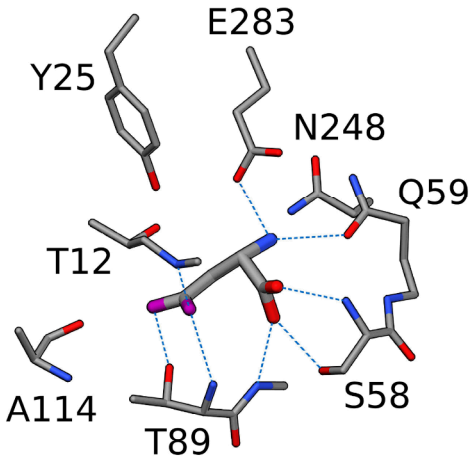
- *E. coli* L-ASP deamidates Asn and Gln



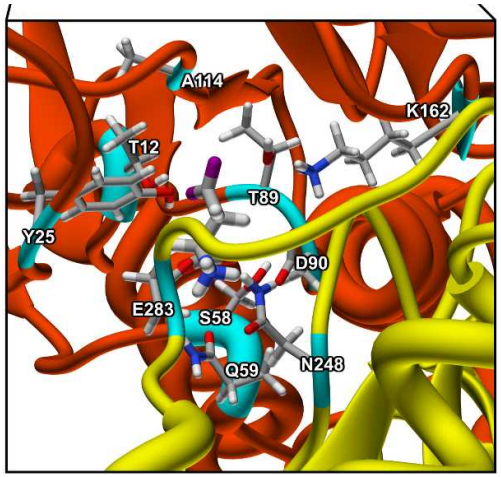

- L-ASP in bloodstream starves certain cancer cells that are auxotrophic for Asn
- Large catalytic difference for Asn vs Gln degradation despite only one $CH_2$ difference
- L-ASP has been used to treat acute lymphoblastic leukemia for 40+ years – yet complete reaction mechanism remains unknown!!

# L-ASP structure and function

- Homotetramer – active site at interface between two subunits
- Two threonines identified as possible nucleophiles – T12 and T89
  - T89V and T12A single mutants both reduce $k_{cat}$ ~100,000 fold
  - T89V forms covalently bound acyl-enzyme dead-end state with substrate (crystal structure)
- K162 mutants also reduce $k_{cat}$
  - May have unusually low $pK_a$ – proton shuttle?
- Y25 suggested as possible proton shuttle to E283 during nucleophilic attack
  - E283 mutations show little change in $k_{cat}$

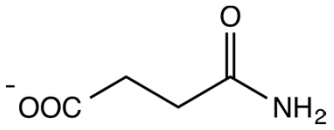



X-ray structure with Asp ligand



# Substrates and kinetics

- L-ASP can catalyze many substrates
  - Asn analogs such as succinamate and small peptides with carboxy-terminal Asn – absolute requirement of carboxyl 2-3 carbons from amide

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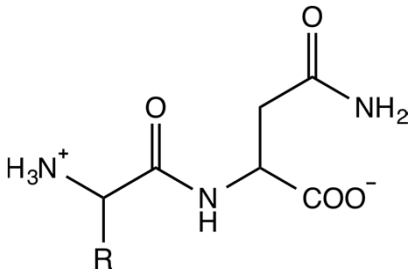


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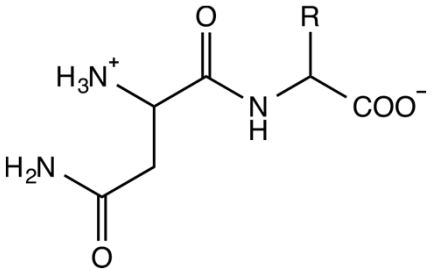


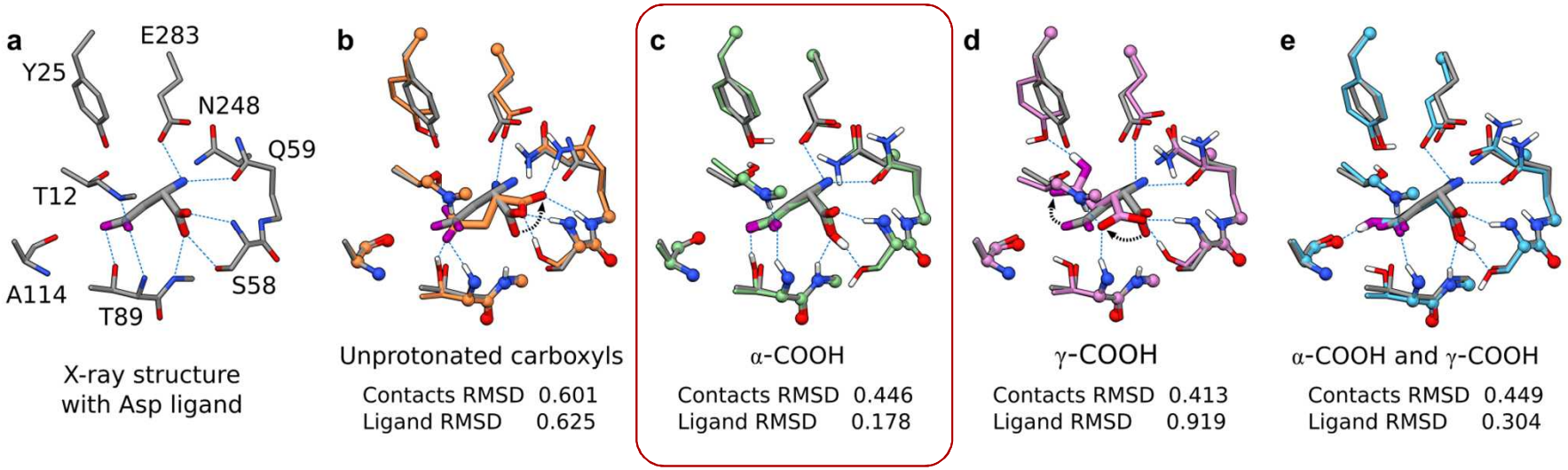

✖

- Kinetic studies support a double displacement mechanism – $NH_3$ is cleaved first followed by $H_2O$ nucleophilic attack

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow[\mathrm{NH_3}]{k_2} \mathrm{ES'} \xrightarrow[\mathrm{H_2O}]{k_3} \mathrm{EP} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{E} + \mathrm{P}$$

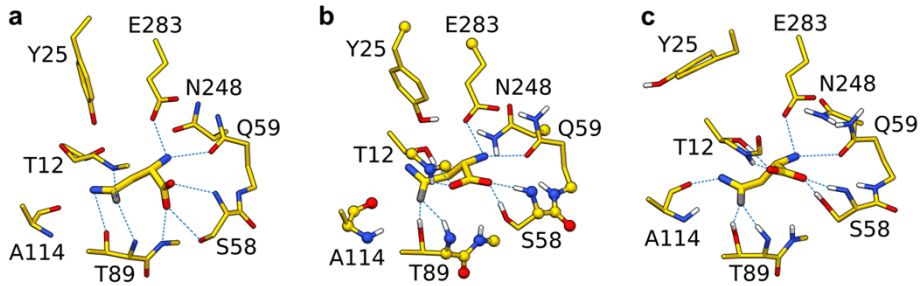
# L-ASP crystal structure

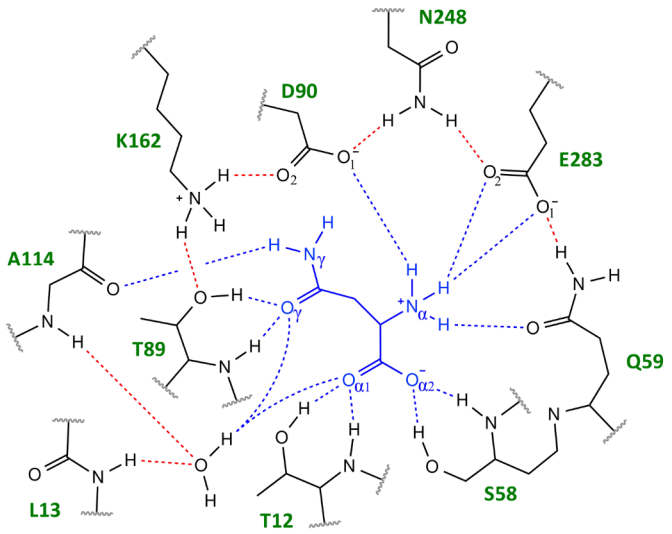
- Proposed L-ASP reaction mechanisms have been based on crystal structure with final **Asp product**
- Hydrogen bonding geometry suggests unusual protonation of α-carboxyl (H-bonded to S58)
- Classical MD simulations with Asp product in different protonation states (protein backbone restrained)



# L-ASP with natural substrates

- Asn substrate quickly rearranges in L-ASP active site
  - T12, S58 and T89 “clamp” onto oxygens of Asn



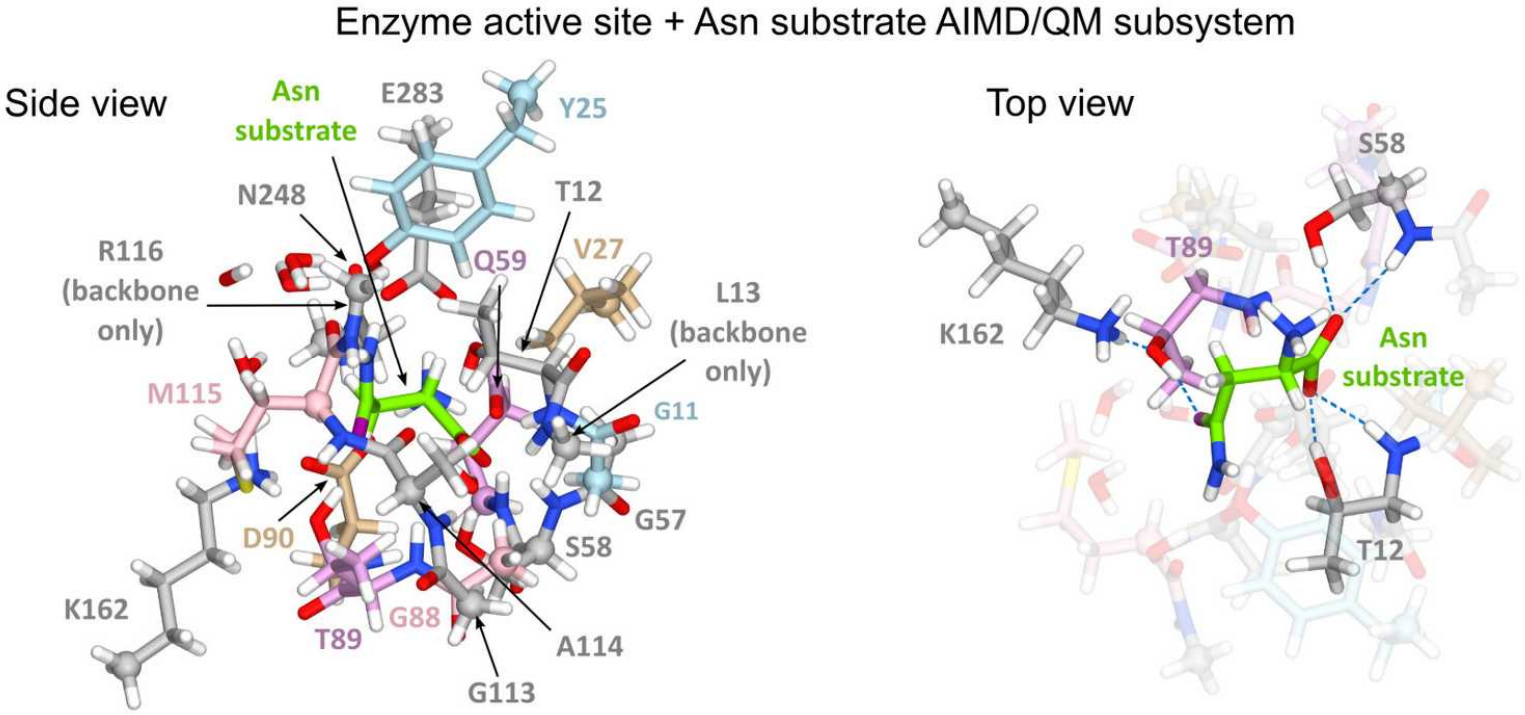




Probability of finding heavy atoms within 3 Å

| | ASN | | | | GLN | | | |
|---|---|---|---|---|---|---|---|---|
| | Lig. 1 | Lig. 2 | Lig. 3 | Lig. 4 | Lig. 1 | Lig. 2 | Lig. 3 | Lig. 4 |
| T12−OH----$O_{\alpha 1}$ | 0.53 | 0.91 | 0.96 | 0.68 | 0.00 | 0.98 | 0.96 | 0.00 |
| T12−NH----$O_{\alpha 1}$ | 0.92 | 0.87 | 0.89 | 0.63 | 0.00 | 0.07 | 0.58 | 0.00 |
| S58−OH----$O_{\alpha 2}$ | 0.99 | 0.99 | 0.99 | 0.66 | 0.96 | 0.52 | 0.99 | 0.49 |
| S58−NH----$O_{\alpha 2}$ | 0.86 | 0.85 | 0.91 | 0.02 | 0.96 | 0.00 | 0.75 | 0.59 |
| Q59−O----$HN_{\alpha}$ | 0.78 | 0.80 | 0.78 | 0.02 | 0.44 | 0.00 | 0.91 | 0.30 |
| Q59−NH----$O_{\alpha 2}$ | 0.00 | 0.00 | 0.00 | 0.00 | 0.26 | 0.00 | 0.00 | 0.29 |
| T89−OH----$O_{\gamma(\delta)}$ | 0.50 | 0.83 | 0.40 | 0.53 | 0.00 | 0.29 | 0.01 | 0.00 |
| T89−NH----$O_{\gamma(\delta)}$ | 0.89 | 0.86 | 0.82 | 0.50 | 0.00 | 0.69 | 0.56 | 0.00 |
| D90−O----$HN_{\alpha}$ | 0.99 | 0.99 | 0.99 | 0.98 | 0.99 | 0.91 | 0.99 | 0.97 |
| A114−O----$HN_{\gamma(\delta)}$ | 0.59 | 0.57 | 0.50 | 0.60 | 0.57 | 0.04 | 0.00 | 0.31 |
| E283−$O_1$----$HN_{\alpha}$ | 0.95 | 0.82 | 0.74 | 0.72 | 0.28 | 0.00 | 0.00 | 0.66 |
| E283−$O_2$----$HN_{\alpha}$ | 0.29 | 0.58 | 0.69 | 0.76 | 0.21 | 0.00 | 0.00 | 0.65 |
| HOH----$O_{\alpha 1}$ | 0.77 | 0.69 | 0.41 | 0.53 | 0.00 | 0.06 | 0.00 | 0.08 |
| HOH----$O_{\gamma(\delta)}$ | 0.12 | 0.09 | 0.42 | 0.33 | 0.96 | 0.00 | 0.00 | 0.67 |
| Q59−NH----$O_1$−E283 | 0.31 | 0.37 | 0.11 | 0.00 | 0.02 | 0.01 | 0.00 | 0.00 |
| K162−NH----OH−T89 | 0.91 | 0.92 | 0.89 | 0.87 | 0.19 | 0.00 | 0.00 | 0.21 |
| K162−NH----$O_2$−D90 | 1.00 | 1.00 | 1.00 | 0.96 | 1.00 | 0.07 | 0.99 | 1.00 |
| N248−NH----$O_1$−D90 | 0.34 | 0.24 | 0.32 | 0.04 | 0.05 | 0.18 | 0.00 | 0.00 |
| N248−NH----$O_1$−E283 | 0.19 | 0.08 | 0.25 | 0.32 | 0.08 | 0.02 | 0.01 | 0.24 |
| N248−NH----$O_2$−E283 | 0.37 | 0.51 | 0.27 | 0.24 | 0.14 | 0.04 | 0.02 | 0.20 |
| A114−NH----$OH_2$ | 0.97 | 0.99 | 0.99 | 0.98 | 1.00 | 0.93 | 0.99 | 0.81 |
| L13−NH----$OH_2$ | 0.99 | 0.98 | 0.97 | 0.84 | 1.00 | 1.00 | 1.00 | 0.41 |

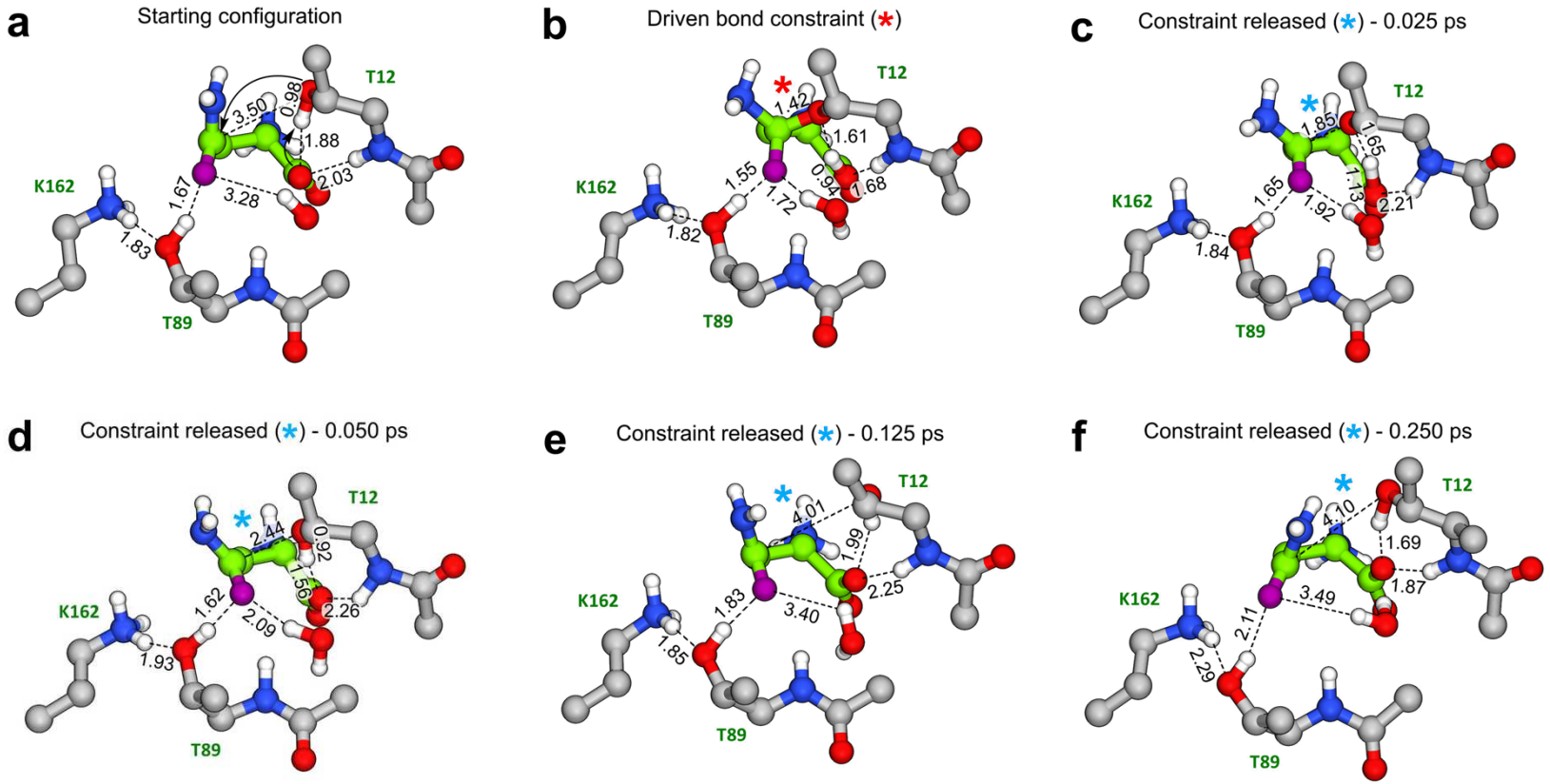
# Testing initial stages of reaction

- Test direct nucleophilic attack by T12 onto γ-carbon of Asn
  - Use *ab initio* MD to look at kinetics + single point energy calculations
  - QM system includes nearby protein residues + waters – 239 atoms
  - α-carbons fixed in order to maintain structure of active site



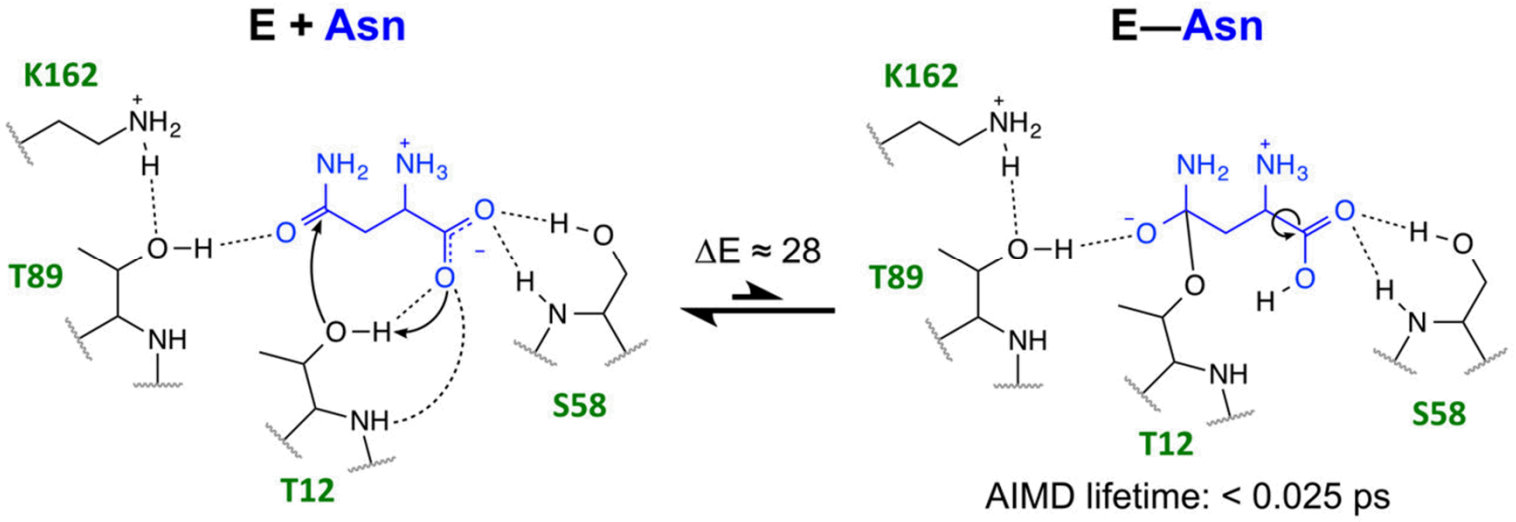
# Direct nucleophilic attack by T12

- Nucleophilic attack is driven by constraining distance between atoms and slowly reducing the distance
- PBE functional + vdW-DF2 dispersion correction (NVT, dt=0.5ps)



# Direct nucleophilic attack by T12

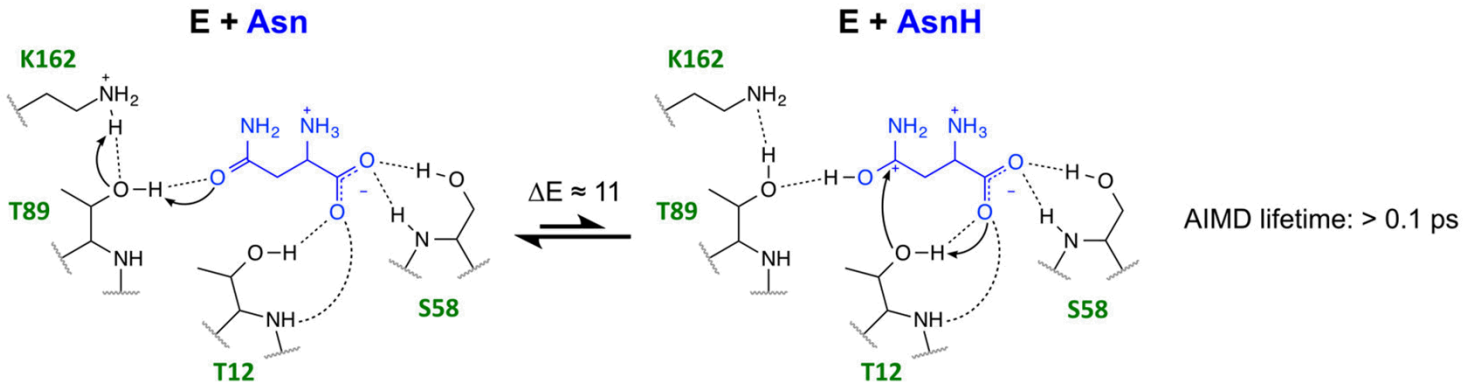
- T12 hydroxyl proton spontaneously transferred to α-carboxyl
- Enzyme-substrate covalent intermediate unstable
- What about single point energies?
  - Optimized end states with B3LYP functional and 6-31G(d) basis set
  - Single point calculations with M062X and ωB97X-D functionals and 6-311G++(2d,2P) basis set




*ΔE* (M062X) = 28 kcal/mol // *ΔE* (ωB97X-D) = 32 kcal/mol

# Pre-protonation by K162-T89

- Tetrahedral intermediate may be stabilized by first protonating amide oxygen through K162-T89 proton bridge

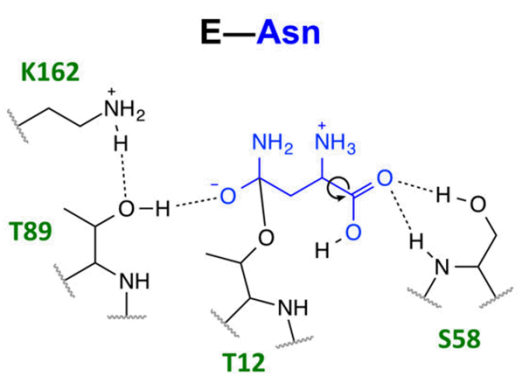



$\Delta E_1$ (M062X) = 11 kcal/mol
$\Delta E_1$ (ωB97X-D) = 12 kcal/mol

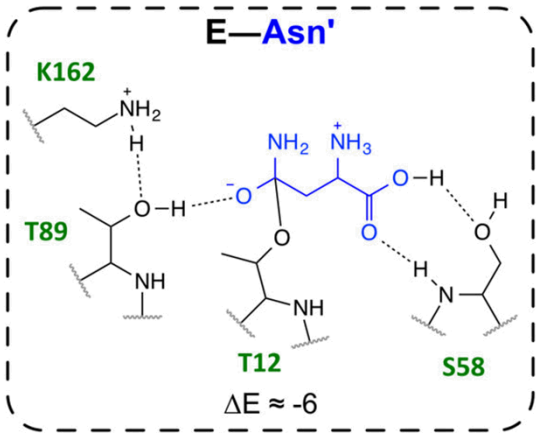
$\Delta E_2$ (M062X) = 16 kcal/mol
$\Delta E_2$ (ωB97X-D) = 22 kcal/mol

# α-COOH – S58 hydrogen bonding

- Protonated α-COOH may prefer a “paired” hydrogen bonding pattern with S58 instead of a “clamp”

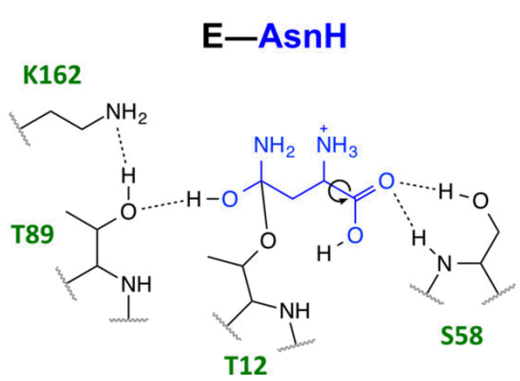



AIMD lifetime: < 0.025 ps

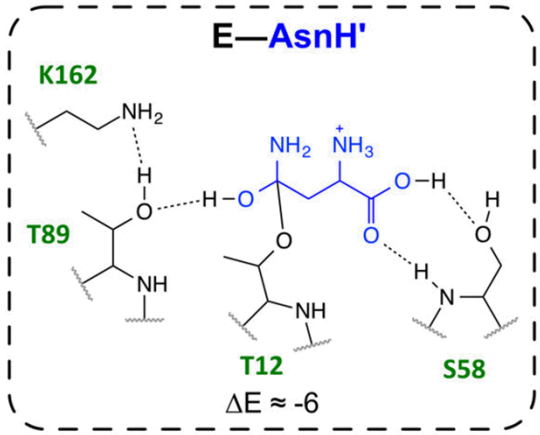



ΔE ≈ -6

*ΔE* (M062X) = -6 kcal/mol
*ΔE* (ωB97X-D) = -6 kcal/mol




AIMD lifetime: > 2.0 ps

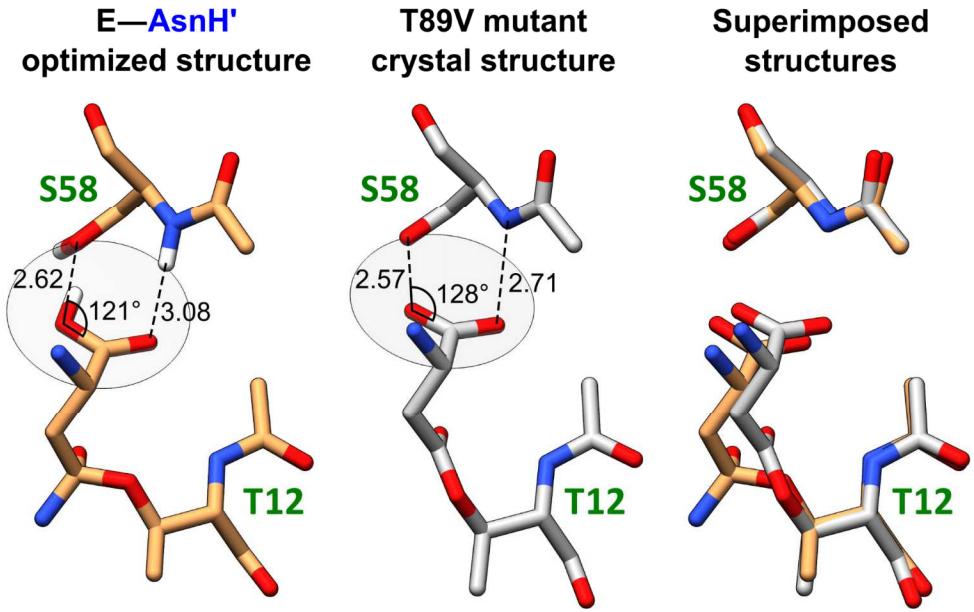



ΔE ≈ -6

*ΔE* (M062X) = -6 kcal/mol
*ΔE* (ωB97X-D) = -7 kcal/mol

# T89V mutant crystal structure

- E-AsnH’ configuration resembles the acyl-enzyme intermediate present in the T89V crystal structure (PDB 4ECA)




- WT + T89V crystal structures may provide details of intermediates, not initial substrate orientation

# Ongoing work

- Use QM/MM approach to allow better optimization of structures
- Determine energetic barriers using freezing string method – typical transition state search methods fail due to # of DOF
- Test nucleophilic attack by T89 and possible subsequent steps

- References:

  - Anishkin, A., **Vanegas, J. M.**, Rogers, D. M., Lorenzi, P. L., Chan, W. K., Purwaha, P., Weinstein, J. N., Sukharev, S., and Rempe, S. B. Catalytic Role of the Substrate Defines Specificity of Therapeutic L-Asparaginase. *J. Mol. Biol.* DOI: 10.1016/j.jmb.2015.06.017 (**2015**)
  - Chan, W. K. *et al.* The Glutaminase Activity Of L-Asparaginase Is Not Required For Anticancer Activity Against Asns-Negative Cell Lines. *Blood* 123**,** 3596–3606 (**2014**).

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- Our external collaborators:
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  - Defense Threat Reduction Agency