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**Supporting information for article:**

**Structural and biophysical aspects of L-asparaginases: a growing family with amazing diversity**

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## Supplementary Tables

**Table S1.** Examples of crystal structures of Class 1 (bacterial-type) L-asparaginases (and their mutants) and glutaminase-asparaginases available in the PDB.

|   | Abbreviation | gene                     | Uniprot        | PDB IDs (examples)                                   |
|---|--------------|--------------------------|----------------|--|
| <b>Class 1 type-1</b>                                 |              |                          |                |  |
| <i>Escherichia coli</i> [1, 2]                        | EcAI         | <i>ansA</i>              | P0A962         | 6NXC, 6NXD, 2P2D, 2P2N, 2HIM                         |
| <i>Pyrococcus furiosus</i> [3]                        | PfAI         | <i>PF2047</i>            | Q8TZE8         | 4RA6, 4RA9, 5CBP, 4NJE, 5B5U, 5B74, 4Q0M             |
| <i>Pyrococcus horikoshii</i> [4]                      | PhAI         | <i>PH0066</i>            | O57797         | 1WNF, 1WLS   |
| <i>Thermococcus kodakarensis</i> [5]                  | TkAI         | <i>TK1656</i>            | Q5JIW4         | 5OT0   |
| <i>Vibrio cholerae</i> [6]                            | VcAI         | <i>VC_1995</i>           | Q9KQK3         | 2OCD   |
| <i>Yersinia pestis</i> [6]                            | YpAI         | <i>ansA</i>              | A0A384KLA7     | 3NTX   |
| <i>Cavia porcellus</i> [7, 8]                         | CpAI         | <i>ASPG</i>              | H0W0T5         | 4R8K, 4R8L, 5DND, 5DNC, 5DNE                         |
| <b>Class 1 type-2</b>                                 |              |                          |                |  |
| <i>Campylobacter jejuni</i> [6]                       | CjAII        | <i>ansA</i> <sup>1</sup> | Q0PC96         | 3NXK   |
| <i>Helicobacter pylori</i> [9]                        | HpAII        | <i>ansA</i> <sup>1</sup> | Q9ZLB9         | 2WT4, 2WLT   |
| <i>Wolinella succinogenes</i> [10, 11] <sup>2</sup>   | WsAII        | <i>ansB</i> <sup>3</sup> | P50286         | 1WSA, 5K45, 5K4G, 5K4H, 6RUE, 6SYH, 6RUF, 5K3O       |
| <i>Erwinia chrysanthemi</i> [12–17]                   | ErAII        | <i>ansB</i>              | P06608         | 5F52, 5I48, 5I3Z, 5I4B, 5HW0, 6PAE, 1JSL, 1JSR, 1HFK |
| <i>Erwinia carotovora</i> <sup>4</sup> [18, 19]       | EwAII        | N/A, <i>lanS</i>         | Q6Q4F4, Q7WWK9 | 2HLN, 2JK0, 1ZCF                                     |
| <i>Escherichia coli</i> <sup>5</sup> [2, 20–25]       | EcAII        | <i>ansB</i>              | P00805         | 3ECA, 1JAZ, 1JJA, 6NX9, 6NX8, 6NX7, 6NX6, 6NXA       |
| <b>Class 1 glutaminase-asparaginases (G-A)</b>        |              |                          |                |  |
| <i>Pseudomonas sp. 7A/putida</i> <sup>6</sup> [26–29] | PGA          | <i>ansB</i>              | P10182/Q88K39  | 4PGA, 3PGA, 1DJP, 1DJO, 6WZ4, 6WZ6, 6WYW, 6WZ8,      |
| <i>Acinetobacter glutaminasificans</i> [30]           | AGA          | <i>ansB</i>              | P10172         | 1AGX   |

<sup>1</sup> Described as product of *ansA* gene; however, the architecture of the active site and/or  $\mu\text{M}$  substrate (L-Asn) affinity (Table S2) suggest classification as a Class 1 type-2 enzyme.

<sup>2</sup> Two variants differing at position 121 (Ser/Pro) are reported.

<sup>3</sup> Ambiguous classification in Uniprot: “gene *ansA* synonym *ansB*”.

<sup>4</sup> In the past classified as: *Pectobacterium atrosepticum*, *Pectobacterium atrosepticum SCRI1043*, *Pectobacterium carotovorum*.

<sup>5</sup> Includes *Escherichia coli* and *Escherichia coli K-12*.

<sup>6</sup> Strains *Pseudomonas sp.* (strain ATCC 29598 / 7A) or *Pseudomonas putida* (strain ATCC 47054 / DSM 6125 / NCIMB 11950 / KT2440).

**Table S2.** Unified nomenclature (residue numbers and **generic labels**) of residues involved in catalysis and important for substrate binding in different Class 1 L-asparaginases. Primed residues are contributed from the complementary subunit of the intimate dimer. A residue label in (front) superscript denotes difference from the consensus residue at this position specified in the column header.

| Unified nomenclature <sup>1</sup>              | ( <sup>1</sup> )Thr | ( <sup>2</sup> )Tyr | ( <sup>3</sup> )Thr | ( <sup>4</sup> )Asp | ( <sup>5</sup> )Lys | ( <sup>6</sup> )Ser | ( <sup>7</sup> )Gln | ( <sup>8</sup> )Ala | ( <sup>9</sup> )Asn' | ( <sup>10</sup> )Glu' |
|--|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|----------------------|-----------------------|
| <b>Class 1 type-1</b>                          |                     |                     |                     |                     |                     |                     |                     |                     |                      |                       |
| <i>Escherichia coli</i>                        | 14                  | 283'                | 91                  | 92                  | 163                 | 60                  | Ser <sup>6</sup> 1  | Ser <sup>11</sup> 7 | 246                  | -                     |
| <i>Yersinia pestis</i>                         | 14                  | 273'                | 91                  | 92                  | 163                 | 60                  | Ser <sup>6</sup> 1  | Ser <sup>11</sup> 7 | 246                  | -                     |
| <i>Vibrio cholerae</i>                         | 14                  | 284'                | 91                  | 92                  | 163                 | 60                  | Ser <sup>5</sup> 1  | Ser <sup>11</sup> 7 | 246                  | -                     |
| <i>Pyrococcus furiosus</i>                     | 11                  | 273'                | 83                  | 84                  | 154                 | 52                  | Thr <sup>5</sup> 3  | Ser <sup>10</sup> 9 | Gly <sup>2</sup> 36  | -                     |
| <i>Pyrococcus horikoshii</i>                   | 11                  | 273'                | 83                  | 84                  | 154                 | 52                  | Thr <sup>5</sup> 3  | Ser <sup>10</sup> 9 | Gly <sup>2</sup> 36  | -                     |
| <i>Thermococcus kodakarensis</i>               | 11                  | 274'                | 83                  | 85                  | 156                 | 54                  | Thr <sup>5</sup> 5  | Ser <sup>11</sup> 1 | Gly <sup>2</sup> 36  | -                     |
| <i>Cavia porcellus</i>                         | 19                  | 308'                | 116                 | 117                 | 188                 | 85                  | Ser <sup>8</sup> 6  | 142                 | 272                  | -                     |
| <b>Class 1 type-2</b>                          |                     |                     |                     |                     |                     |                     |                     |                     |                      |                       |
| <i>Escherichia coli</i>                        | 12                  | 25                  | 89                  | 90                  | 162                 | 58                  | 59                  | 114                 | 248                  | 283                   |
| <i>Wolinella succinogenes</i>                  | 14                  | 27                  | 39                  | 94                  | 166                 | 60                  | 61                  | 118                 | 252                  | 287                   |
| <i>Helicobacter pylori</i>                     | 16                  | 29                  | 95                  | 96                  | 168                 | 62                  | 63                  | 114                 | 255                  | 289                   |
| <i>Erwinia chrysanthemi</i>                    | 15                  | 29                  | 95                  | 96                  | 168                 | 62                  | 63                  | 120                 | Ser <sup>2</sup> 54  | Asp <sup>2</sup> 87   |
| <i>Erwinia carotovora</i>                      | 15                  | 29                  | 95                  | 96                  | 168                 | 62                  | 63                  | 120                 | Ser <sup>2</sup> 54  | Asp <sup>2</sup> 87   |
| <i>Campylobacter jejuni</i>                    | 16                  | 30                  | 95                  | 96                  | 168                 | 62                  | Ser <sup>6</sup> 4  | 120                 | Ser <sup>2</sup> 55  | Asp <sup>2</sup> 89   |
| <b>Class 1 glutaminase-asparaginases (G-A)</b> |                     |                     |                     |                     |                     |                     |                     |                     |                      |                       |
| <i>Pseudomonas sp. 7A/putida</i>               | 20                  | 34                  | 100                 | 101                 | 173                 | 97                  | Glu <sup>6</sup> 8  | Ser <sup>1</sup> 25 | Ser <sup>2</sup> 58  | 294                   |
| <i>Acinetobacter glutaminasificans</i>         | 12                  | 26                  | 92                  | 93                  | 165                 | 59                  | Glu <sup>6</sup> 0  | Ser <sup>1</sup> 17 | Ser <sup>2</sup> 52  | 288                   |

<sup>1</sup> Residues marked with ' originate from a different subunit. (<sup>2</sup>)Tyr originates from the same subunit as the catalytic triad (<sup>1</sup>)Thr-(<sup>2</sup>)Tyr-(<sup>3</sup>)Thr in type-2 enzymes and in G-A, while it is donated by a different subunit in type-1 enzymes. Residues determining substrate preferences (L-Asn/L-Glu): (<sup>9</sup>)Asn' and (<sup>10</sup>)Glu', always come from the complementary subunit.

**Table S3.** Crystal structures of Class 2 (plant-type) asparaginases

| <b>Class 2 (or type-3)</b>      |                     |                   |                |   |
|---------------------------------|---------------------|-------------------|----------------|---|
|                                 | <b>Abbreviation</b> | <b>gene</b>       | <b>Uniprot</b> | <b>PDB IDs (examples)</b>                 |
| <i>Escherichia coli</i> [31–34] | EcAIII              | <i>iaaA, ybiK</i> | P37595         | 2ZAK, 2ZAL, 3C17, 1T3M, 1JN9, 1K2X        |
| <i>Lupinus luteus</i> [35]      | LIAIII              | <i>lla</i>        | Q9ZSD6         | 2GEZ                                      |
| <i>Phaseolus vulgaris</i> [36]  | PvAIII(K)           | <i>PvASPG1</i>    | V7CU13         | 4PV3                                      |
| <i>Cavia porcellus</i> [37]     | CpAIII              | <i>ASRGL1</i>     | A0A286XYM2     | 4O48, 4O47                                |
| <i>Homo sapiens</i> [38–42]     | HsAIII              | <i>ASRGL1</i>     | Q7L266         | 4PVR, 4PVS, 4PVP, 4PVQ, 4OSX, 4OSY, 4ZM9, |

**Table S4.** Numbering of homologous residues and conserved structural elements in crystal structures of Class 2 asparaginases. A residue label in (front) superscript denotes difference from the consensus residue at this position specified in the column header.

| <b>Unified nomenclature</b> | <sup>(100)</sup> Gly | <sup>(101)</sup> Thr | <sup>(102)</sup> Thr | <sup>(103)</sup> Thr | <sup>(104)</sup> Arg | <sup>(105)</sup> Asp | <sup>(106)</sup> Gly | <sup>(107)</sup> Gly | <sup>(108)</sup> Glu | <sup>(K)</sup> His | <sup>(K)</sup> Arg | <sup>(K)</sup> Glu | <sup>(K)</sup> Ser* | <sup>(Na)</sup> Asn |
|-----------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|--------------------|--------------------|--------------------|---------------------|---------------------|
| <i>Escherichia coli</i>     | 178                  | 179                  | 197                  | 230                  | 207                  | 210                  | 231                  | 233                  | 234                  | 119                | 207                | 234                | <sup>Val</sup> 120  | 67                  |
| <i>Homo sapiens</i>         | 167                  | 168                  | 186                  | 219                  | 196                  | 199                  | 220                  | 222                  | 223                  | 114                | 196                | 223                | <sup>Cys</sup> 115  | 62                  |
| <i>Lupinus luteus</i>       | 192                  | 193                  | 211                  | 243                  | 221                  | 224                  | 244                  | 246                  | 247                  | 118                | 221                | 247                | <sup>Ile</sup> 118  | 66                  |
| <i>Phaseolus vulgaris</i>   | 195                  | 196                  | 214                  | 246                  | 224                  | 227                  | 247                  | 249                  | 250                  | 117                | 224                | 250                | 118                 | 65                  |
| <i>Cavia porcellus</i>      | 167                  | 168                  | 186                  | 219                  | 196                  | 199                  | 220                  | 222                  | 223                  | 114                | 196                | 223                | <sup>Cys</sup> 115  | 62                  |

\*Ser is present at this position in K-dependent enzymes, while in other enzymes Val, Cys or Ile was found in the corresponding position.

**Table S5.** *Rhizobium etli* (Class 3) asparaginases

| <b>Class 3 (<i>R. etli</i>-type)</b> |                    |                    |                |                           |
|--------------------------------------|--------------------|--------------------|----------------|---------------------------|
|                                      | <b>Abbrviation</b> | <b>gene</b>        | <b>Uniprot</b> | <b>PDB IDs (examples)</b> |
| <i>Rhizobium etli</i>                | ReAIV              | <i>RHE_CH01144</i> | Q2KB35         | -                         |
| <i>Rhizobium etli</i>                | ReAV               | <i>ansA</i>        | Q2K0Z2         | -                         |

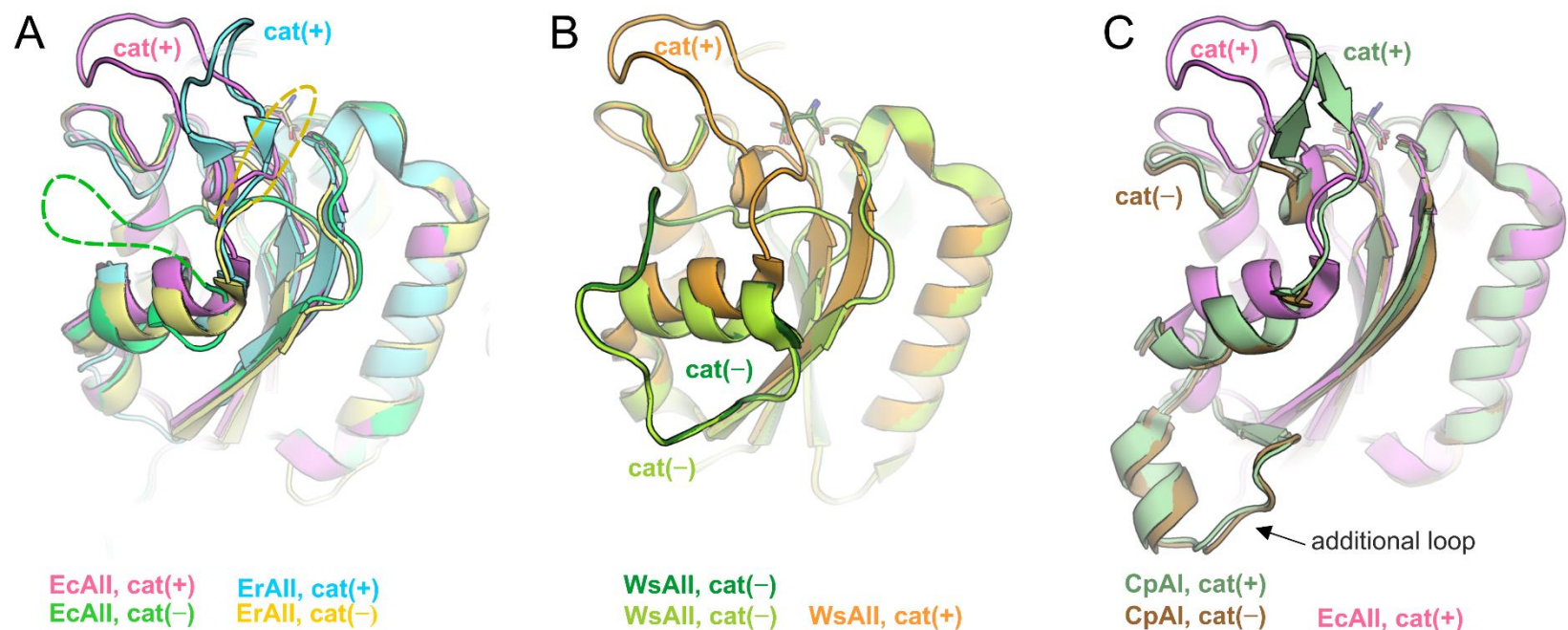
**Table S6.** Kinetic parameters of selected asparaginases reviewed in this paper. The kinetic parameters are specified for L-Asn substrate.

|  | $K_M$ [M]             | $k_{cat}$ [ $s^{-1}$ ] | $k_{cat}/K_M$ [ $M^{-1}s^{-1}$ ] | tested pH, (maximum) | tested temp., $T_m$ , (optimal) |
|--|-----------------------|------------------------|----------------------------------|----------------------|---------------------------------|
| <b>Class 1 type-1</b>                                      |                       |                        |                                  |                      |                                 |
| <i>Escherichia coli</i>                                    | $1.2 \times 10^{-3}$  | no data                | no data                          | no data              | no data                         |
| <i>Pyrococcus furiosus</i> [43]                            | $12 \times 10^{-3}$   | 870                    | 72 500                           | 4.0-10.5, (9.0)      | 37-95°C, 85°C, (no data)        |
| <i>Thermococcus kodakarensis</i> [44]                      | $5.5 \times 10^{-3}$  | 1397                   | 254 000                          | 6.0-11.0, (9.5)      | 30-95°C, no data, (85°C)        |
| <i>Cavia porcellus</i> [7]                                 | $57.7 \times 10^{-6}$ | 38.6                   | 800 000                          | no data              | no data                         |
| <b>Class 1 type-2</b>                                      |                       |                        |                                  |                      |                                 |
| <i>Helicobacter pylori</i> [9]                             | $19 \times 10^{-6}$   | no data                | no data                          | no data              | no data                         |
| <i>Wolinella succinogenes</i> (S121)[10]                   | $21.7 \times 10^{-6}$ | 97.8                   | 4 500 000                        | no data              | no data                         |
| <i>Wolinella succinogenes</i> (P121)[10]                   | $23.6 \times 10^{-6}$ | 86.7                   | 3 700 000                        | no data              | no data                         |
| <i>Erwinia chrysanthemi</i> [45]                           | $58 \times 10^{-6}$   | 23 800                 | 411 800                          | no data              | 30-70°C, 46.4°C, (no data)      |
| <i>Erwinia carotovora</i> [46]                             | $85 \times 10^{-6}$   | 31 400                 | 369 000 000                      | 4-10, (6.5-9.0)      | no data                         |
| <i>Escherichia coli</i> [47–49]                            | $35 \times 10^{-6}$   | 29                     | 830 000                          | 3-11, (6.0-8.0)      | 40-80°C, 49°C, (no data)        |
| <b>Class 1 glutaminase-asparagiases</b>                    |                       |                        |                                  |                      |                                 |
| <i>Pseudomonas sp. 7A / putida</i> [50]                    | $4.4 \times 10^{-6}$  | no data                | no data                          | 4-10, (not detected) | no data                         |
| <i>Acinetobacter glutaminasificans</i> [51]                | $40 \times 10^{-6}$   | no data                | no data                          | no data              | no data                         |
| <b>Class 2 (or type-3)</b>                                 |                       |                        |                                  |                      |                                 |
| <i>Escherichia coli</i> [52]                               | $3.90 \times 10^{-3}$ | 0.28                   | 71.8                             | no data              | no data, 59.2°C, (no data)      |
| <i>Homo sapiens</i> [42, 53]                               | $2.09 \times 10^{-3}$ | 3.19                   | 1 520                            | 3-10, (5.5-6.0)      | no data, 70°C, (no data)        |
| <i>Lupinus luteus</i> [52]                                 | $4.90 \times 10^{-3}$ | 0.32                   | 66.7                             | no data              | no data                         |
| <i>Phaseolus vulgaris</i> [54] <sup>1</sup>                | $3.43 \times 10^{-3}$ | 37.4                   | 10 903                           | 5.5-10.0 (7.5)       | 5-95°C, 48.5/48.0°C, (no data)  |
| <i>Cavia porcellus</i> [37]                                | $2.24 \times 10^{-3}$ | 3.95                   | 1 760                            | no data              | no data                         |
| <b>Class 3 (<i>R.etli</i>-type)</b>                        |                       |                        |                                  |                      |                                 |
| <i>Rhizobium etli</i> (inducible enzyme) [55] <sup>2</sup> | $8.9 \times 10^{-3}$  | 106                    | 12 000                           | 7.5-11.0             | 50-60°C, no data, (37°C)        |

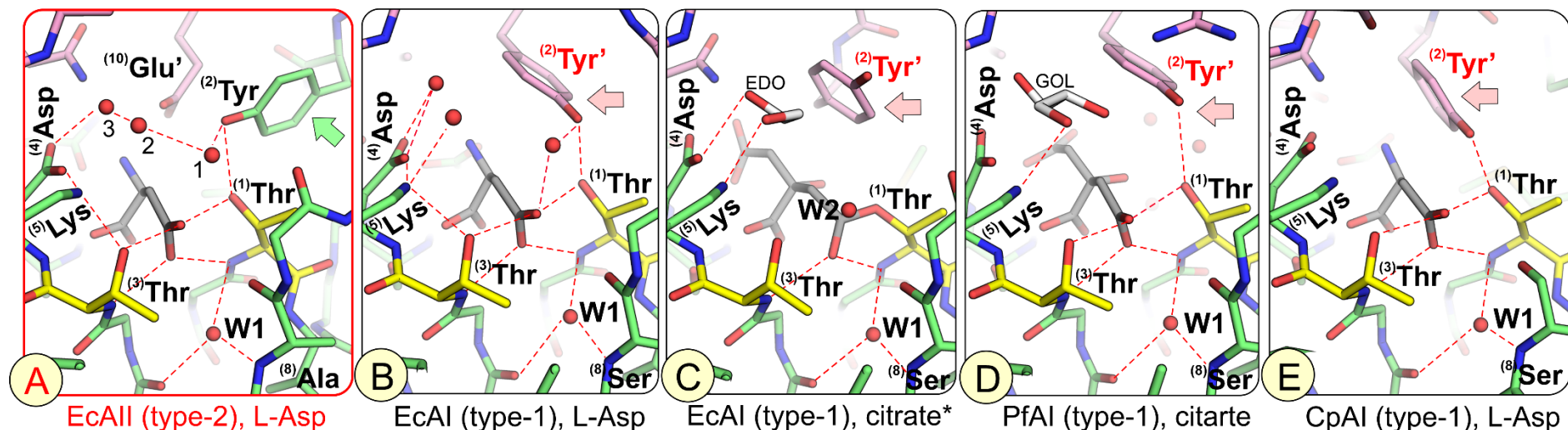
<sup>1</sup> Determined in the presence of potassium cations.

<sup>2</sup> Recently determined kinetic parameters indicate that the  $K_M$  is lower, in the range of  $\sim 4 \times 10^{-4}$  [M] (Loch & Sliwiak, unpublished).

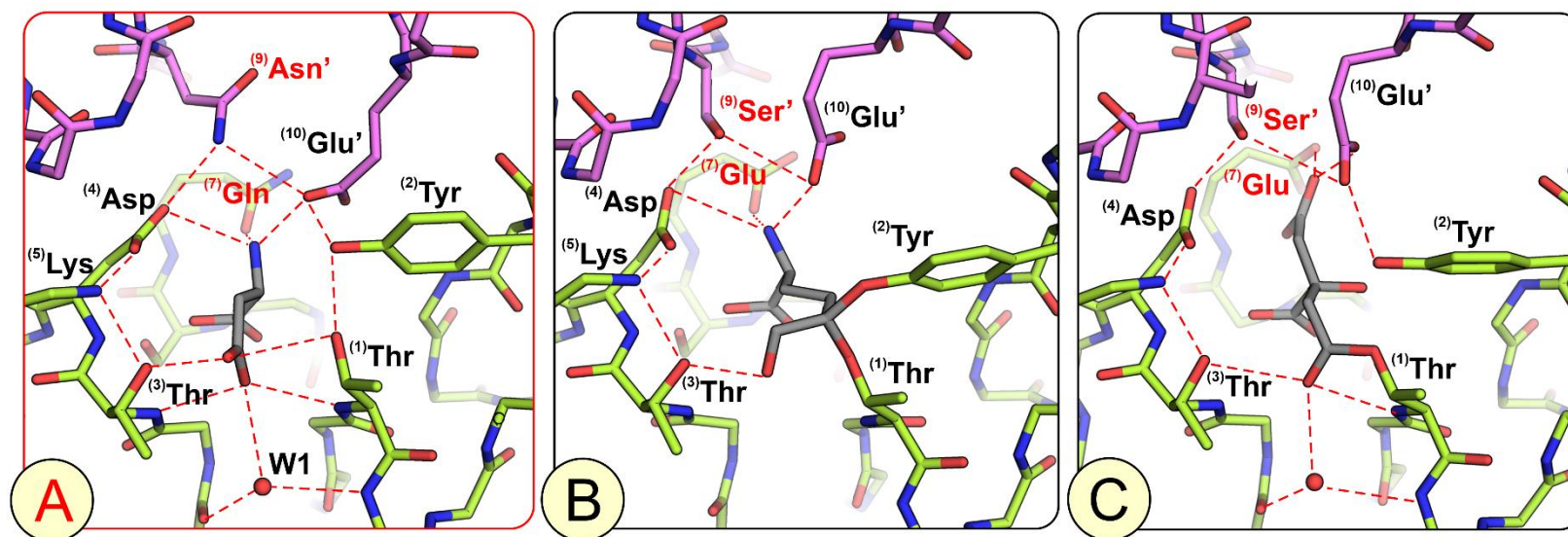
## Supplementary Figures



**Fig. S1.** Comparison of conformational states of FGE in different Class 1 asparaginases. (A) EcAII with the state of FGE in *cat*(-) (green, PDB ID 6v23) and (in the presence of L-Asp) *cat*(+) (pink, PDB ID 6pab), ErAII (in the presence of L-Gln) in *cat*(-) (yellow, PDB ID 1hfw), PhAI (in the absence of ligands) in *cat*(+) (cyan, PDB ID 1wsl). (B) WsAII (in the presence of L-Asp) in *cat*(+) (orange, PDB ID 5ko3), WsAII (in the presence of L-Asp) in *cat*(-) (light green, PDB ID 5k4g) and (in the presence of L-Gln) in *cat*(+) (dark green, PDB ID 5k45). (C) CpAI (in the presence of L-Asp) in *cat*(+) (pale green, PDB ID 4r8l) and (in the absence of ligands) in *cat*(-) (brown, PDB ID 4r8k). The CpAI model was superposed on EcAII in the *cat*(+) state (pink, PDB ID 6pab). The additional loop in the N-terminal domain of the mammalian enzymes is marked by arrow. Green and yellow dashed lines in panel A mark a missing (disordered) fragment of the FGE in EcAII and ErAII in the *cat*(-) state.

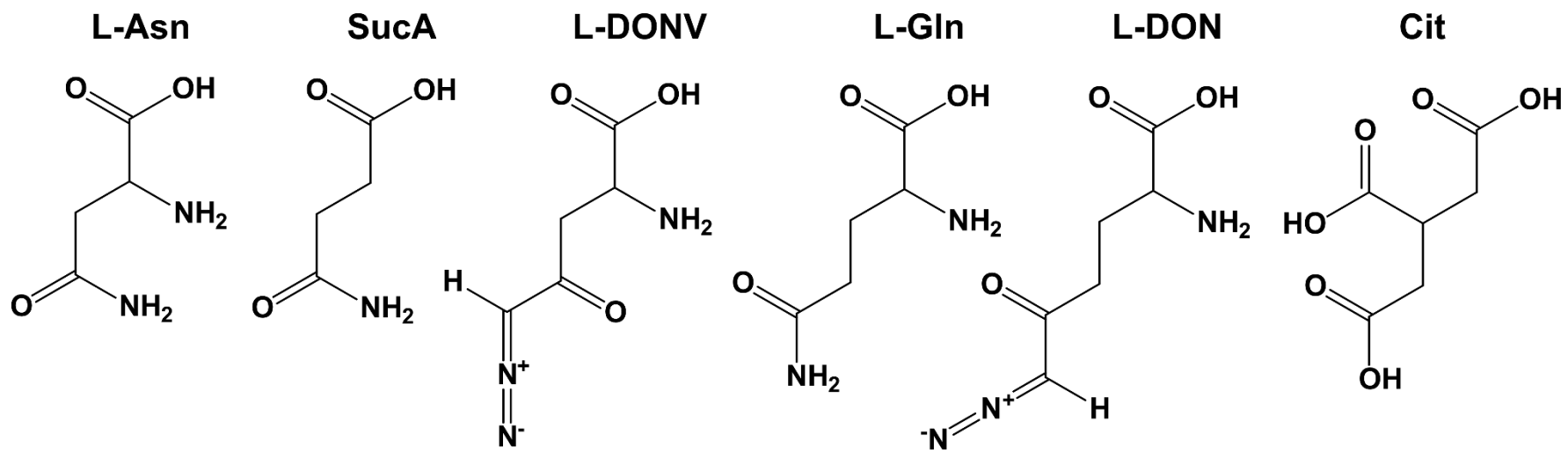


**Fig. S2.** Comparative analysis of the of active site structure in Class 1 L-asparaginases. (A) L-Asp bound in the active site of the model bacterial type-2 L-asparaginase EcAII (PDB ID 3eca). (B) L-Asp bound in the active site of EcAI (PDB ID 4nje).  $(2)\text{Tyr}'$ , which activates  $(1)\text{Thr}$ , is contributed by the complementary protomer (marked by '). (C) A similar situation is observed in EcAI trapped in the acyl-enzyme intermediate state in the presence of citrate ions; however, in this case  $(2)\text{Tyr}'$  is rotated away from  $(1)\text{Thr}$  (PDB ID 6nxc). (D) Citrate ion in the active site of the type-1 enzyme from *P. furiosus*;  $(2)\text{Tyr}'$  from the other protomer activates  $(1)\text{Thr}$ , just as in (B). (E) L-Asp bound in the active site of the mammalian (*C. porcellus*) type-1 enzyme (PDB ID 4r8l). W1 and W2 are conserved in all bacterial enzymes, while waters 1, 2 and 3 are typical for bacterial type-2 enzymes. Fat green arrow marks the position of  $(2)\text{Tyr}$  in bacterial type-2 enzymes, while fat pink arrows mark the position of  $(2)\text{Tyr}'$  in type-1 enzymes. In (C) and (D), in close vicinity of  $(2)\text{Tyr}'$  small ligands such as ethylene glycol (EDO) or glycerol (GOL) were identified.

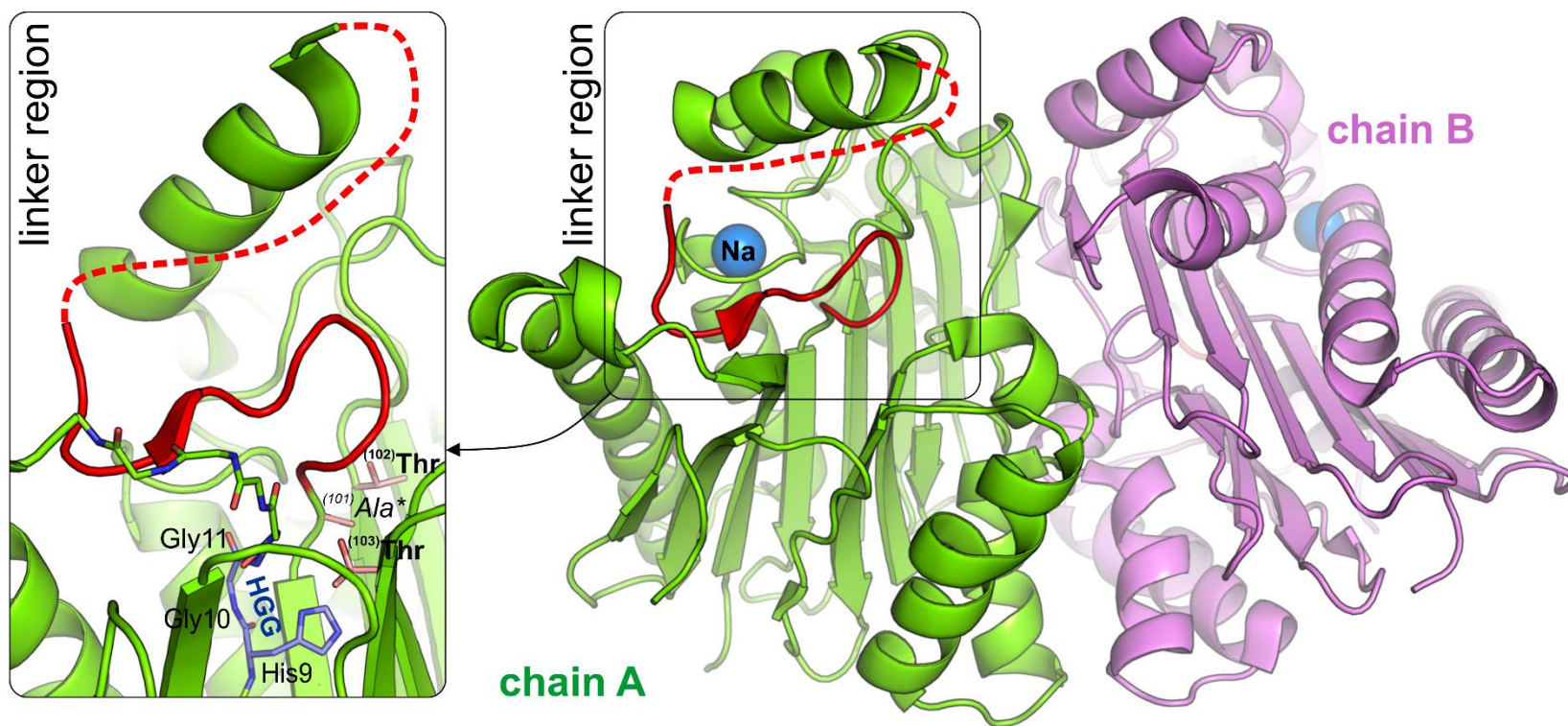


**Fig. S3.** Comparison of the active site structure in EcAII and in selected glutaminase-asparaginases (G-A) with FGE in cat(+) conformation. Chains colored green and violet belong to different subunits. To date, there is no crystal structure of a complex between G-A and L-Gln/L-Glu bound in the active site, with FGE in cat(+) conformation. (A) EcAII (PDB ID 3eca) with L-Asp in the active site. Residues determining L-Asn affinity, <sup>(7)</sup>Gln and <sup>(9)</sup>Asn', are marked in red. (B) G-A from *Pseudomonas sp.* 7A (PDB ID 1djp) with the inhibitor L-DON covalently bound to <sup>(1)</sup>Thr and <sup>(2)</sup>Tyr. (C) G-A from *Pseudomonas putida* (PDB ID 6wyw) with citrate covalently bound to <sup>(1)</sup>Thr. In G-A structures, similarly to bacterial type-2 asparaginases, <sup>(2)</sup>Tyr, which covers the active site, is located in the same subunit as the catalytic threonines.

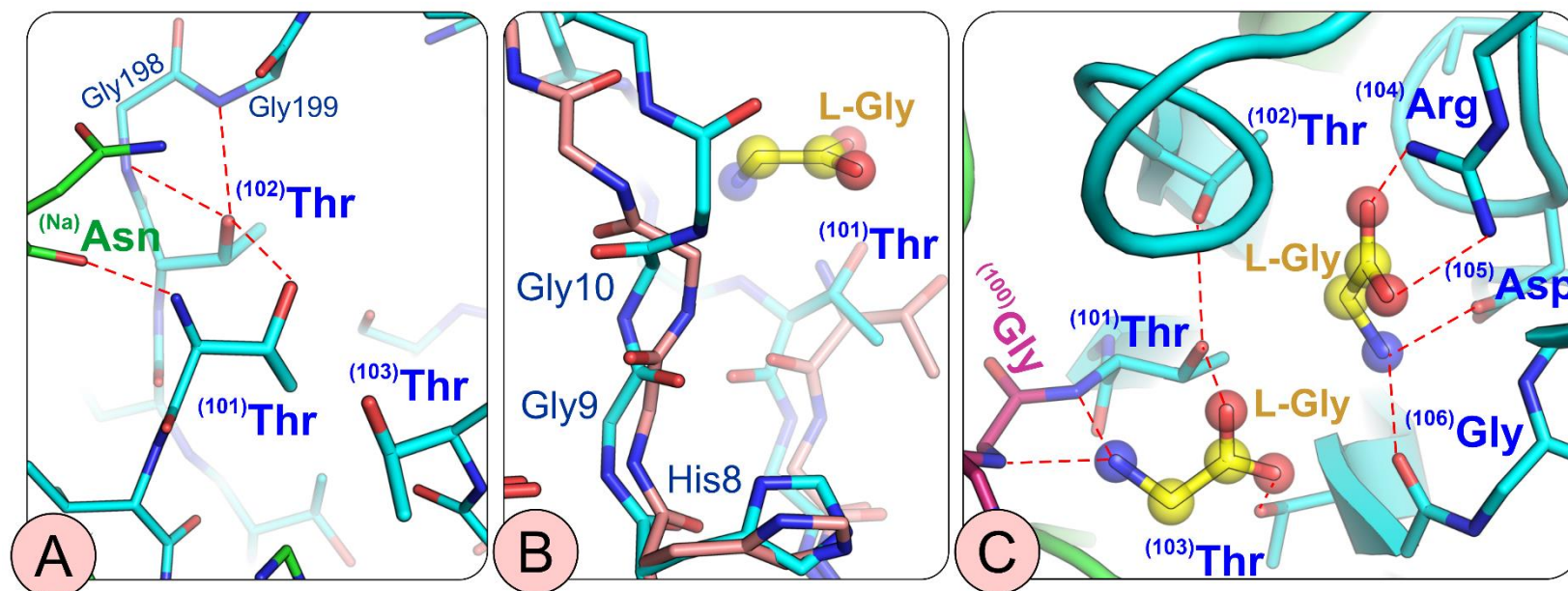




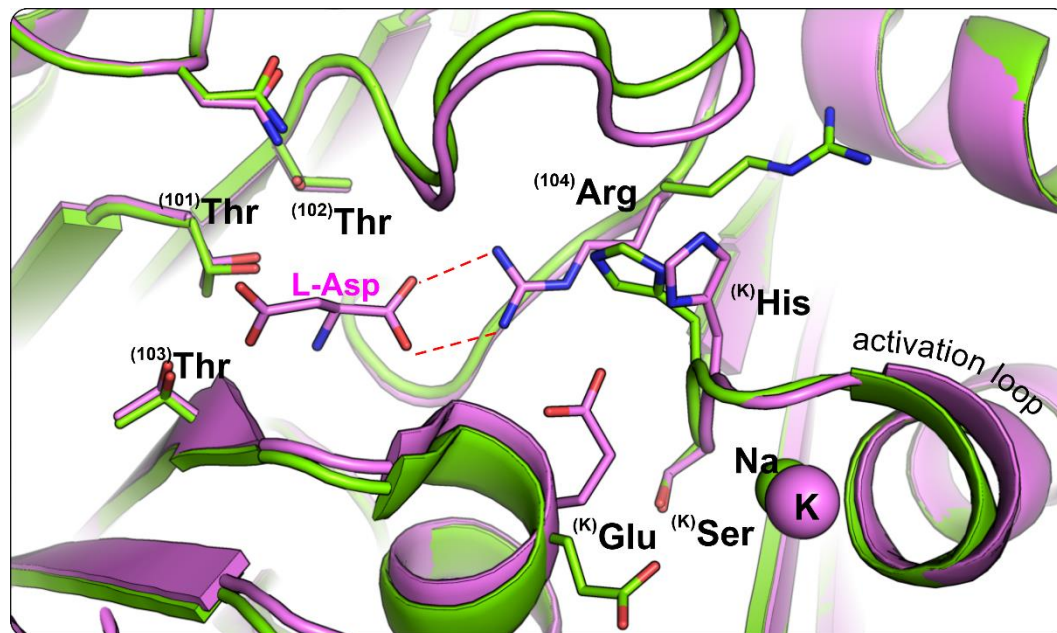
**Fig. S4.** Structures of L-asparaginase substrates and inhibitors: L-Asn (L-asparagine), SucA (succinic monoamide), L-DONV (5-diazo-4-oxo-L-norvaline), L-Gln (L-glutamine), L-DON (6-diazo-5-oxo-L-norleucine), Cit (citric acid).



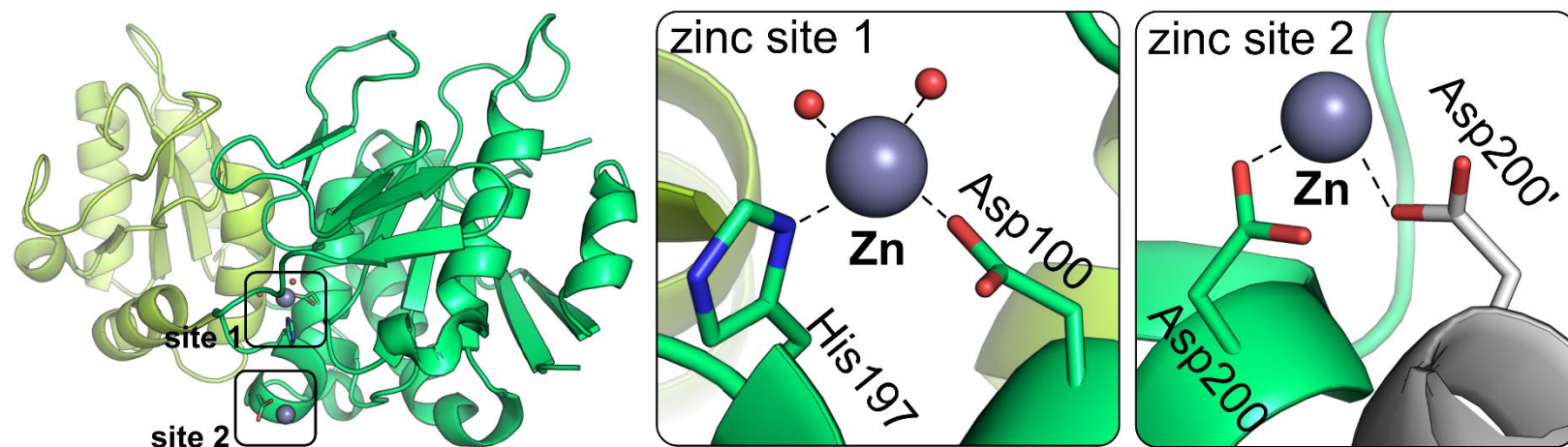
**Fig. S5.** Crystal structure of uncleaved precursor of a typical Class 2 L-asparaginase, EcAIII (PDB ID 3c17), with T179A mutation, comprised of chain A (green) and chain B (violet). The linker region (red) is only partially visible in the crystal structure (the missing part is marked with red dashed line). The inset represents a zoom in view of the linker region and the active site with the catalytic triad  $(^{101})\text{Thr}$ - $(^{102})\text{Ala}^*$ - $(^{103})\text{Thr}$  ( $(^{102})\text{Thr}$  replaced with  $(^{102})\text{Ala}^*$ ) and the HGG loop (blue).



**Fig. S6.** Detailed view on the active site of Class 2 L-asparaginases. (A) H-bonds between  $^{(101)}\text{Thr}$ - $^{(102)}\text{Thr}$  and carbonyl oxygen of  $^{(\text{Na})}\text{Asn}$  in EcAIII (PDB ID 2zal). (B) Differences in conformation of the HGG loop (His8, Gly9, Gly10) in the cleaved (PDB ID 4o0g, salmon) and uncleaved (PDB ID 4osx, cyan) HsAIII. (C) Glycine molecules (yellow) bound in uncleaved HsAIII (PDB ID 4osx); a fragment of the linker region is colored dark pink.



**Fig. S7.** Potassium-dependent asparaginase from *P. vulgaris* in ON (PDB ID 4pu6, violet) and OFF (PDB ID 4pv3, green) states. In the absence of K<sup>+</sup> ions, the activation loop coordinates Na<sup>+</sup> ions.



**Fig. S8.** Zinc binding sites in the EcAII structure PDB ID 1jaz. The N-terminal domain is colored dark green, while the C-terminal domain is colored light green. The zinc binding site 2 is composed of Asp200 and Asp200' from a symmetry-related molecule (gray).



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