Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.


**Electronic Supplementary Information**

# The role of conserved Cys residues in Brassica rapa auxin amidohydrolase: the Cys139 is crucial for the enzyme activity and the Cys320 regulates enzyme stability

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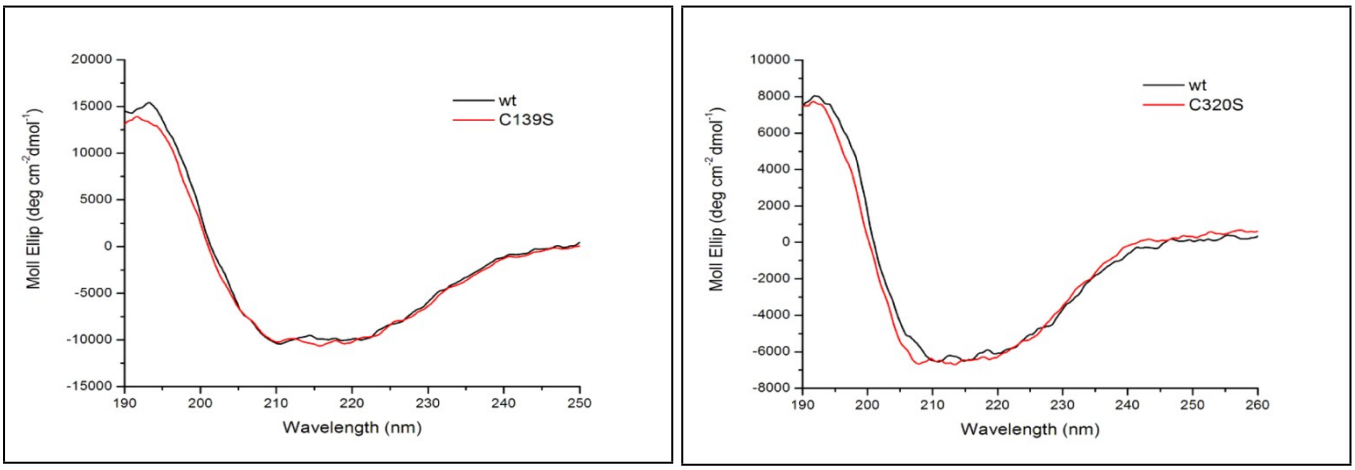


**Figure S1**. Circular dichroism spectropolarimetry (CD) revealed preservation of the secondary structure of the mutant proteins BrILL2C139S and BrILL2C320S compared to the BrILL2 wild type enzyme.

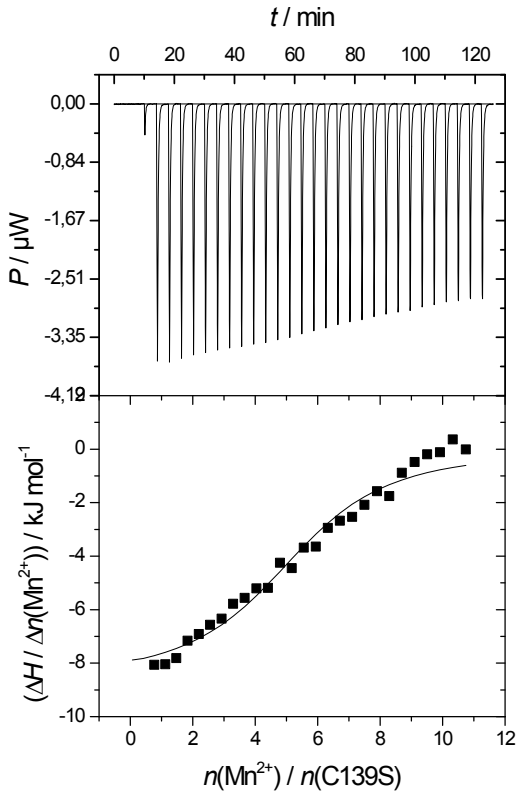


**Figure S2.** Representative figure for ITC titration of the mutant BrILL2C139S ($c = 5 \cdot 10^{-6}$ mol dm$^{-3}$) with $MnCl_2 \cdot 4H_2O$ ($c = 2.5 \cdot 10^{-4}$ mol dm$^{-3}$).

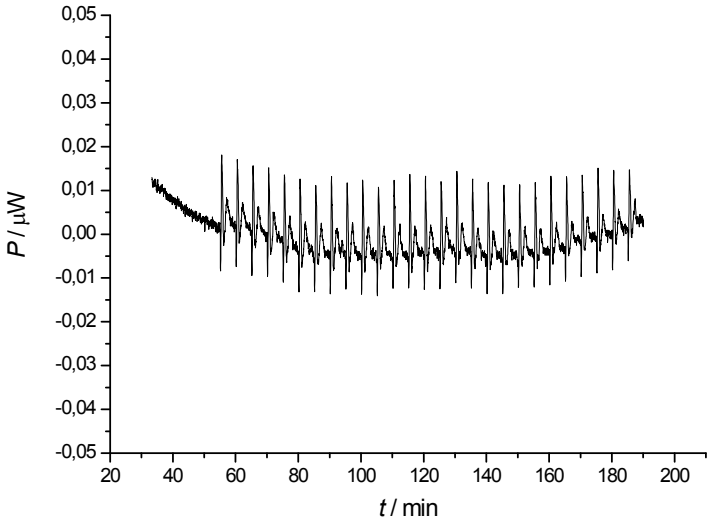


**Figure S3**. Raw ITC data of a blank titration (titration of $MnCl_2$ into the buffer solution).

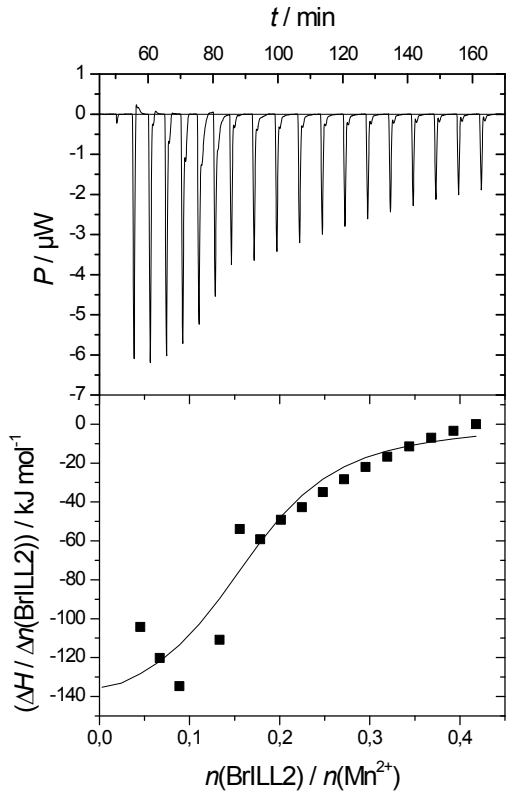


**Figure S4**. Representative ITC titration profile of $MnCl_2 \cdot 4H_2O$ ($4.5 \times 10^{-5}$ mol dm$^{-3}$) with BrILL2 wt ($9.11 \times 10^{-5}$ mol dm$^{-3}$) with fixed $N = 6$ (reverse titration).

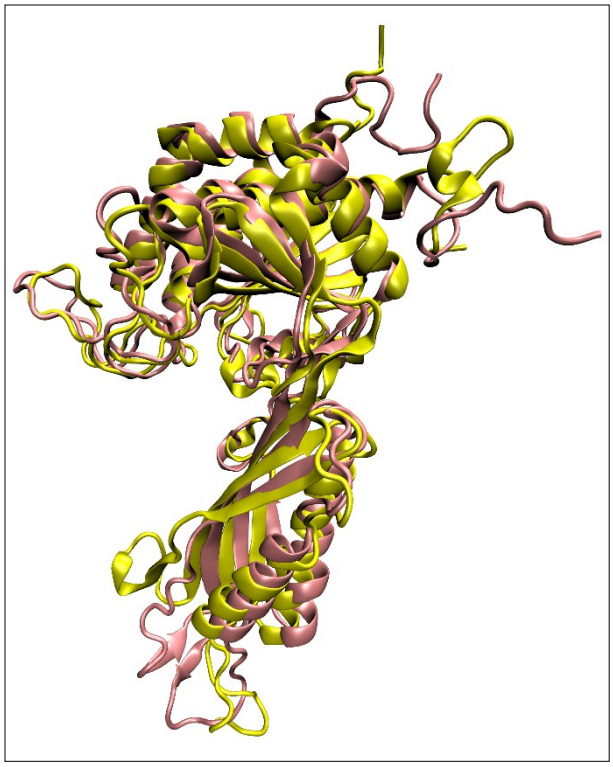

**Figure S5.** Alignment of theBrILL2wt (pink) and the BrIll2C139S$^{-}$ mutant (yellow); protein structures obtained after 200 ns of MD simulations. Simulations were performed in the presence of manganese ions in the active site.

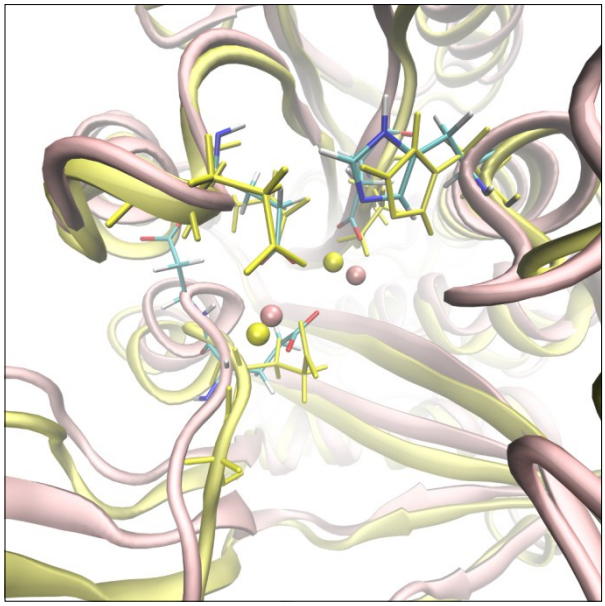

**Figure S6.** Alignment of the active sites of the BrILL2wt enzyme (the amino acid residues in the stick representation are coloured by the atom type and the rest of the protein shown as cartoon pink) and the BrILL2C139S$^{-}$ mutant, (deprotonated Ser139, C139S-, coloured yellow) structures obtained after 200 ns of MD simulations.

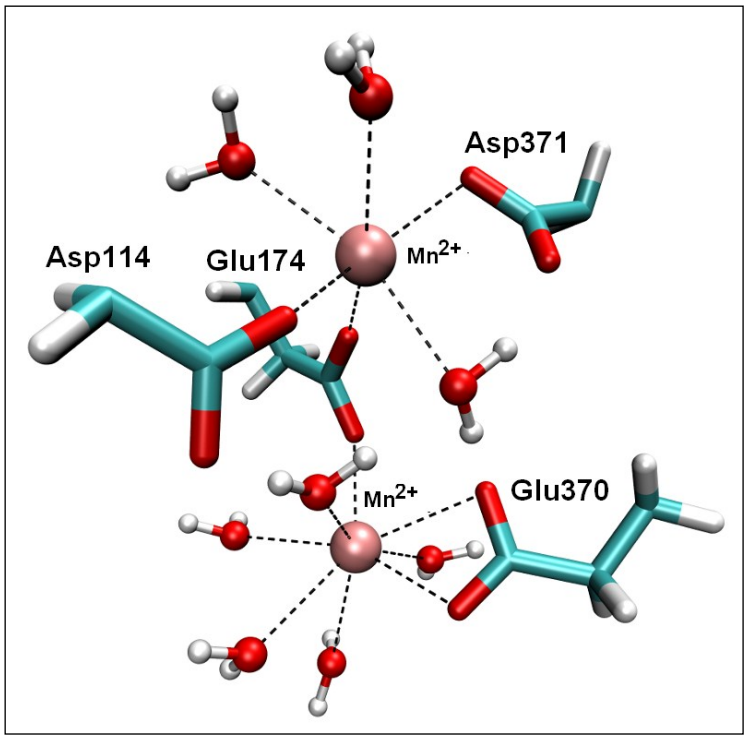


**Figure S7.** Details of the $Mn^{2+}$ surrounding in the BrILL2C139S mutant. For simplicity only side chains of amino acids are shown. $Mn^{2+}$ ions are coloured pink. All residues (side chains) and water molecules within 3 Å of metal ions are displayed. For details of the ligand participating in the metal ions coordination see ESI: Table S2, S3.

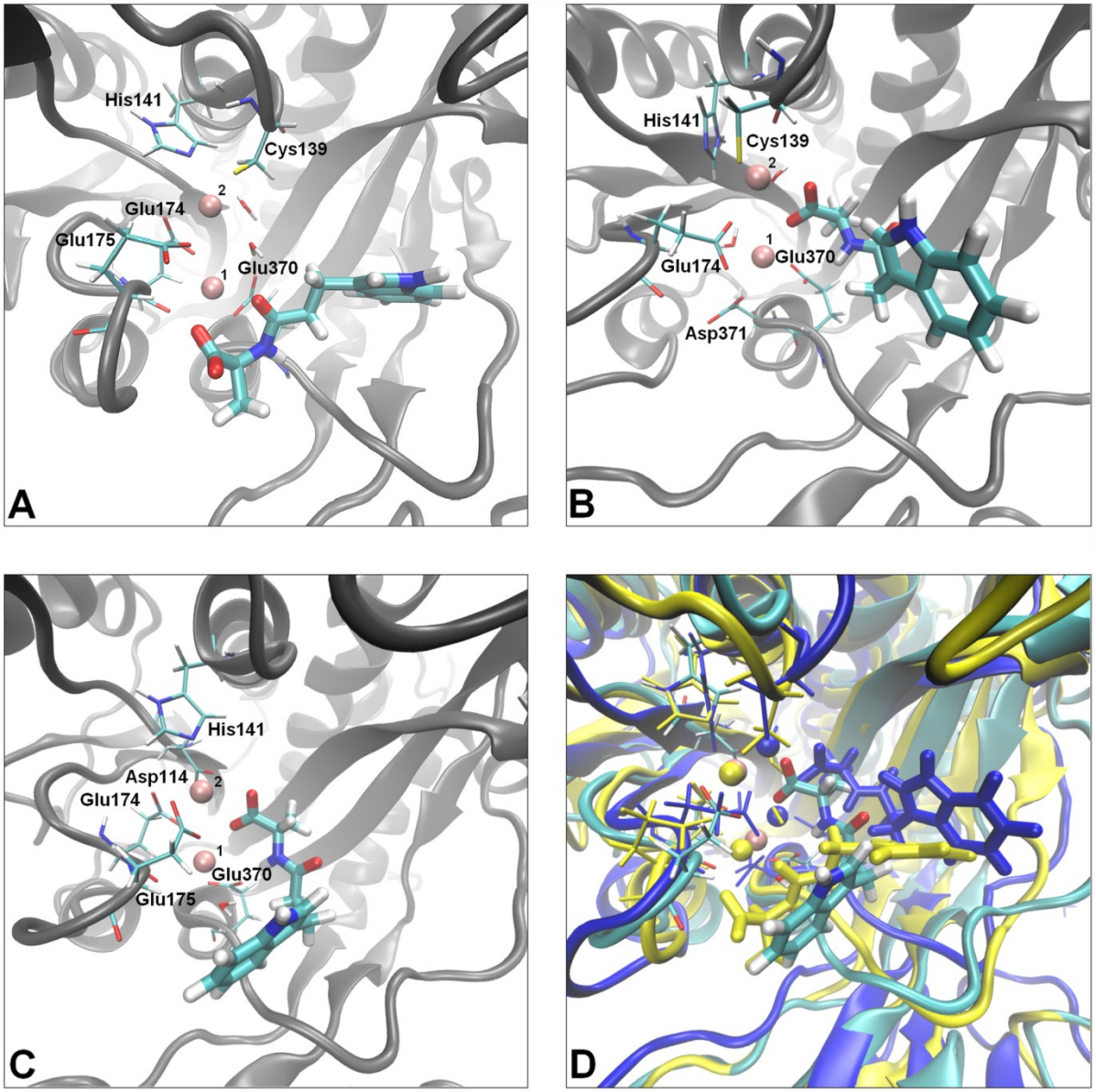


**Figure. S8.** The $Mn^{2+}$ coordination in the complexes between the BrILL2 variants and substrate IPA-Ala obtained after 200 ns of MD simulations at room temperature. Two manganese ions are shown as violet spheres. IPA-Ala is shown in thicker sticks representation. The amino acid residues and water molecules participating in the manganese ions coordination are represented as thin sticks.

**A)** Complex BrILL2wt-IPA-Ala, binding mode 1 (BM1). $Mn^{2+}$ number 1 is coordinated with Glu174, Glu 175, Glu370, one water molecule and substrate (bidentantely with carbonyl and carboxyl oxygens distance about 2.5 Å). $Mn^{2+}$ number 2 is coordinated with Cys139, His141, Glu174, Glu175 and one water molecule.

**B)** Complex BrILL2wt-IPA-Ala, binding mode 2 (BM2). Both metal ions are coordinated by substrate ($Mn^{2+}$ number 2 bidentantely with carboxyl oxygens and $Mn^{2+}$ number 1 with one carboxyl oxygen, distances about 2.6 Å). Besides, $Mn^{2+}$ at position 1 is coordinated with Glu174, Glu370, Asp371 and one water molecule and $Mn^{2+}$ at position 2 is coordinated with Cys139, His141, Glu174 and one water molecule.

**C)** Complex mutant BrILL2C139S$^{-}$-IPA-Ala. $Mn^{2+}$ number 1 is coordinated with Glu174, Glu175, Glu370, one water molecule and one carboxyl oxygen from substrate (distance about 2.6 Å). $Mn^{2+}$ number 2 is coordinated with Asp114, His141, Glu174, Glu175 and substrate (bidentantely with carboxyl oxygens, distances about 2.6 Å).

**D**) Alignment of the active sites of the structures of the BrILL2wt-IPA-Ala BM1 (yellow), BrILL2wt-IPA-Ala BM2 (blue), and the mutant BrILL2C139S$^{-}$-IPA-Ala (coloured by atom names) complexes obtained after 200 ns of MD simulation in water.

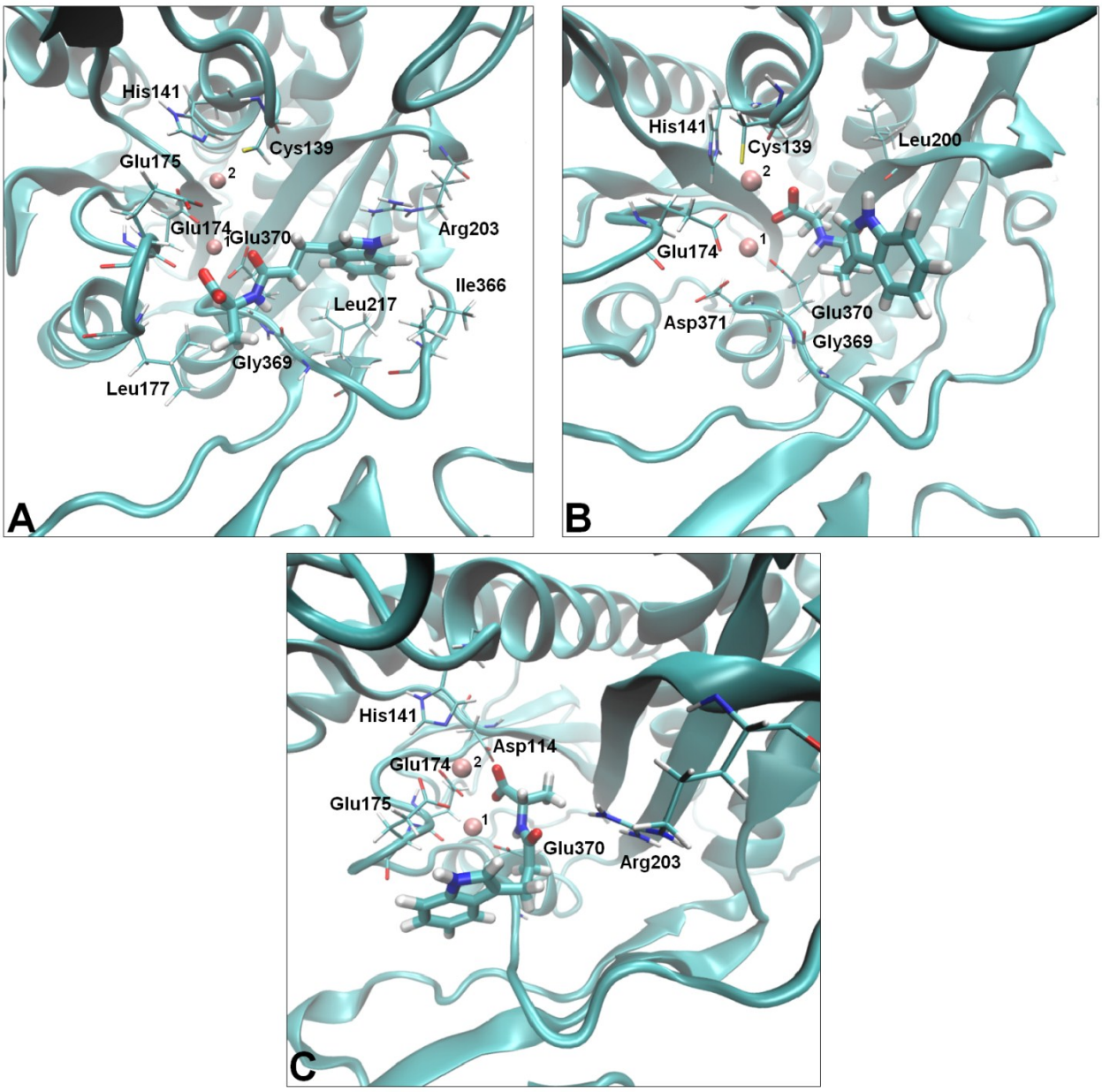


**Figure S9.** The substrate IPA-Ala stabilization in the active site of the BrILL2-IPA-Ala complexes: **A)** wt, BM1; B) wt, BM2; and C) C139S.

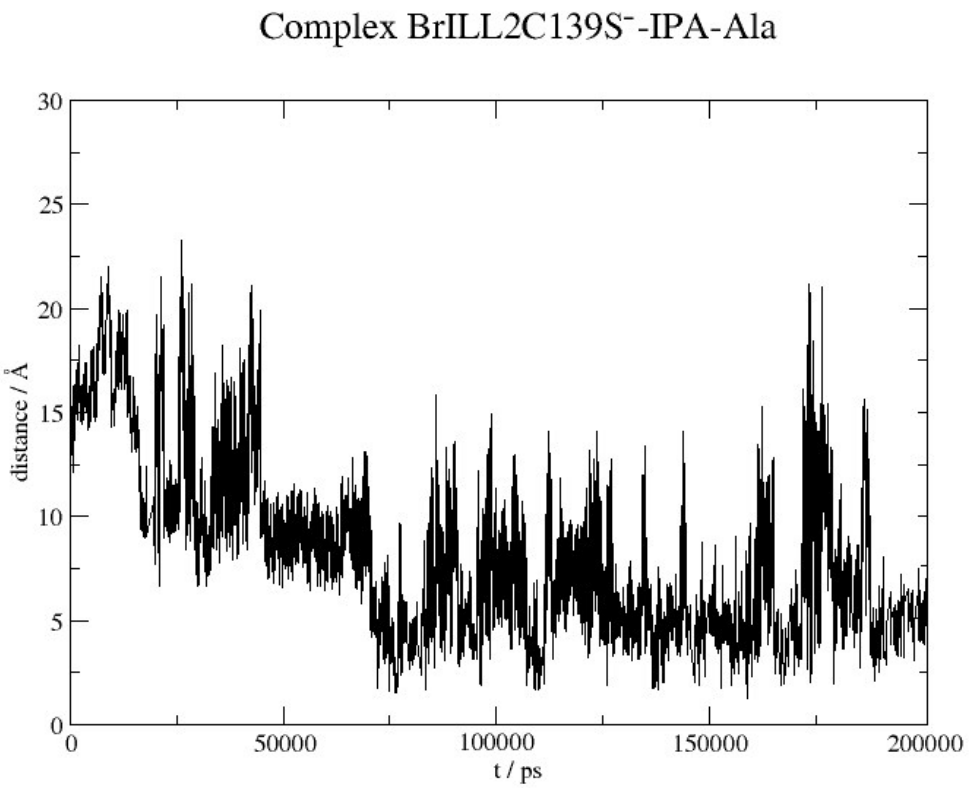


**Figure S10.** The Arg203- IPA-Ala distance during 200 ns of MD simulations of the BrILL2C139S⁻-IPA-Ala complex.

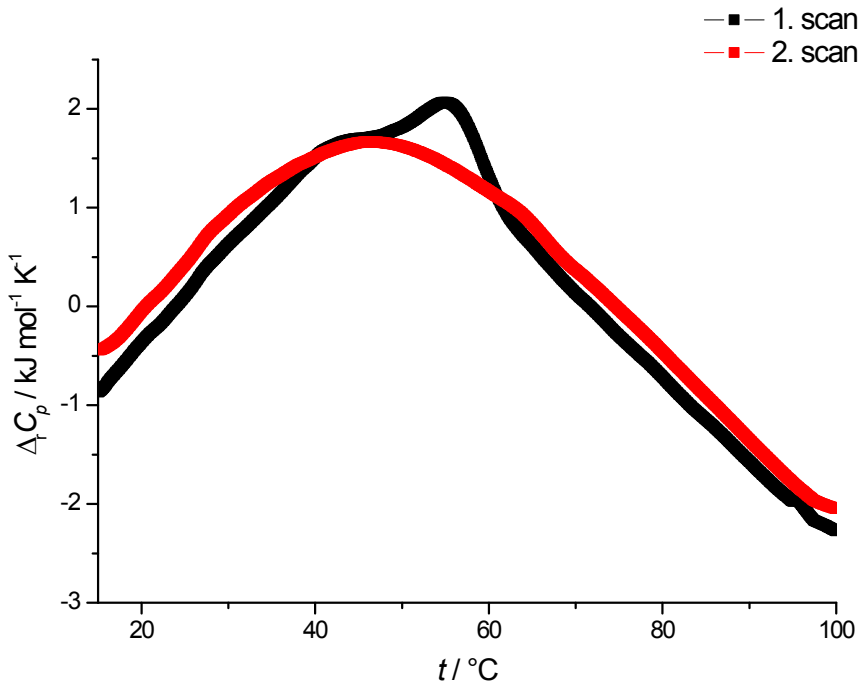


**Figure S11**. Representative DSC thermogram of a BrILL2wt enzyme at the scanning rate 1 °/min

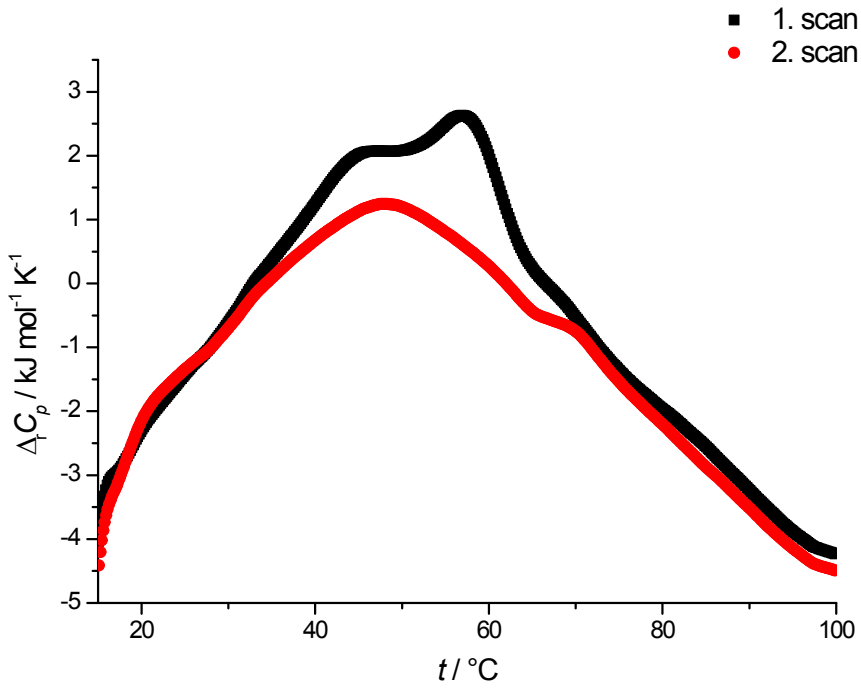


**Figure S12**. Representative DSC thermogram of a BrILL2wt enzyme at the scanning rate 2 °/min

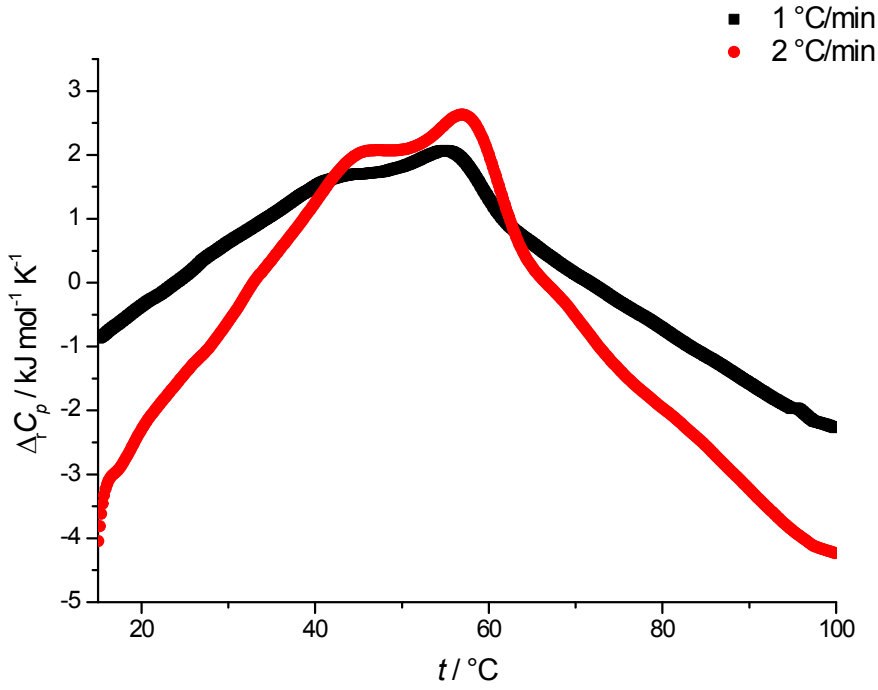


**Figure S13**. Comparison of a BrILL2wt DSC thermograms at different scanning rates: 1 °/min (black line); 2 °/min (red line).

**Table S1**: Ratio $Mn^{2+}$/enzyme of BrILL2 determined by HR-ICP-MS. The apoenzyme was obtained after purification by dialysis in EDTA (10 mM, 3h). Additional dialysis in 50 mM Tris buffer was done to remove excess of EDTA. The enzyme saturated with $Mn^{2+}$ was prepared by dialysis of the apoenzyme in MnCl2 and then 50 mM Tris buffer.

| Enzyme form | Molar ratio $Mn^{2+}$/protein |
|---|---|
| Apoenzyme, EDTA | 0.068 |
| Purified enzyme, no treated | 0.114 |
| Enzyme saturated with $Mn^{2+}$ | 5.446 |

**Table S2.** Average distances and standard deviations (Å) between the metal ions during 200 ns of MD simulations of the ligand free enzymes in water.

BrILL2wt (run 1) (4.10±0.20) Å
BrILL2wt (run 2) (4.05±0.51) Å
BrILL2C139S (5.03±1.36) Å distance continuously increased during MD simulation, and at the end of 200 ns it is 6.91 Å
BrILL2C139S$^-$ (4.33±0.47) Å
BrILL2wt-IPA-Ala BM1 (3.77±0.75) Å
BrILL2wt-IPA-Ala BM2 (4.01±0.28) Å
BrILL2C139S$^-$-IPA-Ala (3.97±0.23) Å

**Table S3.** $Mn^{2+}$ coordination. The average distances and standard deviations (Å) between the protein residues and the metal ions obtained during 200 of MD simulations. The residues within 3.6 Å from the metal ions are listed wherein the residues with the average distance above 2.86 Å (weak coordination) are given in grey.

| system | $Mn^{2+}$1 | $Mn^{2+}$2 |
|---|---|---|
| initial | Cys139 (2.92)<br>Glu175 (2.61)<br>Glu174 (2.45)<br>Glu370 (2.52) | Cys139 (2.74)<br>Asp114 (2.42)<br>Glu370 (2,56)<br>Glu174 (2.67)<br>His141 (2.57)<br>His199 (2.51) |
| BrILL2wt-IPA-Ala BM1 | Glu174(OE1) (3.01±0.88)<br>Glu175(OE2) (2.83±0.90)<br>Glu370(OE2) (2.42±0.48)<br>Glu370(OE1) (2.68±0.56) | Cys139(S) (2.88±0.40)<br>His141(NE2) (2.86±0.30)<br>Glu174(OE1) (2.56±0.52)<br>Glu174(OE2) (3.01±0.91)<br>Glu175(OE2) (2.89±0.99) |
| BrILL2wt-IPA-Ala BM2 | Glu174(OE1) (3.21±0.82)<br>Glu174(OE2) (2.85±0.67)<br>Glu370(OE1) (2.64±0.16)<br>Glu370(OE2) (2.81±0.47)<br>Asp371(OD2) (3.39±1.66) | Cys139(S) (2.75±0.10)<br>His141(NE2) (2.71±0.13)<br>Glu174(OE2) (2.78±0.52) |
| BrILL2C139S$^-$-IPA-Ala | Glu174(OE2) (2.53±0.21)<br>Glu175(OE2) (3.59±1.16)<br>Glu370(OE2) (2.70±0.34)<br>Glu370(OE1) (2.60±0.26) | Asp114(OD2) (2.53±0.20)<br>His141(NE2) (2.81±0.61)<br>Glu174(OE1) (2.63±0.24)<br>Glu174(OE2) (2.65±0.25)<br>Glu175(OE1) (3.59±1.16)<br>Glu175(OE2) (3.71±1.49) |

**Table S4.** Relative binding free energies determined by MM_PBSA calculations. The trajectory sampled the last 8 ns of 200 ns long MD simulations in water, at room temperature, was used in the calculations.

| **Complex** | **ΔΔG** (kcal/mol) |
|---|---|
| BrILL2wt-IPA-Ala BM1 | **0.0±5.6** |
| BrILL2wt-IPA-Ala BM2 | **5.9±7.9** |
| BrILL2C139S$^-$-IPA-Ala | **7.9±3.2** |

**Table S5.** Average distances and standard deviations (Å) between the substrate (IPA) and the closest enzyme residues, as well as the metal ions, during MD simulations.

BrILL2wt-IPA-Ala BM1
Distance between $Mn^{2+}$ 1 and IPA(O2) = **(2.53±0.17) Å**
Distance between $Mn^{2+}$ 1 and IPA(O3) = **(2.80±0.75) Å**
Distance between Gly369 (O) and IPA-Ala(HN) = **(2.51±0.58) Å**
Distance between Leu217(HD11) and IPA(H4) = **(5.58±2.00) Å**, distance continuously decreased during MD simulation, and in the final structure it is 2.72 Å
Distance between Leu217(HD22) and IPA(H5) = **(5.22±2.33) Å**, distance continuously decreased during MD simulation, and in the final structure it is 2.38 Å
Distance between Ile366(HD12) and IPA(H6) = **(5.35±2.49) Å**, distance continuously decreased during MD simulation, and in the final structure it is 2.64 Å
Distance between Ile366(HD12) and IPA(H5) = **(5.99±1.73) Å**, distance continuously decreased during MD simulation, and in the final structure it is 2.90 Å
Distance between Arg203(NH1) and IPA(C7) = **(5.53±2.27) Å**, distance continuously decreased during MD simulation, and in the final structure it is 3.23 Å
Distance between Arg203(NH1) and IPA(C6) = **(5.67±1.77) Å**, distance continuously decreased during MD simulation, and in the final structure it is 3.40 Å
Distance between Leu177(HD22) and IPA(H92) = **(4.27±1.08) Å**, distance continuously decreased during MD simulation, and in the final structure it is 2.36 Å
Distance between Leu177(HD22) and IPA(H91) = **(4.21±1.12) Å**, distance continuously decreased during MD simulation, and in the final structure it is 2.49 Å

BrILL2wt-IPA-Ala BM2
Distance between $Mn^{2+}$ 1 and IPA(O1) = **(2.96±0.72) Å -** distance continuously decreased during MD simulation, and in the final structure it is 2.47 Å (Fig. S9)
Distance between $Mn^{2+}$ 2 and IPA(O1) = **(3.99±1.64) Å -** distance continuously decreased during MD simulation, and in the final structure it is 2.61 Å (Fig. S9)
Distance between $Mn^{2+}$ 2 and IPA(O3) = **(3.18±1.05) Å –** distance continuously decreased during MD simulation, and in the final structure it is 2.54 Å (Fig. S9)
Distance between Glu370 (OE1) and IPA(HN) = **(2.70±0.99) Å -** distance continuously decreased during MD simulation, and in the final structure it is 1.99 Å (Fig. S9)
Distance between Gly369 (O) and IPA(H111) = **(3.97±1.03) Å**
Distance between Leu200 (O) and IPA(HN1) = **(5.25±3.28) Å**

BrILL2C139S$^-$-IPA-Ala
Distance between $Mn^{2+}$ 2 and IPA(O1) = **(4.30±2.46) Å -** distance continuously decreased during MD simulation, and in the final structure it is 2.56 Å (Fig. S9)
Distance between $Mn^{2+}$ 2 and IPA(O3) = **(3.86±1.77) Å -** distance continuously decreased during MD simulation, and in the final structure it is 2.68 Å (Fig. S9)
Distance between Glu370(OE2) and IPA(HN) = **(3.55±1.61) Å** – distance in the final structure is 2.68 Å

Distance between Arg203(HH22) and IPA(O2) = **(5.36±2.54) Å**

**Table S6.** Average distance between manganese ions and water molecules that coordinate the metal in the final (optimized) structures during the last 2 ns of 200 ns of MD simulations of the complexes

BrILL2wt-IPA-Ala BM1
Mn1: WAT4456 = (2.98±0.30) Å (calculated for the last 2 ns)
Mn2: WAT7163 = (2.60±0.25) Å (calculated for the last 2 ns)

BrILL2wt-IPA-Ala BM2
Mn1: WAT9866 = (2.90±0.82) Å (calculated for the last 2 ns)
Mn2: WAT3901 = (3.05±0.61) Å (calculated for the last 2 ns)

BrILL2C139S$^{-}$-IPA-Ala
Mn1: WAT9165 = (3.01±1.21) Å (calculated for the last 2 ns)

**Table S7.** Metal ion parameters used to simulate different BrILL2 structures (variants).

| $R_{vdw}$ (Å) | $\varepsilon_{0vdw}$(kcal/(mol Å)) | q($e^{+}$) |
|---|---|---|
| 1.80 | 0.0613 | 1.5 |