A fundamental catalytic difference between zinc and manganese dependent enzymes revealed in a bacterial isatin hydrolase

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The catalytic mechanism of the cyclic amidohydrolase isatin hydrolase depends on a catalytically active manganese in the substrate-binding pocket. The Mn$^{2+}$ ion is bound by a motif also present in other metal dependent hydrolases like the bacterial kynurenine formamidase. The crystal structures of the isatin hydrolases from Labrenzia aggregata and Ralstonia solanacearum combined with activity assays allow for the identification of key determinants specific for the reaction mechanism. Active site residues central to the hydrolytic mechanism include a novel catalytic triad Asp-His-His supported by structural comparison and hybrid quantum mechanics/classical mechanics simulations. A hydrolytic mechanism for a Mn$^{2+}$ dependent amidohydrolases that disfavour Zn$^{2+}$ as the primary catalytically active site metal proposed here is supported by these likely cases of convergent evolution. The work illustrates a fundamental difference in the substrate-binding mode between Mn$^{2+}$ dependent isatin hydrolase like enzymes in comparison with the vast number of Zn$^{2+}$ dependent enzymes.

The catalytic activity of metallohydrolase enzymes strongly depends on the identity of the metal and on the nature of its binding site. Zn$^{2+}$ bound hydrolytic enzymes, in particular metallo-β-lactamases1, probably constitute the most extensively studied cases for such a group. Depending on the enzymatic class, hydrolytic enzymes with both a mononuclear or dinuclear Zn$^{2+}$ sites have been identified as widely distributed throughout all kingdoms of life3. The catalytic mechanism for the mononuclear and dinuclear Zn$^{2+}$ sites have been debated for years3–5, and only recently the collection of accumulated knowledge allowed the proposition of a plausible mechanism6–9. Despite Zn$^{2+}$ being the most widely distributed hydrolytic cation, it has been shown that Zn$^{2+}$ also possesses an inhibitory potential for certain metallohydrolases as, for example, in carboxypeptidase A10.

The specialized metallo-enzyme amidohydrolase superfamily (AHS) is able to carry out a diverse range of chemical reactions including the degradation of metabolic precursors like pyrimidine (dihydropyrimidinases) and hydantoin, (hydantoinases)11. The AHS members are often categorised as both Zn$^{2+}$ and Mn$^{2+}$ dependent hydrolases. However, amidohydrolases involved with purines degradation in both bacteria and plants are mainly Mn$^{2+}$ dependent allantoinases12,13, even though Zn$^{2+}$ dependent allantoinase activity has been reported14.

The compound isatin is a metabolic precursors and was originally identified as an intermediate product of the indole-3-acetic acid (IAA) degradation pathway in Bradyrhizobium diazoefficiens15. Hydrolytic activity of isatin hydrolase (IH) was first demonstrated in various symbiotic rhizobial species16. Recent bioinformatics studies

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indicates that IH are found widespread in bacteria including pathogens specific to the human gut and plants, e.g., *B. enterica* and *R. solanacearum* [12]. Isatin hydrolase A from *Labrenzia aggregata* (LaIHA) and *R. solanacearum* (RsIHA) share 59% sequence identity while the two putative orthologues LaIHA and LaIHB in *L. aggregata* share 51% sequence identity. Both LaIHA and LaIHB contain the central metal binding motif His[C[T/A]HxDxP]H in each protomer of the catalytically active dimer. Structural characterization of LaIHB revealed a novel fold, which later was also attributed to other amidohydrolases such as bacterial kynurenine formamidase B (KynB) (EC 3.5.1.9) [13]. The fold is generally described as an α/β hydrolase with a central scaffold resembling the swivel-3/α/β fold [14], while the majority of AHS members contains a monomeric (3/α/β)₆-TIM like-barrel structural fold [15]. A notable difference between LaIHB and kynurenine formamidase from *Bacillus anthracis* (BaKynB) is the presence of a mononuclear manganese-binding site in LaIHB, while the structure of KynB accommodates a bi-nuclear zinc site [16]. LaIHB shows a strict specificity for Mn²⁺ over Zn²⁺, as reported by kinetic measurements [17], and rationalised by computer simulations [18].

Here we present two crystal structures of LaIHA, one bound to the hydrolytic product isatinate, and one bound to benzyl benzoate, as well as the apo structure of homologous RsIHA. Together with results from enzyme kinetic analysis, the resolved structures are used to describe the molecular basis for the Mn²⁺-dependent mechanism of hydrolysis, showing that IHA is a true orthologue of IHB. For the first time, the mechanism of a Mn²⁺ dependent hydrolysis reaction is characterised in detail by computer simulations using multiscale quantum mechanics/molecular mechanics (QM/MM) [19] and ab initio molecular dynamics (MD) simulations [20]. The simulations were crucial to unravelling the essential catalytic role of the conserved residues His79 and His207 for the formation of the product. Based on the identification of mechanistically important residues presented in this work, including the Mn²⁺ binding residues in the LaIHA active site, we further present evidence that the Mn²⁺ dependent allantoinase amidohydrolase (AHS) (EC 3.5.1.9) displays the activity in the active site geometry despite the differences in the overall protein fold. As this likely represents a case of convergent evolution, we propose that the generalised catalytic mechanism described here for the IHs may also apply to the AAH family and other similar Mn²⁺ dependent hydrolases.

**Materials and Methods**

**Cloning, expression and purification of LaIHA and RsIHA.** Chemicals were purchased from Sigma-Aldrich (Norway) unless stated otherwise. The open reading frame encoding LaIHA (UniProtKB: A0P0F0) was amplified by PCR from a boiled colony of *L. aggregata* IAM12614. DNA fragments were isolated and digested with BamHI and EcoRI and cloned into the T7-RNA polymerase dependent *E. coli* expression plasmid pT7H7G [21]. The 6xHis containing *LaIHA* was expressed in *E. coli* BL21 AI cells (Invitrogen) in 2xTYE-medium for 4 hours at 37°C. Cells were harvested by centrifugation, re-suspended, and lysed by sonication in a buffer containing 50 mM Tris-HCl pH 8.0 and 0.5 M NaCl supplemented with a protease inhibitor tablet (Roche Complete, EDTA-free). Insoluble material was removed by centrifugation (4000 g, 10 min.). The soluble protein extract was batch-adsorbed onto 25 mL Ni-NTA agarose resin (Qiagen) per litre of original culture and loaded into liquid chromatography columns. The protein loaded Ni-NTA columns were washed with >20 column volumes (CV) of equilibration buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 50 mM imidazole). The protein was eluted from the resin with elution buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM Na₂EDTA). Fractions containing the recombinant proteins were pooled, and the buffer changed into a low salt buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, and 1 mM Na₂EDTA) on a Sephadex G-25 column (GE Healthcare). Before crystallisation and kinetic experiments, LaIHA was further treated with 10 mM EDTA and dialysed (500 fold dilution) into a stability buffer (5 mM bis-tris pH 7.0, 100 mM NaCl and 1 mM DTT). The protein solutions were centrifuged at 180,000 g for 10 minutes at 4°C, and the supernatant could hereafter be stored in a stable condition at 4°C for at least 12 months.

The RsIHA (UniProtKB: Q8XYC3) was ordered from Genscript and inserted into the expression vector pET-M11. The six-histidine containing construct was expressed in *E. coli* BL21 gold cells, in LB-medium (containing 50 μg/mL kanamycin) overnight at 18°C. Cells were harvested by centrifugation, re-suspended, and lysed by a bead-beater (Biospec) in a lysis buffer (50 mM Bis-Tris pH 7.0 and 0.1 M NaCl), protease inhibitor tablet (Roche Complete, EDTA-free). Insoluble material was removed by centrifugation, and the lysate was supplemented with 20 mM imidazole before loaded onto a pre-equilibrated HisTrap HP (GE). The column was washed with 10 CV with equilibration buffer (50 mM Tris-HCl pH 8.0, 0.1 M NaCl, and 20 mM imidazole) and eluted with elution buffer (50 mM Tris-HCl pH 8.0, 0.1 M NaCl, and 300 mM imidazole). The pooled fractions were supplemented with a molar 1:50 ratio of tobacco etch virus (TEV) protease and dialysed overnight against dialysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl). The RsIHA was again loaded to the HisTrap HP column and the flow-through was collected. RsIHA was concentrated to 20 mg/mL and loaded onto a Superdex 200 10/300 GL size exclusion column (GE) pre-equilibrated in SEC buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl). Pure RsIHA were pooled, concentrated, and stored at −80°C. RsIHA was treated like LaIHA before crystallisation and kinetic experiments.

**Crystallization of LaIHA and RsIHA ligand complexes.** The pre-treatment of LaIHA and RsIHA with an excess of EDTA and dialysis was mainly carried out to remove any potential trace of metals, leaving an estimated EDTA concentration at 0.02 mM. Prior to crystallisation 1 mM MnCl₂ was added. LaIHA and RsIHA were crystallized at 22°C using a protein concentration of 15 mg/mL in 5 mM Bis-Tris pH 7.0, 100 mM NaCl, and 1.0 mM MnCl₂. LaIHA was crystallised by vapour diffusion using sitting drops 1 + 1 μL (LaIHA: Reservoir) over 100 μL reservoir solution. The best diffraacting crystals were obtained in 20% glycerol and 20% PEG 1500. The LaIHA:isatinate structure was obtained by soaking the crystals for approximately 1 min with 500 μM isatin before flash-freezing the crystals in liquid nitrogen (LN₂). This condition also served as cryo-protection. RsIHA was also crystallised with vapour diffusion using hanging drops, 1 + 1 μL (RsIHA: Reservoir) over 400 μL reservoir
solution sealed with immersion oil (56822, Sigma). The best diffracting crystals of \( RS_{iHA} \) grew in 1 M succinate, 100 mM HEPES pH 6.5. Cryo-protection was achieved by adding 1 \( \mu L \) of 60% w/v PEG400 suspended in reservoir solution to the drop. The crystals were mounted and flash-cooled immediately after. Crystals of \( LaiHA:benzyl benzoate \) were obtained in 28% PEG 1500 and 1 mM \( MnCl_2 \), with a hanging drop setup using 250 \( \mu L \) reservoir and 1 + 1 \( \mu L \) drops. With a protein concentration of 20 mg/ml used. The hanging drop wells were sealed with immersion oil. Crystals appeared within one week. Cryo-protection was obtained by adding glycerol to the reservoir solution to a final concentration of \( \sim 12\% \), which after 12 hours would allow additional vapor diffusion to take place before mounting and flash-cooling in LN\(_2\).\(^{25}\)

**Data collection and structure determination.** All datasets were collected at 100 K. For \( LaiHA:Benzy1 benzoate \) a dataset extending to \( 1.50 \) \( \AA \) resolution was collected at a wavelength of 1.0004 \( \AA \). The space group was determined to be \( P1\), and a collection strategy was calculated by iMOSFLM\(^{28}\). The data were processed with XDS. A test set of 5% was used for \( R_{wp} \) calculation\(^{28}\). Phasing was performed by molecular replacement (MR) using \( La_{HB} \) (PDB ID: 4J0N) as a search model for Phaser\(^{30}\). Initial model building and refinement were performed in Phenix\(^{30}\). The final model was built using Coot\(^{31}\) with small ligand models and restraints produced using eLBOW\(^{30}\). For \( LaiHA:isatinate \), a diffraction dataset was collected at beamline P13 at PETRA III (Hamburg, Germany)\(^{32}\). The space group was determined and would only allow a \( P1 \) lattice. A complete dataset was collected at 1.79 \( \AA \) resolution with and oscillation range of \( \Omega = 0.01^\circ \) and processed with XDS. The structure was solved with MR using a \( LaiHA:Benzy1 benzoate \) monomer as the search model and performed with Phaser in Phenix. Diffraction data on \( Rs_{iHA} \) was collected at beamline ID30B at ESRF (Grenoble, France). The space group was determined and allowed \( P \ 3\ 2\ 1 \), a complete dataset with at \( \Omega = 0.05^\circ \) over 126\% was collected. The data extended to 2.65 \( \AA \) and was processed with XDS. The structure was solved with MR using \( LaiHA:Benzy1 benzoate \) monomer as the search model in Phenix. Model building and refinement was performed using coot\(^{31}\) and phenix. Refine within the phenix package\(^{30}\). NCS restraints were applied throughout the refinement, however released in the final round (Number of molecules in the asymmetric unit of each crystals structure have been added in the Supplementary Table S1)\(^{34}\). All structure validations were performed using MolProbity\(^{30}\). Structure analysis and figure preparation were done using PyMOL Molecular Graphics System, Version 1.5.0.3, Schrödinger, LLC. The finalised model and structure factors were deposited to Protein Data Bank (PDB) and given the PDB accession codes (\( LaiHA:Benzy1 benzoate \) PDB ID 5NNA; \( Rs_{iHA} \) PDB ID 5NMP; \( LaiHA:isatinate \) PDB ID 5NNB).

**Enzymatic assay.** The measurement of the activity, and its metal dependence, were performed as described by Bjerregaard-Andersen et al.\(^ {17} \). The method is based on an increased absorption of the product isatinate at 368 nm. This can be followed using a standard spectrophotometer. Measurements were performed using a JASCO V-630 spectrophotometer with a cuvette light path of 1.0 cm. An extinction coefficient of \( 4.5 \times 10^3 \) cm\(^{-1}\)mol\(^{-1}\)L was used for isatinate. The data were fitted with the Michaelis-Menten equation assuming one binding site using the software GraphPad Prism 6.

**Sequence analysis.** Structural homologues of \( LaiHA \) and \( E. coli \) allantoino amidohydrolase (\( EcAAH \) ) were identified using the DaliLite v. 3.\(^{35} \), followed by inspection in PyMoL. The sequences of the identified homologues were then imported in Jalview\(^{46} \) using the fetch from PDB option and combined with sequences of \( LaiHA, \ Rs_{iHA}, \) and \( Rhodococcus rhodochrous \) HpoH (\( RrHpoH \)). Sequences with the following UniProt accession codes (in parentheses) were used: \( LaiHA \) (A0P0F0), \( La_{HB} \) (A0NY7), \( Rs_{iHA} \) (Q8XCY3), \( Ec_{KynB} \) (B4E919), \( Pu_{KynB} \) (Q91234), \( Ba_{KynB} \) (Q81PP9), \( Ba_{AHD} \) (P81432), \( Mj_{AHD} \) (Q58193), \( Rh_{HpoH} \) (B5MAD9). For AAH alignment, the following accession codes were used: \( Ec_{AAH} \) (P77425), \( At_{AAH} \) (Q8VXY9), \( Bv_{HYD} \) (A4QQA0), \( Bm_{HYD} \) (A0A0H3KRF1), \( Bc_{HYD} \) (B4EHA1), \( Ge_{RAC} \) (Q53389), \( Sk_{SYN} \) (Q96W94). Sequence alignments were performed using the Clustal algorithm with default settings in Jalview. Phylogenetic trees were calculated in Jalview as average distance using identity percentage and exported to TreeDyn 198.\(^{33,7} \) for visualisation. The sequences were included in alignment as supplied by the PDB.

**Molecular modelling.** The system consisting of \( LaiHA, \) isatin, 47331 water molecules, and 19 Na\(^{+} \) ions was placed in a box of \( 105 \times 126 \times 118 \) \( \AA^3 \) simulation box. The initial geometry of the protein was taken from the X-ray structure of \( LaiHA:isatinate \) resolved by us and described in this text. Isatin was introduced in the system by molecular replacement, fitting the aromatic moiety of isatinate. The Mn\(^{2+} \) ion, isatin, the catalytic water, the side chains of the ligating amino acids (His69, His73, Asp75, Gln219), and of the neighbouring His79, His207, Asp193 residues were treated at a quantum-mechanical level (QM) using density functional theory with the Becke and Lee-Yang-Parr (BLYP) approximations for the exchange-correlation functional\(^{38,39} \). The Kohn-Sham orbitals for the valence electrons were expanded over a DZVP Gaussian basis set\(^8 \) and an auxiliary plane-wave basis set with a cut-off of 240 Ry. The core electrons were integrated out using Goedecker-Teter-Hutter pseudopotentials\(^{41} \). The remainder of the system was described at the molecular-mechanics (MM) level using the Amberff14SB force field\(^{42} \).

In a first step, the system was equilibrated by full classical molecular dynamics (MD) simulations in the NpT ensemble (\( T = 300 \) K, \( p = 1 \) atm), constraining the QM region to its initial positions, and using a time-step of 1.5 fs. Thereafter, QM/MM Born-Oppenheimer MD simulations were performed at 300 K within the NVT ensemble using the Nosé–Hoover thermostat\(^{43,44} \) and a time-step of 0.25 fs. The simulations were run using the CP2K package (https://www.cp2k.org/)\(^{40,41} \). The mechanism of hydrolysis was sampled by coupling QM/MM MD to metadynamics simulations\(^ {8,9,36} \) using collective variables defined as differences between coordination numbers (Visualized in Fig. S1, in the supporting information):
Km activity in the presence of Mn$^{2+}$ two homologues. Thus, a metal dependence analysis was only performed on La$^{3+}$ (Supplementary Fig. S3). The motif is absent in Rs$^{3+}$.

Both refinement statistics are collected as described in Supplementary Table S1. Both La$^{3+}$ and Rs$^{3+}$ similar to the one of Methanococcus jannaschii$^{(\text{AHD})}$ and Bacillus stea-

Results
IHA contains a mononuclear manganese binding site involving an additionally conserved glutamine. The structures of LalIHA:benzyl benzoate and LalIHA:isatinate were determined at 1.50 Å and 1.79 Å resolution, respectively, while the one of RsIHA was determined at 2.65 Å resolution. Data collection and model refinement statistics are collected as described in Supplementary Table S1. Both LalIHA structures are highly similar to the one of LalIHB with a root mean square deviation (rmsd) value of ~0.7 Å computed on all main chain atoms. Both LalIHB and LalIHA are dimers with two exchanged regions – a small N-terminal α-helical exchange and a larger β-hairpin exchange (Fig. 1a). A hallmark of this fold is the contribution of two conserved residues of one monomer in the formation of the substrate-binding pocket located in the other monomer. For the IHs these two residues are two tryptophans (Fig. 1b).

The highly conserved Mn$^{2+}$ binding site is located at the bottom of the substrate-binding pocket. The Mn$^{2+}$ is found in an octahedral complex coordinated by His69, His73, Asp75, and two water molecules (Fig. 1c). Asp75 forms a bidentate contact in LalIHA:Benzyl benzoate and a mono-dentate electrostatic contact in the LalIHA:isatinate structures. The mono or bi-dentate coordination results in two or one water molecules as additional ligands, respectively, preserving the octahedral geometry. The side chain carbonyl of Gln219 in LalIHA (Ala224 in LalIHB) is found in alternative conformations, one of which coordinates Mn$^{2+}$. The non-binding conformation allows additional water to approach a Mn$^{2+}$ (W1025 in LalIHB, Fig. 1c). Sequence analysis reveals that Gln219 is part of a motif, conserved as either GLAS (e.g. in LalIHB) or GLQC (e.g. in LalIHA) (Figs 1 and 2a). The short residues motifs AS and QC are conserved as sequence pairs, which could indicate a functional dependency. Interestingly, the residues S or C are found to be the key residue involved in regulating the proton flow through the water channel. A mutation of the serine residue to a cysteine to form a GLAC motif caused a gain in activity in LalIHB$^{37}$. The GLQC/GLAS sequence, combined with the conserved manganese binding site appears to be a signature motif for isatin hydrolase activity. In KynB, the comparable position holds a conserved I(I/L/E) motif, which is also found in the two uncharacterized structures of amidohydrolases (AHID) from Bacillus stearothermophilus (BsAHID) and Methanococcus jannaschii (MjAHID) (Fig. 2a, Full sequence alignment shown in Supplementary Fig. S3). The motif is absent in RsHpoI, which has confirmed cyclase activity. Whether the I(I/L)E motif and perhaps, in particular, the glutamate is indicative of activity on linear amides or a binuclear metal site remains uncertain.

Activity measurements confirm that both LalIHA and RsIHA have isatin hydrolase activity (Fig. 1d,f). The determination of the catalytic parameters assuming Michaelis-Menten kinetics yields $K_m$ values for LalIHA and RsIHA of 16μM and 10μM, respectively. Structural superposition revealed identical metal binding sites in the two homologues. Thus, a metal dependence analysis was only performed on LalIHA. LalIHA shows the highest activity in the presence of Mn$^{2+}$ confirming that this enzyme is Mn$^{2+}$ dependent. However, the relative activity of 15.1 ± 2.2% is also observed in the presence of Cd$^{2+}$. Notably, LalIHA could not be activated in the presence of both Zn$^{2+}$ and Cu$^{2+}$ (Fig. 1e).

QM/MM calculations reveal the importance of an Asp-His dyad in the proton abstraction. In LalIHA, both the carbonyl group of the substrate and the catalytic water coordinate to the Mn$^{2+}$ ion. Like in other hydrolytic enzymes, the nucleophilic water is activated by a pre-organized base, which can efficiently extract a proton to form hydroxide (OH$^-$). In LalIHA, His207 acts as such base. The positively charged protonated His207-H$^+$ is stabilised by the presence of a hydrogen-bonded partner, i.e., Asp193. The water-His207-Asp193 H-bond network closely resembles the Ser-His-Asp triad common to serine proteases. His79, also H-bonded to the catalytic water, completes a complex His79-water-His207-Asp193 proton-shuttle system (Fig. 2b,d). Water deprotonation in LalIHA is extremely efficient and occurs spontaneously during the QM/MM simulations at room temperature$^{21}$. Activation of the catalytic water is favoured by its isolation from the water channel that

$$CN(i;j) = 1 - \frac{(d_{ij}/d_0)^p}{1 - (d_{ij}/d_0)^{p+q}}$$
Figure 1. (a) Overall structure of the *La*IHA dimer found in the asymmetric unit, the protomers are colored green and cyan and shown in two different orientations rotated by 90 degrees. Manganese and highlighted residues a location of active sites in the dimer. (b) Isatinate and benzyl benzoate in the substrate binding pocket of *La*IHA. Isatinate is coordinating bidentate directly to the manganese. Parts of the pocket, residues Trp61 and Trp59, are contributed by the opposing monomer via the hairpin swap. Key binding pocket residues of the *La*IHB binding pocket (grey) are functionally conserved. (c) Catalytically important residue in the active site of *La*IHA: benzyl benzoate. The corresponding site of *La*HA: benzyl benzoate is superposed (transparent). The manganese is found in octahedral coordination similarly to that described in17. Gln219 resides in a double conformation and only partially coordinates to the manganese. Also, W1025 is found in a double conformation (denoted A and B in Fig. 1). Note that Asp75 is coordinating bidentate in *La*IHA:isatinate while monodentate in *La*IHA: benzyl benzoate. (d) The isatin hydrolysis by *La*IHA (circles) and *Rs*IHA (triangles) follows Michaelis-Menten kinetics, and the parameters are collected in f). (e) Both *La*IHA and *Rs*IHA display strong manganese dependency. (f) Kinetic parameters of *La*IHA and *Rs*IHA collected with previous values from *La*IHB and activated mutant *La*IHB S225C. All measurements in (d) and (e) were performed in triplicates.
Figure 2. (a) Alignment of selected sequences of IHA and AAH homologues with known structures. Conserved residues are highlighted in blue. Residues involved in metal coordination are indicated by a dot, residues presumed to be involved in proton transfer are indicated with an asterisk. All are completely conserved, apart from the LaIHA Asp193 (yellow) which is functionally conserved. The orthologue is defining aromatic binding pocket motif for isatin, WXW/FXW, is highlighted in orange. The key GLQC/GLAS motif of IHA and IHB, indicating isatin hydrolase activity, is highlighted in green, with a similarly conserved motif in KynB homologues highlighted in red. (b) Phylogram of IHA homologues from A indicates IHA-like and KynB-like sequences, RrHpoH proposed to carry a novel cyclase activity seems to belong to neither of these. An example of the fold (dark and light grey) and active site position is shown. (c) Phylogram of AAH homologues. Note the large diversity between EcAHH (prokaryote) and AtAAH (eukaryote). An example of the fold (green and cyan) and active site position is shown, His226 (AtAAH) is delivered from opposing monomer through domain swap of AtAAH. (d) Active site residues of BaKynB (Yellow, black numbers) superposed with LaIHA (Grey, grey numbers), the mononuclear Mn$^{2+}$ in LaIHA is labelled (Mn$_1$) and the binuclear Zn$^{2+}$ sites in BaKynB are labelled Zn$_1$ and Zn$_2$. (e) Active site residues of EcAAH (green and cyan, black numbers) superposed manually with LaIHA (grey, grey numbers), the mononuclear Mn$^{2+}$ from EcAAH labelled Mn$_1$. 


connects the substrate cavity to the exterior. Confinement of the catalytic water has been reported as an important structural requirement in other hydrolytic (metallo)-enzymes\(^{51-55}\). Figure 3 depicts the molecular details of the hydrolytic reaction. The nucleophilic attack of OH\(^-\) to the carbonyl of isatin leads to the formation of a tetrahedral intermediate. Our model predicts an activation barrier of a few (i.e., less than 6) kcal mol\(^{-1}\). Thus, as in other metallohydrolases (i.e., zinc-\(\beta\)-lactamases), this step is not rate-limiting for the reaction. The tetrahedral intermediate is only metastable and it can easily recombine into the reactant state. In fact, the following separation of the scissile C-N bond constitutes the rate-limiting step of the reaction with a barrier height of ~12 kcal mol\(^{-1}\).

Our data report that C-N separation occurs through a dissociative pathway. The transition state is stabilised by formation of two hydrogen-bonds (H-bonds), the first between the nucleophilic OH\(^-\) and the N\(\delta\) atom of His79, and the second between the isatin N atom and the N\(\epsilon\)-H moiety of His207 (Fig. 3, Supplementary movie). The final product formation of isatinate is accompanied by synchronous proton exchange in both of these two H-bonds. Our simulations indicate that the proton transfers occur after the transition state has been reached and the C-N bond is broken. Kinetic measurements in a deuterated environment did not reveal any observable kinetic isotope effect on the reaction, providing an additional indication that proton transfers are not involved in the rate-limiting step of the enzymatic process (data not shown).

The preference of a dissociative pathway over an associative or concerted mechanism may be a consequence of the fact that the N-atom in isatin acts as a poor base. In general, the \(\pi\) conjugation between the lone pair of N and the aromatic ring present in aryl-amines decreases the \(pK_a\) value by few pH units. As a result, we observe that formation of a stable H-bond with His207, and consequently the proton transfer, occurs only upon a major elongation of the isatin C-N bond, which is associated to increased localization of the electron density at the N atom. Additional stabilization of the moiety into the product is obtained by the deprotonation of the nucleophile OH\(^-\) group by His79 to produce a carboxylate group chelating the Mn\(^{2+}\) as observed in the crystal structure.

The isatinate conformer produced by hydrolysis is not the most stable one. In fact, the \(\alpha\)-carbonyl can isomerise via a bicycle motion, similar to that described in excited state isomerisation of retinal in rhodopsin\(^{36}\). This isomerised product found in our simulations is in agreement with the binding geometry of the product, isatinate, described in the crystal structure (Fig. 1b). In particular, the final binding state of isatinate shows bidentate coordination to the manganese ion, and similar stacking interactions with the indole rings of Trp59, Trp61, Trp80 and Tyr204 (Fig. 1b).

The strong exothermic isomerisation step (~10 kcal mol\(^{-1}\)) yields a structure of isatinate with a strong intra-molecular H-bond between the amino and the \(\alpha\)-carbonyl groups. This possible intermediate most likely constitutes the driving force of the reaction and traps the isatinate as a ligand to Mn\(^{2+}\) in its product state before it is replaced by water and released from the binding pocket (Fig. 3).

**Figure 3.** Reaction mechanism describing the hydrolysis of isatin. The reaction is visualised from the initial nucleophilic attack of OH\(^-\) to the carbonyl of isatin until the final substrate isatinate is formed. The final step involves isatinate isomerization, promoted by the strong intra-molecular H-bond between its amino and \(\alpha\)-carbonyl groups, as well as the reformation of the hydrogen bond between Asp193 and His207. Free energy differences were estimated from metadynamics simulations.
Isatin hydrolase and kynurenine formamidase active-site conservation indicates a similar mechanism. The recently determined crystal structure of kynurenine formamidase from *Bacillus anthracis* (BaKynB) contains a binuclear Zn\(^{2+}\) site and thus is part of the EC 3.5 class that has a similar overall protein fold when compared with *LaIHA* (sequence identity 24%). Figure 2d shows the superposition of the metal binding sites for *LaIHA* isatinate and BaKynB (PDB ID: 4COG). The structural architecture of the metal binding sites is highly conserved between both enzymes (Fig. 2d). The most striking difference is the position of Gln219 in *LaIHA*, which is occupied by glutamate (Glu172) in BaKynB. This residue is conserved for all verified KynB enzymes. In BaKynB, the His60 (equivalent to His79 in enzymes. In *BaMovie M1*). In isatin hydrolysis presented here, both His79 and His207 are involved in the catalytic mechanism (Supplementary Movie M1). In BaKynB, the Zn\(^{2+}\) in position (I) Zn\(^{2+}\) is octahedrally coordinated and bound in an equivalent position as Mn\(^{2+}\) in *LaIHA*. In a homologous structure of KynB from *Burkholderia cepacia* (RsKynB), this position is occupied by Cd\(^{2+}\). The Zn\(^{2+}\) in position (II), Zn\(_{II}\)\(^{2+}\) is also found in an octahedral conformation.

Convergent evolution of the active-site geometry in *LaIHA* and *EcAAH*. Amidohydrolases with known manganese dependency where identified through literature searches and examined manually for structural similarity. This approach revealed two candidates that, upon close inspection, shared metal binding sites residues and residues identified as catalytically important. The allantoate amidohydrolases (AAHs) are part of the purine degradation pathway and hydrolyse the allantoate to ureidoglycolate, carbon dioxide, and ammonium in the process, thus stressing the importance of identifying the relevant metal\(^{58}\). The crystal structure of BaKynB from *Bacillus anthracis* (PDB ID: 4COG) presents a glutamate (Glu173, Fig. 2d) adopting an equivalent conformation as that of Gln219 in *LaIHA*. In conjunction with the conserved His161 and Asp56 (BaKynB numbering), it forms a secondary Zn\(^{2+}\) site (Zn\(_{II}\)) binding site in the presented binuclear metal binding (Fig. 2d). The remaining metal-coordinating residues are identical to both RsIHA and *LaIHA*. It cannot be completely ruled out that, in certain conditions, Gln219 would allow the formation of a secondary metal-binding site in *LaIHA*. This is however unlikely, due to the different Lewis-acid/base properties of an amide group, compared to those of a carboxylate. The structure of *LaIHA*, which features an alanine residue in the position of Gln219, does not have this option and thus is expected to be strictly mononuclear, with the highest hydrolytic activity measured in the presence of Mn\(^{2+}\), as also observed for *LaIHA* presented in this manuscript.

In the case of the amidohydrolase diaminopimelate desuccinylase (dapE), it was found that the compound L-captopril only binds to the Zn\(^{2+}\) bound form of the enzyme and not to the physiologically relevant Mn\(^{2+}\) bound form, thus stressing the importance of identifying the relevant metal\(^{58}\). Different metallo-\(\beta\)-lactamases can bind and be catalytically active in the presence of both one and two Zn\(^{2+}\) metals in the binding site. In both cases, the reaction mechanisms were characterized by computational modelling in the past (1 Zinc: B1 BcII from *Bacillus cereus* and B2 CphA from *Aeromonas hydrophilia*, 2 Zinc: B1 CcrA from *Bacteroides fragilis*)\(^{39–41}\). The mono and binuclear reaction scheme for Zn\(^{2+}\) binding beta-lactamases has recently been revised in Lisa MN et al.\(^{20}\). The mechanism of hydrolysis in *LaIHA* shares similarities with those found in single-Zn\(^{2+}\) enzymes. In particular, the reaction proceeds through a two-step mechanism, where the nucleophilic attack on the amide carbonyl of isatin is followed by the breaking of the C-N bond. On the contrary, in CcrA from *B. fragilis*, the reaction follows a concerted mechanism where the nucleophilic attack of the hydroxyl takes place simultaneously with the opening of the \(\beta\)-lactam ring.

Discussion

The Baltic sea bacteria *Labrenzia aggregata* genome includes two homologous open reading frames for isatin hydrolases A and B that share 51% sequence identity (SI). The more distant homolog from *Ralstonia solanacearum* (RsIHA) shares 59% SI with *LaIHA* and 51% SI with *LaIHB*. Unlike the whole sequence, the active site and substrate binding pocket are highly conserved among these proteins. Even though the key substrate binding pocket residues originally identified in the structure of *LaIHB* - Phe63, Trp65 and Trp84 and Phe209\(^{27}\) do not share equivalent positions in the sequences of *LaIHA* or *RsIHA*, their aromatic side chains do adopt an identical structural conformation in the respective binding pockets, thus indicating functional conservation (Fig. 1b). Compared to *LaIHB*, the Mn\(^{2+}\) binding sites in both *RsIHA* and *LaIHA* include Gln219, also responsible for increased metal specificity\(^{39}\). The crystal structure of BaKynB from *Bacillus anthracis* (PDB ID: 4COG) presents a glutamate (Glu173, Fig. 2d) adopting an equivalent conformation as that of Gln219 in *LaIHA*. In conjunction with the conserved His161 and Asp56 (BaKynB numbering), it forms a secondary Zn\(^{2+}\) site (Zn\(_{II}\)) binding site in the presented binuclear metal binding (Fig. 2d). The remaining metal-coordinating residues are identical to both *RsIHA* and *LaIHA*. It cannot be completely ruled out that, in certain conditions, Gln219 would allow the formation of a secondary metal-binding site in *LaIHA*. This is however unlikely, due to the different Lewis-acid/base properties of an amide group, compared to those of a carboxylate. The structure of *LaIHB*, which features an alanine residue in the position of Gln219, does not have this option and thus is expected to be strictly mononuclear, with the highest hydrolytic activity measured in the presence of Mn\(^{2+}\), as also observed for *LaIHA* presented in this manuscript.
As the calculations produced a slightly endothermic profile (+2 kcal), the excess energy released by deprotonation of the protein may also constitute the driving force for the enzymatic process. Though, we stress that due to high computational costs, the free energy profile was obtained with a resolution that is of the same order as the reaction free energy balance.

The sequence alignment and structural superposition confirm that not only the metal coordinating residues (Fig. 2a–c, dots), but also the residues involved in the proton transfer (Fig. 2a–c, asterisk) are highly conserved. Only the position corresponding to LaIHA residue Asp193 (Fig. 2a) alternates between aspartate and glutamate, thus showing anyway functional conservation. The conservation suggests functional importance and, supported by the structural superposition and mechanistic elucidation, points to a common catalytic role. This is particularly interesting in the light of the sequence variation within the HH-like and AAH-like folds as illustrated in Fig. 2b,c,e. Here, the phylegetic tree of proteins belonging to the respective folds indicates a large sequence variation, perhaps best exemplified by the significant divergence of EcAAH compared to Arabidopsis thaliana AAH (AtAAH), i.e., prokaryotic and eukaryotic. However, both the overall fold, displaying domain-swapped dimers, and the active site geometry are highly conserved (Fig. 2c–e).

It remains to be established whether the catalytic mechanism exemplified in LaIHA by our structural and in silico studies is specific to the HH-like enzymes, or could represent a broader case of convergent evolution across a range of AHS metallo-hydrolases. In the case of the AAH homologues, the Mn2+ binding site is structurally completely conserved, including the binding residues in the Mn site (His69, His73 and Asp75 for LaIHA), and the proton transfers triad (His79, His203 and Asp193) in LaIHA compared to Glu127, His382 and His226 in EcAAH, with the peculiar difference that in EcAAH the Mn2+ occupies site I (See comparison in Fig. 2c,e). The HH, KYNb and AAH families are not the only ones to have divergent metal site constellation, despite similar metal binding residues, the ureidoglycolate amidohydrolase from Arabidopsis thaliana IHA (A001005617) has a binuclear Mn2+ site66,67 (PDB ID 4PXB), while the putative hydantoinases (PDB ID 5I4M, 5THW and 4WJB) and β-alanine synthase from Saccharomyces kluyveri R243 (PDB ID 2VL1) have binuclear Zn2+ sites, the L-N-carbamoylase from Geobacillus stearothermophilus has a mononuclear Co2+ site44 (PDB ID 3N5F). The model for hydrolysis presented here would only allow a Mn2+ in site I, and a Zn2+ would act as an inhibitor. In particular, according to previous simulations, Zn2+ is not able to bind both isatin and the water, disrupting the preorganization of the active site and preventing the initial nucleophilic attack21. Thus, it remains an open question whether the Mn2+ in site II also act as an inhibitor or merely adopt an alternative position in the structure of EcAAH due to the crystal conditions. It is a reoccurring problem for metal dependent hydrolases that the majority of the information is collected from in vitro experiments and that conflicting conclusions are reached when it comes to metal specificity. As in the case of KYNb were a binuclear Zn2+ is described in the structure39 and Zn2+ dependent activity is presented, however it still remains an open question whether this is the physiological metal, as in other systems such as Ralstonia metallidurans a 15 to 20-fold activation of kynurenine formamidase was detected after treatment with Mn2+68.

The major difference between the Michaelis complexes in LaIHA/B and zinc-β-lactamases is constituted by the binding mode of the substrate. β-lactam substrates, bind in the proximity of the metal(s), but their amide moiety do not participate directly to their coordination sphere(s) as is the case for the reacting cyclic amide in the IH. It is carboxylate groups of the carpapenem family, that later during the catalysis interact directly with the Zn2+ moiety do not participate directly to their coordination sphere(s) as is the case for the reacting cyclic amide in the IH.

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Author Contributions
T.S. and K.B.A. performed most of the wet lab experiments, analysed the data and co-wrote the manuscript with J.P.M. and M.C. L.U. performed the molecular simulations studies supervised by M.C., J.G. and G.D. M.E. designed constructs and performed the initial expression and purification studies, J.P.M. supervised the optimization of protein expression, crystallization and data collection. All analysed the data and edited the manuscript. J.P.M., M.C., G.D. and J.G. obtained the funding.

Additional Information
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