In silico design of potentially functional artificial metallo-haloalkane dehalogenase containing catalytic zinc

Thiau-Fu Ang^{1,3} · Abu Bakar Salleh^{2,3,4} · Yahaya M. Normi^{1,3} · Thean Chor Leow^{1,3,4}

Received: 28 February 2018 / Accepted: 2 July 2018 / Published online: 12 July 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Artificial metalloenzymes are unique as they combine the good features of homogeneous and enzymatic catalysts, and they can potentially improve some difficult catalytic assays. This study reports a method that can be used to create an artificial metal-binding site prior to proving it to be functional in a wet lab. Haloalkane dehalogenase was grafted into a metal-binding site to form an artificial metallo-haloalkane dehalogenase and was studied for its potential functionalities in silico. Computational protocols regarding dynamic metal docking were studied using native metalloenzymes and functional artificial metalloenzymes. Using YASARA Structure, a simulation box covering template structure was created to be filled with water molecules followed by one mutated water molecule closest to the metal-binding site to metal ion. A simple energy minimization step was subsequently run using an AMBER force field to allow the metal ion to interact with the metal-binding residues. Long molecular dynamic simulation using YASARA Structure was performed to analyze the stability of the metal-binding site and the distance between metal-binding residues. Metal ions fluctuating around 2.0 Å across a 20 ns simulation indicated a stable metal-binding site. Metal-binding energies were predicted using FoldX, with a native metalloenzyme (carbonic anhydrase) scoring 18.0 kcal/mol and the best mutant model (C1a) scoring 16.4 kcal/mol. Analysis of the metal-binding site geometry was performed using CheckMyMetal, and all scores for the metalloenzymes and mutant models were in an acceptable range. Like native metalloenzymes, the metal-binding site of C1a was supported by residues in the second coordination shell to maintain a more coordinated metal-binding site. Short-chain multihalogenated alkanes (1,2-dibromoethane and 1,2,3-trichloropropane) were able to dock in the active site of C1a. The halides of the substrate were in contact with both the metal and halide-stabilizing residues, thus indicating a better stabilization of the substrate. The simple catalytic mechanism proposed is that the metal ion interacted with halogen and polarized the carbon–halogen bond, thus making the alpha carbon susceptible to attack by nucleophilic hydroxide. The interaction between halogen in the metal ion and halide-stabilizing residues may help to improve the stabilization of the substrate–enzyme complex and reduce the activation energy. This study reports a modified dynamic metal-docking protocol and validation tests to verify the metal-binding site. These approaches can be applied to design different kinds of artificial metalloenzymes or metal-binding sites.

Keywords Artificial metallo-haloalkane dehalogenase · Artificial metalloenzyme · Dynamic metal docking

Abu Bakar Salleh and Yahaya M. Normi have contributed equally to this work.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13205-018-1333-9>) contains supplementary material, which is available to authorized users.

✉ Thean Chor Leow
adamleow@upm.edu.my

Extended author information available on the last page of the article

Introduction

Halogenated compounds are widely used in industry and agriculture, and as components (i.e., solvents) in daily household items (Chaudhry and Chapalamadugu 1991; Belkin 1992; Bidmanova et al. 2010). Organic compounds are resistant to degradation, and the accumulation of these compounds can lead to serious environmental and health issues (ATSDR 1989, 2001; Belkin 1992; U.S. EPA 2004, 2009). Some halogenated organic compounds are degraded through naturally available or laterally evolved dehalogenases. Some of the most studied dehalogenases are

haloalkane dehalogenases (HLDs), which are categorized under the hydrolytic dehalogenases group. HLDs belong to a family of α/β -hydrolase fold and perform catalysis using an S_N2 (nucleophilic substitution in which the rate-determining step involves two components) mechanism that requires only water as a cofactor. This catalytic mechanism involves the catalytic triad of Asp–His–Asp/Glu. The carboxylate oxygen of aspartate initially launches a nucleophilic attack on the partially positive carbon atom of the halogen-bound substrate to produce a halide ion and alkyl-enzyme intermediate with an ester bond. The nearby His–Asp/Glu (acid–base pair) subsequently hydrolyzes a water molecule to produce a nucleophilic hydroxide that will attack the carbon of the ester bond. This generates a tetrahedral intermediate that immediately decomposes to form RCH_2O^- and grabs a proton from the nucleophile to form RCH_2OH (Franken et al. 1991; Verschuere et al. 1993; Prokop et al. 2003; Janssen 2004; Gehret et al. 2012; Koudelakova et al. 2013b). HLDs possess halide-binding residues, also known as halide-stabilizing residues, which is their unique feature (Chovancová et al. 2007). These residues are critical for the catalytic activity of HLDs as they help to stabilize the halide during formation of the enzyme–substrate complex. They are also important for leaving-group stabilization (Kennes et al. 1995; Krooshof et al. 1998). HLDs generally have broad and robust substrate specificities that cover chlorinated, brominated, and some iodinated substrates. They also have enzyme-dependent preferences for substrate length (Janssen 2004; Damborský et al. 2010; Koudelakova et al. 2013a). Koudelakova et al. (2013a) reported that general HLDs are effective when catalytic activity is in the 10^4 – 10^5 $M^{-1} s^{-1}$ range; however, for anthropogenic multihalogenated compounds, such as the possibly carcinogenic 1,2,3-trichloropropane (U.S. EPA 2009), the catalytic activity is very low ($40 M^{-1} s^{-1}$) (Koudelakova et al. 2013a). In addition, HLDs cannot degrade compounds with multiple halogens on a single carbon or generally fluorinated compounds (Kamachi et al. 2009; Koudelakova et al. 2013a). Interestingly, the only degradable fluoroalkane is fluoroacetate, which is degraded by fluoroacetate dehalogenase (Liu et al. 1998; Janssen 2004; Koudelakova et al. 2013a). The reason that fluoroacetate dehalogenase can degrade fluoroacetate (CH_2FCOO^-) is likely due to the presence of three halide-stabilizing residues that interact with fluoride and two positively charged arginine residues that are hydrogen bonded with the negatively charged carboxyl group. This contributes to binding the substrate and stabilizing the ester intermediate, thus reducing the activation energy for breaking carbon–fluoride (C–F) bonds (Kamachi et al. 2009).

A metallodehalogenase, atrazine chlorohydrolase (AtzA), was recently crystallized and its structure elucidated (Peat et al. 2015). The proposed catalytic mechanism involved metal in the stabilizing atrazine and water bound to the metal

being deprotonated to form nucleophilic hydroxide, thus leading to nucleophilic attack and the release of a chloride ion (Peat et al. 2015). Until 2002, no dehalogenases were known to be metalloenzymes; however, Seffernick et al. proved that AtzA was a metalloenzyme that could remove chloride from atrazine, a widely used herbicide of the last century (Seffernick et al. 2002). Instead of using halide-stabilizing residues to stabilize the halide, AtzA uses a promoter–water pathway to degrade atrazine. The metal helps to stabilize the halide and also promotes the formation of hydroxide as a nucleophile instead of aspartate or glutamate in nonmetallo-HLDs.

The recent successful design of artificial metalloenzymes using a computational approach as the first step has shown that these studies are able to predict if a protein will function in a wet lab experiment (Yeung et al. 2009; Zastrow et al. 2012; Khare et al. 2012; Miner et al. 2012). Yeung et al. (2009) turned whale myoglobin into a functional nitric oxide reductase by modifying the enzyme pocket with several point mutations to allow binding of the iron ion (Yeung et al. 2009). They first tested out the capability of metal ion binding in the designed binding site via molecular dynamic (MD) simulation. This study proposes that grafting a metal-binding site into the active site of HLD (with a slight modification of the active site) may create a functional metallo-HLD. This paper presents computational approaches to designing metallo-HLDs that are not found in nature.

Results and discussion

Creating artificial metallo-HLDs in silico

General HLDs only use halide-stabilizing residues to stabilize halogen in the haloalkane and they cannot break down haloalkanes containing multiple halogens (more than two halogens on different carbons or multiple halogens on the same carbon) and C–F bonds. Until now, only fluoroacetate was known to be defluorinated by fluoroacetate dehydrogenase (Liu et al. 1998). This occurred due to stabilization of the halide and carboxyl group and strongly polarized C–F bonds, thus greatly reducing the activation energy for defluorination (Kamachi et al. 2009). This suggests that stabilization of the electrophile (carboxyl group) and leaving group (fluoride ion) is crucial for the catalysis to work. Among all dehalogenase enzymes, only two are metalloenzymes: AtzA and TrzN, which is an atrazine chlorohydrolase (Seffernick et al. 2002, 2010; Peat et al. 2015). These enzymes require the metal to be catalytically functional. For AtzA, a metal is involved in stabilizing the halogen atom and no halide-stabilizing residues are involved (Peat et al. 2015). This project creates an artificial metallo-HLD in silico that may degrade short-chain multihalogenated haloalkanes

using both metal and halide-stabilizing residues to stabilize multiple halogens and polarize the carbon–halogen bond to reduce the activation energy (Peat et al. 2015).

Template selection

This study first assessed all available HLDs, and those with elucidated structures are shown in Table 1. DhaA, an HLD from *Rhodococcus rhodochrous* UniProt No. P0A3G2; (Kulakova et al. 1997), was selected as the template. This enzyme was crystallized and its structure solved at high resolution 1.26 Å; PDB ID: 4E46; (Stepankova et al. 2013). Using a high-resolution structure as a template will create more structurally valid mutants in silico and thus increase the reliability of the computer simulation result. DhaA contains a moderate active site (255 Å³) (Koudelakova et al. 2013a) that can be modified to include a metal-binding site. DhaA has advantages over other enzymes due to its wider pH range and its ability to be overexpressed, and remains soluble up to 120 mg/L using the commercial expression host, *Escherichia coli*.

The structure of DhaA contains a conserved α/β hydro-lase fold superfamily with a lid domain formed by six helices (Newman et al. 1999). The active site cavity is formed between the core and the lid domain, and this limits the entry of solvent molecules (Stsiapanava et al. 2010). The active site consists of Asp106 (nucleophile), His-272/Glu-130 (acid–base pair), and Asn-41/Trp-107 (halide-binding residues), and is surrounded by hydrophobic residues that help it to interact with the hydrophobic parts of substrates (Newman et al. 1999). Most artificial metalloenzymes have their metal-binding site built in the active site pocket or vacant space in the protein with water freely accessible into that site (Sigman et al. 2000; Ueno et al. 2007; Johnson 2014); however, the metal-binding site in DhaA was designed in an active site where solvent entry was more restricted. Zinc binding most likely occurs during protein folding; therefore, the possibility that zinc needs to diffuse

via the narrow tunnel into the metal-binding site is omitted. In addition, if the mutant is expressed insoluble, the protein can be extracted from inclusion bodies using mild solubilizing agents before being refolded together with the metal ion to ensure zinc binding in the active site (Yeung et al. 2009).

Design and creation of metal-binding sites

Residues in the active site pocket were first studied using HotSpot Wizard (Bendl et al. 2016), and the data are compounded in Supplementary Info S1. Highly conserved residues were avoided; however, in most cases, nucleophilic aspartic acid (Asp-106) was mutated to alanine to disable the enzymatic activity catalyzed by Asp-106. Tunnel residues were low priority as these residues were too far away from the center of the active site. Unlike studies that used a program to identify these residues (Hellinga and Richards 1991; Hellinga et al. 1991; Clarke and Yuan 1995), probable metal-binding residues were mainly selected via observation. Residues with side chains pointing towards each other and located close to each other were selected. A total of 24 models were designed (Table 2) and mutated in silico using FoldX. However, after multiple in silico point mutations, some of the side chains pointed away from their original direction to follow the most favorable conformation. The stability of the mutants was calculated, and changes in free energy ($\Delta\Delta G$) for all mutants were > 1.84 kcal/mol, which is highly destabilizing. However, the same result was obtained for both functional mutated whale myoglobin and nonmutated whale myoglobin. Therefore, mutant stability is not overly relevant as the mutants will most likely be expressed in the inclusion bodies and refolding will be required (Yeung et al. 2009).

Dynamic metal docking

The dynamic metal-docking setup was primarily based on the method used by Yeung et al. (2009). Zinc was chosen

Table 1 Biochemical and structural properties of HLDs

	Haloalkane dehalogenases (HLDs)						
	DbjA	DhaA	DhlA	DmbA	DmmA	DppA	LinB
pH profile	9.7	8.0–9.5	8.2	9	ND	8.0–9.0	8.2, 8.8
Expression in <i>E. coli</i> (mg/L)	50	120	70	10	150	ND	50
Volume of active site (Å ³)	237	255	132	239	358	351	295
PDB ID	3AFI	4E46	2YXP	2QVB	3U1T	2XT0	1MJ5
Resolution (Å)	1.75	1.26	1.53	1.19	2.20	1.90	0.95

These are HLDs with published crystallized structures. The information are obtained from Koudelakova et al. (2013a) and Protein Data Bank (PDB). pH profile refers to range of pH for enzyme to have activity more than 90%. The bold numbers indicate preferable and acceptable value to be qualified as good template ND not determined

Table 2 Metal-binding site models

Metal-binding site	Models	Control mutation	Metal-binding residues	Proton acceptor	Dynamic metal docking
Control 1	1CA2	NA	H94, H96, H119	NA	Success
Control 2	1JP6	NA	L29H, F43H, H64, V68H	NA	Success
A	A1	D106A	L246H, I132H, L209E	H272H	Success
	A2	D106A	L246H, I132H, L209D	H272H	Success
	A3	D106A	L246H, I132D, L209H	H272H	Fail
	A4	D106A	L246H, I132H, L209H	H272H	Fail
	A5	D106A	L246H, I132H, L209H	H272H	Success
	A6	D106A	L246D, I132H, L209H	H272H	Fail
	A7	D106A	L246E, I132H, L209H	H272H	Fail
B	B1	D106A	L246H, W141H, L209H	H272H	Fail
	B2	D106A	L246H, W141H, L209E	H272H	Fail
	B3	D106A	L246H, W141E, L209H	H272H	Fail
	B4	D106A	L246H, W141Y, L209H	H272H	Fail
C	C1a	NA	D106D, L246H, H272H	L209D	Success
	C1b	NA	D106D, L246H, H272H	L209E	Fail
D	D1	D106A	H272H, L246H, V245H	NA	Fail
	D2	D106A	H272E, L246H, V245H	NA	Fail
	D4	D106A	H272E, L246C, V245C	NA	Fail
E	E1	NA	D106D, L246H, I132H	NA	Fail
	E2	NA	D106H, L246D, I132H	NA	Fail
	E3	NA	D106H, L246H, I132D	NA	Fail
	E4	NA	D106D, L246C, I132S	NA	Fail
	E5	NA	D106D, L246C, I132C	NA	Fail
F	F1	NA	D106D, L246H, L209H	NA	Fail
	F2	NA	D106H, L246H, L209E	NA	Fail
	F3	NA	D106H, L246E, L209H	NA	Fail

The controls are referring to positive control where the structures are used to validate the dynamic metal-docking protocol

NA not available

as the catalytic metal as it is the most commonly involved metal in enzymatic hydrolysis (Andreini et al. 2008). For catalytic zinc metalloenzymes, the most common zinc-binding residues are histidine, glutamate, aspartate, and cysteine, with histidine being the most commonly chosen (McCall et al. 2000). Instead of mutating crystallized water near the metal-binding site to a metal ion, water neutralization simulation was performed. Here, waters were put into the simulation box and, after the energy minimization step, the water closest to the metal-binding site was mutated to a metal ion. Two different methods of dynamically docking the metal on carbonic anhydrase were attempted. In the first attempt, immediately after swapping in the zinc ion, the energy minimization was run to let the zinc bond with the metal-binding residues (Supplementary Info S2a). The final structure closely resembled the original carbonic anhydrase but contained zinc; however, when the experiment was repeated to convert whale myoglobin to artificial nitric oxide reductase, His-43 did not bind with the zinc ion in four of the

expected metal-binding residues but instead made contact with water molecules. Thus, a second method of dynamic docking was performed to remove all water molecules and counter ions before the energy minimization step, and the four expected metal-binding residues made contact with the zinc ion. The lack of water molecules made the protein structure to move more flexibly, and no water molecules were standing in between the metal-binding residues and the metal ion. When this second method was repeated on carbonic anhydrase, instead of three metal-binding residues interacting with the zinc ion, the proton acceptor, Glu-106, became the fourth metal-binding residue due to the absence of water (Supplementary Info S2b). Despite this, the second method was used on all models to obtain more potentially successful metal-docking models. The metal-binding pattern for the artificial nitric oxide reductase was also found to be different if there was no haem group bound to the pocket (Supplementary Info S3). The metal-binding pattern was closer to the actual pattern if haem was in the pocket

when dynamic metal docking was performed. This suggests that metal binding is affected by the presence of cofactors or compounds (other than water molecules) located close to the metal-binding site.

In the end, only a few models and both controls had metal successfully docked to the metal-binding site (Table 2; Fig. 1). Most models did not have metal docked successfully due to some of the residue's side chains facing away

from the center of the metal-binding site after mutation. In a few models, metal-binding residues were attracted to the metal ion at the beginning of the metal-binding simulation; however, by the end, one or two residues had slowly moved back to their original position. It is crucial to achieve a good distance between electronegative atoms of metal-binding residues to obtain the preferred metal-binding conformation. Those with long side chains have some advantages as

Fig. 1 Metal-binding site after dynamic metal docking of controls and metal-docked mutant models. Metal-binding site of controls, carbonic anhydrase (**a**) and mutated whale myoglobin (**b**): model A2 (**a**), A5 (**b**), and C1a (**c**). The metal-binding residues are shown in stick-shaped. Original structure (green) and structure after mutation followed by dynamic metal docking (yellow). Zinc ion in grey sphere. The distance between two atoms denoted as yellow-dotted line, and labelled in Å. The standard color for the atom, oxygen (red), and nitrogen (blue)

the side chain can be extended to get closer to the metal ion; however, short side-chain residues (i.e., cysteine) are at a disadvantage due to restricted movement unless all metal-binding residues are close to each other. Two limitations of dynamic metal docking are: if the expected metal-binding residues are not facing toward each other and if the water molecule is not located at the center of the probable metal-binding site. If this occurs, dynamic metal docking will most likely fail.

Computational validation of the metal-binding site

Metal-binding site stability

MD simulation is a useful tool to check the metal-binding capability (Auffinger et al. 2003) and it can also be used to test the metal-binding stability (Fig. 2). The standard distance between Zn^{2+} and an electronegative atom is 1.5–3.0 Å (Barkigia et al. 1990; Koike et al. 1996). For three mutant models (A2, A5, and C1a) and the two controls (1CA2 and m1JP6), the bond distance between the metal ion and the corresponding atom of the metal-binding residue fluctuated around 2 Å. This can be interpreted as a stable metal-binding site, where binding of the metal to the metal-binding residue was favorable and no dissociation occurred. However, for mutant model A1, the bond distance between the metal and His-132 fluctuated vigorously throughout the simulation, and the NE2 atom of His-132 was pulled away from the metal ion after approximately 13 ns. This indicates that the metal-binding site of mutant A1 was not stable and deformed over time. Some metal-binding sites had four metal-binding residues instead of three. Positively charged cations attract aspartate or glutamate better than histidine. In some cases, the metal was positioned in the center between the three metal-binding residues; however, after dynamic metal docking, the nearby aspartate or glutamate moved in and bound with the metal. This sometimes resulted in dissociation of the original metal-binding site. As seen in the dynamic metal docking in carbonic anhydrase, the absence of water allows the zinc ion to bind with four residues due to the structural flexibility. In another study, several models showed that the acid (Asp or Glu) can cause both of the OE atoms (instead of only one) to bind to the metal, as seen in one model of zinc hydrolase, carboxyl peptidase A (Wu et al. 2010).

Metal-binding site geometry

The typical geometry of a catalytic Zn^{2+} -binding site (i.e., the one in carbonic anhydrase) is a tetrahedral shape, as metal ions bond with three metal-binding residues and one water molecule (McCall et al. 2000). However, when MD simulation was performed on carbonic anhydrase and metal-redocked carbonic anhydrase, extra water molecules

bound the metal and, thus, an octahedral-shaped geometry was formed when assessed using CheckMyMetal (Zheng et al. 2014). Tetrahedral zinc complexes generally show up as structural models as they provide more stability and the least restrained conformations (Dudev and Lim 2000). Meanwhile, octahedral zinc complexes provide more flexible conformations; however, this may also be due to the MD simulation setup that allows more ligands to bind to the metal ion instead of restricting intermolecular interactions.

All models showed octahedral-shaped Zn^{2+} -binding sites with acceptable generalized root-mean-squared deviation (gRMSD) and zero vacancy (Table 3). gRMSD indicates whether the observed geometry angle is the same as the ideal geometry angle. gRMSD is most likely in the acceptable range as the MD simulation will eliminate clashes between atoms and allow the atoms to interact in the favorable position. The vacancy gives the percentage of the unoccupied site in the coordinate sphere for the given geometry (Zheng et al. 2014). The backbone dihedral angle of all structures were analyzed, and it was concluded that the metal-binding residues remained in a favorable position after dynamic metal docking (Lovell et al. 2003) (Supplementary Info S4).

Metal-binding prediction and energy analysis

Schymkowitz used FoldX to predict the water- and metal-binding sites of known metalloenzymes with an accuracy of 90–97% (Schymkowitz et al. 2005). Metal-binding energy is the calculation of free energy when a metal binds to a protein, taking into account the desolvation of the ion, electrostatic interaction, and the charge on the metal. The software first places a metal in the expected metal-binding site that is predicted to bond with metal-binding residues (i.e., water) to form hydrogen bonds with protein residues. The metal-binding energy is subsequently calculated and can be differentiated as a low- or high-binding affinity. The metal-binding prediction results for the native metalloenzymes and the three successfully metal-docked mutants showed positive metal binding in the expected metal-binding site with high-binding energy, as seen in the literature (Schymkowitz et al. 2005).

FoldX was used to perform metal-binding analysis on protein structures after metal docking and MD simulation (snapshots every 1 ns). Not every protein structure in the snapshot tested positive for metal binding (Fig. 3). The metal was put into the metal-binding site, so precisely that any slight distortion could cause unsuccessful docking. All residues moved around throughout the simulation, and the distance between the metal and metal-binding residues could sometimes move too far or too close. This may be the reason why the metal could not be placed properly into the metal-binding site. Of the positive controls, m1JP6 seemed to have a less distorted metal-binding site than 1CA2. This may be

Fig. 2 Metal-binding stability analysis. **a** Zinc-redocked carbonic anhydrase, **b** mutated whale myoglobin (artificial nitric oxide reductase), **c** A1 mutant model, **d** A2 mutant model, **e** A5 mutant model, and **f** C1a mutant model. The distance between metal and metal-binding residue is referred as the distance between metal ion and the closest electronegative atom of metal-binding residues

Table 3 Metal-binding site geometry analysis

Model	Geometry	gRMSD	Vacancy
A2	Octahedral	Acceptable	0
A5	Octahedral	Acceptable	0
C1a	Octahedral	Acceptable	0
Control-m1JP6	Octahedral	Acceptable	0
Control-1CA2	Octahedral	Acceptable	0

Metal-binding site geometry was analyzed using online server, programme CheckMyMetal (CMM)

Geometry, arrangement of ligands around the ion, as defined by the NEIGHBORHOOD algorithm

gRMSD, R.M.S. deviation of observed geometry angles (L–M–L angles) compared to ideal geometry, in degrees

Vacancy, percentage of unoccupied sites in the coordination sphere for the given geometry

Fig. 3 Metal-binding energy analysis using FoldX. The command MetalBinding from the software helped to predict the possible binding of metal in the protein structure. The structure used are two positive control which are native metalloenzyme, carbonic anhydrase (PDB ID:1CA2) and artificial metalloenzyme, m1jp6 (original template PDB ID:1JP6), and three mutant models of artificial metallo-haloalkane dehalogenase. The protein structures used are from different snapshots during MD simulation of 1 ns interval

due to the presence of haem close to the metal-binding site, which helped to prevent strong fluctuations in the metal-binding site. For the mutant models, C1a had an overall better metal-binding site with a higher predicted metal-binding energy.

Structural analysis of the mutants

The second coordination shell of metal-binding sites supports the formation of precise and stable metal-binding sites (Lovell et al. 2003; Dudev and Lim 2008). These shells are referred to as residues that form hydrogen bonds with metal-binding residues (known as first shells) to provide a better coordination for the metal-binding residues interacting with the metal ion. Aspartate/glutamate in the second coordination shell grabs a proton from histidine in the first shell, so

that the histidine will be more flexible when bound with the metal ion (Dudev and Lim 2008). In the original structure of carbonic anhydrase retrieved from the PDB database, all three metal-binding residues were coordinated with corresponding residues in the second shell. The same result was shown for the zinc-redocked carbonic anhydrase, with the exception of an extra metal-binding residue (Fig. 4a, b). The metal-binding site in the mutated whale myoglobin only had one second-shell residue supporting the metal-binding site, while the same structure with a cofactor in the binding site produced a better metal-binding site with all three histidines stabilized by second-shell residues (Fig. 4c, d). This experiment indicated that the presence of a secondary shell is necessary for the formation of a successful, stable, and better coordinated metal-binding site. The C1a mutant model received one second-shell residue, but His-272 was supported with its own backbone (Fig. 4e). This suggests that this model has a good and stable metal-binding site coordinated with residues in the second shell.

The root-mean-square deviation (RMSD) and root-mean-square fluctuation (RMSF) of the C1a mutant were analyzed and compared with the native template (Supplementary Info S5). The RMSD of the C α backbone of the template and mutant was plotted. The graph showed that the RMSD increased and fluctuated smoothly around 1.5 Å, thus indicating that the overall structures of the native template and mutant were not destabilized with the simulation. The RMSF results were similar for both protein structures, and the metal-binding residue did not fluctuate after 20 ns with MD simulation. Hence, the C1a mutant maintained its structure throughout the simulation and the mutated residues did not cause any fluctuation in protein structure.

Substrate docking

1,2,3-Trichloropropane and 1,2-dibromoethane were chosen as they are common pollutants. More specifically, 1,2,3-trichloropropane is an anthropogenic substrate that is difficult to degrade using common HLDs. The docking result for the C1a mutant was selected for this paper as its result was better than that of the other two mutants. In Fig. 5a, b, both the wild-type and C1a mutant bound 1,2,3-trichloropropane; however, the binding patterns were different. For example, one chloride atom was stabilized by two halide-stabilizing residues in the wild type, but in C1a, the first and the third chloride atoms of 1,2,3-trichloropropane interacted with the metal ion that was a distance of 3.0 and 2.8 Å away, respectively. Conversely, for 1,2-dibromoethane (Fig. 5c, d), one bromide atom was stabilized by two halide-stabilizing residues for both receptors. In addition, C1a had a metal ion to stabilize the second bromide atom of 1,2-dibromoethane. Like the one shown by fluoroacetate dehalogenase, stabilization of both ends of a substrate will greatly help to

reduce the activation energy (Goldman 1965; Liu et al. 1998; Kamachi et al. 2009). The metal ion is strongly charged and can polarize the carbon–halogen bond to make the alpha carbon more electropositive (Valdez et al. 2014). Both of these dockings show that a metal ion in the C1a mutant can help to stabilize the substrate by bonding with the halogen atom. The water molecule bonded to the metal ion seems to be readily deprotonated to form a nucleophile (hydroxide) by aspartate in the second shell of the metal-binding site (Fig. 5b, d).

Tunnel analysis

For proteins with buried active sites, tunnels are the pathways that allow substrates or cofactors to enter and leave the pocket (Petrek et al. 2006; Chovancová et al. 2012). Native HLD was used as a control to assess whether tunnels allowed the substrate to diffuse through after the grafting of metal-binding sites. Figure 6 shows that multiple tunnels were created and that these could be grouped into two major clusters. Tunnels created in both native HLD and mutant C1a showed that substrates could diffuse into the active site with a tunnel bottleneck of 0.9 Å, which is the standard bottleneck size for tunnel formation in HLDs (Chovancová et al. 2012). This suggests that the metal-binding site does not block the substrate from entering and leaving. The tunnels formed in the cluster group of the C1a mutant appeared to be less spread out and more concentrated. The metal-binding residues were located in the lid and core domain; hence, when the metal ion docked, the overall structure and tunnels became more rigid.

Proposed mechanism

The substrate docking result was used to propose a probable catalytic mechanism for the artificial metallo-HLD. This artificial metalloenzyme might only work best with short multihalogenated haloalkanes. Two probable catalytic mechanisms were proposed. The first mechanism was based on the docking of 1,2-dibromoethane to the active site of the C1a mutant. In this mechanism, terminal halogen is stabilized by halogen-stabilizing residues and Zn^{2+} . The water coordinated with the metal ion is deprotonated by the proton acceptor, Asp-209. Activated hydroxide will subsequently attack the alpha carbon of 1,2-dibromoethane. The hydroxide is located 2.8 Å from C₁ and 3.2 Å from C₂; therefore, it is possible that the nucleophilic hydroxide can attack either carbon atom. If it attacks C₁, the halide-stabilizing residues will stabilize the bromide ion (Fig. 7a); however, Zn^{2+} will stabilize the bromide ion if C₂ is under attack (Fig. 7b).

The second mechanism is independent of halide-stabilizing residues. Figure 7c shows that the two chloride atoms bound to C₁ and C₂ interact with the metal ion and that no

Fig. 4 Second coordination sphere of metal-binding site. The metal-binding site from structure: **a** original carbonic anhydrase, **b** zinc-redocked carbonic anhydrase, **c** mutated whale myoglobin without haem, **d** mutated whale myoglobin with haem, and **e** C1a mutant model. The metal-binding residues (first shell) are shown in stick and residues in the second shell are shown in line. Zinc ion in grey sphere. The hydrogen bond denoted as black-dotted line. The haem (HEM) color in orange. The standard color for the atom, oxygen (red), and nitrogen (blue)

halogen is in contact with the halide-stabilizing residues. The zinc ion is bidentately coordinated with the two chloride atoms of 1,2,3-trichloropropane. Both mechanisms require water to be deprotonated by Asp-209, which is a known proton acceptor. Coordination of the zinc ion with water will greatly polarize the O–H bond and speed up the deprotonation of water. Positively charged metal cannot only help to stabilize the electronegative part of the leaving group and intermediate, but it can also polarize the carbon–halogen bond and make the alpha carbon more electropositive and vulnerable to nucleophilic substitution. Unlike native HLDs that use only halide-stabilizing residues, the addition of metal ions to mutant models can stabilize one more halogen atom. This will greatly stabilize the substrate–enzyme conformation and hence lower the activation energy. Enzyme–substrate intermediates are formed for native HLDs, and activated hydroxide is used to hydrolyze the alkyl–enzyme intermediate. For the C1a mutant, activated hydroxide will directly attack the alpha carbon and replace the halogen atom. Instead of a two-step nucleophilic substitution, only a one-step nucleophilic substitution is needed for C1a.

Artificial metalloenzymes have been created via various trial-and-error methods. Computational design will improve over time, hence reducing the cost and time for the successful design of artificial proteins. Since the work presented here are purely in silico, the mutant will be created in the laboratory to test its metal-binding capability and catalytic activity.

Materials and methods

Creation of metal-binding sites

Protein structures

All protein structures were obtained from the Protein Data Bank (PDB) database. The PDB IDs for the protein structures used were carbonic anhydrase = 1CA2, whale myoglobin = 1JP6, and DhaA = 4E46. All protein structures were initially repaired by modifying residues with bad torsion angles or Van der Waals's clashes to a position with minimum energy using the RepairPDB command

(Supplementary Info S6) in FoldX (Van Durme et al. 2011). The molecular viewing software, PyMOL (Makarewicz and Kaźmierkiewicz 2013), was used to view and generate high-quality 3D protein structures.

Template analysis

The online server, HotSpot Wizards 2.0 (Bendl et al. 2016) (<http://loschmidt.chemi.muni.cz/hotspotwizard/>), was used to identify mutation hot spots. The 3D protein structure was viewed and studied using YASARA Structure (YASARA Biosciences, Austria; Krieger et al. 2002 and PyMOL Schrödinger, USA; Makarewicz and Kaźmierkiewicz 2013) software.

Residue selection for the creation of metal-binding sites

The active site was carefully studied to choose the residues to be mutated to generate metal-binding residues. The active site pocket was analyzed using HotSpot Wizard 2.0 software created by Pavelka et al. (2009). Selecting suitable residues to form the metal-binding site was based on rational explanations as well as the characteristics of metal-binding sites in other native metalloenzymes. Several metal-binding sites were designed and grouped together based on the residues selected; for example, mutant model group A was formed by mutating residues L246, I132, and L209. Different amino acid combinations were used when forming these metal-binding sites.

Metal-binding sites were created with different combinations of metal-binding residues. The choice of metal-binding site fulfilled three criteria: (1) the three residues were located within 15 Å of each other; (2) the center of the metal-binding site was around 10 Å from the center of the halide-binding residues; (3) the side chains of the residues pointed roughly towards the center of the metal-binding site.

Metal-binding sites A–F represent the three residues in the template to be mutated to form the metal-binding site. The models in each metal-binding site (A–F) are formed of different combinations of residues.

In silico mutations

The template structure was first repaired in silico using the protein design software, FoldX. The command, BuildModel in FoldX, was used to create different variations of metal-binding sites (Supplementary Info S6).

Fig. 5 Docking of substrate into wild-type DhaA and C1a mutant model. Docking of 1,2,3-trichloropropane (123TCP) into active site of native HLD (**a**) and C1a mutant model (**b**); docking of 1,2-dibromoethane (12DBE) into active site of native HLD (**c**); C1a mutant

model (**d**). Zinc ion in grey sphere. The distance between two atoms was denoted as yellow-dotted line and labelled in Å. The standard color for the atom, oxygen (red), nitrogen (blue), bromine (brown), and chlorine (green)

Dynamic metal docking

Simulation box setup

The repaired structures or models were put into a simulation cell box that created 10 Å around all atoms. The default setup of the cell neutralization experiment in YASARA Structure was subsequently used to fill the cell with water

and counterions (i.e., Na^+ and Cl^-). Force Field, AMBER03, was applied to every atom, with a cutoff of 7.86 Å for non-bonded interactions. pK_a values were predicted for every residue, and protonation states were assigned according to a pH of 9.4 (the optimum pH of DhaA).

The simulation box was then filled with water molecules, and some of the water molecules were randomly replaced with counterions (i.e., Na^+ and Cl^-) until the

Fig. 6 Tunnel formation created using Caver 3.0. **a** Native haloalkane dehalogenase, PDB ID:4E46, and **b** C1a mutant model. The blue lines refer to tunnel cluster 1 and the green line refers to tunnel cluster 2

cell was neutral and reached 0.9% of NaCl concentration. Simulation continued with the deletion of water molecules until the water density in the box reached 0.9953 g/L. Energy minimization was subsequently performed to remove bumps and to correct the covalent geometry.

Metal ion incorporation

The water molecule closest to the designed metal-binding site was mutated to form a Zn^{2+} ion. All water and counterions were subsequently removed. The simulation was continued using experiment energy minimization. Metal docking was considered successful if the Zn^{2+} ion bound to the three metal-binding residues. Successful metal docking was characterized as the metal being able to interact (2 Å between the electronegative atom of metal-binding residues and the metal ion) with all three metal-binding residues.

Molecular dynamic (MD) simulation

After metal docking, the structure was refilled with water, and cell neutralization was performed. Long MD simulation was performed after metal docking to assess the stability of the metal-binding site. In YASARA Structure, all organic molecules were parameterized using the AutoSMILES algorithm, which is an automatic force-field parameter assigned to organic molecules. The algorithm involves the assignment of bond orders and adding in missing hydrogen, AM1BCC atoms, bond types, and general AMBER force-field atom

types and its parameters. Energy minimization steps were performed using the Particle Mesh Ewald algorithm (to treat long-range electrostatic interactions within 10 Å of the nonbonded cutoff) and the LINS algorithm (to constrain all bonds involving hydrogen atoms to enable short timesteps). Simulation started at 303 K and ran for 20 ns with structural restraint. Simulation snapshots were saved every 25 ps for further analysis.

Computational validation of metal-binding sites

To assess the validity of the designed metal-binding sites, several analyses (e.g., structural geometry, metal-binding energy, and metal-binding stability over time) were performed. The native metalloenzyme (carbonic anhydrase) and artificial nitric oxide reductase created using whale myoglobin as a template were used as controls when validating the metal-binding sites.

Metal-binding stability analysis

Data generated from long-term MD simulation can be used to assess the stability of the metal-binding site and to see if it falls apart over time or remains intact. The distance between the metal and metal-binding residues was recorded and plotted graphically as a function over time. Models with docked metals were run for 20 ns using the MD simulation to check if the metal-binding site disintegrated at the end of the simulation.

Fig. 7 Possible catalytic mechanism of artificial metallo-haloalkane dehalogenase (C1a mutant model). **a** C1a mutant model vs 1,2-dibromoethane—nucleophilic attack at C₁ atom, **b** C1a mutant model

vs 1,2-dibromoethane—nucleophilic attack at C₂ atom, and **c** C1a mutant model vs 1,2,3-trichloropropane. Black curved arrow represents motion of electrons

Fig. 7 (continued)

Metal-binding site geometry analysis

CheckMyMetal (Zheng et al. 2014) (http://csgid.org/csgid/metal_sites/) was used to assess the geometry of the metal-binding site and the vacancy of the metal.

Metal-binding energy analysis

Metal-binding energy of the designed metal-binding site was checked using the MetalBinding analysis command in FoldX (Supplementary Info S6) (<http://foldx.crg.es/>).

Substrate docking

Preparation of receptors and ligands

The receptors were the native DhaA and mutant models with validated metal-binding sites. During metal docking, these receptors had pairs of halide-stabilizing residues fixed in position, while the MD simulation was running. The ligands (1,2,3-trichloropropane CID: 7285 and 1,2-dibromoethane CID: 7839) were obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>), and their structural format was converted from .sdf to .pdb using YASARA Structure.

Substrate docking

Dehalogenase (haloalkanes) substrates were docked in silico into the active site of the mutant and native models using AutoDock software (Morris et al. 1998) embedded in YASARA Structure. Docking of 1,2,3-trichloropropane and 1,2-dibromoethane was run 25 times, and the substrates were set to be flexible. The native or mutated DhaA receptor and ligand were parameterized according to AutoSMILES. Force Field, AMBER03, was applied for the charge assignment. The substrate docking box was defined as the binding pocket that was 2 Å from the center residues (41, 106, 107, 149, 168, and 272) with dimensions of 16.13 Å × 12.77 Å × 16.47 Å. In the 25 runs, the substrate that was in contact with halide-stabilizing residues (Trp-107 and Asn-41) or metal ions and had the highest binding energy was selected for further analysis.

Tunnel analysis

Tunnels formed in mutant and native enzymes were checked using Caver3.0 (Chovancová et al. 2012). The structures used were from the protein structure snapshot taken during MD simulation. Protein structure snapshots with intervals of 50 ps to 1 ns (20 snapshots) were taken to analyze the tunnel formation. The probe radius for the tunnel bottleneck was set at 0.9 Å. Tunnels with an RMSD of 4.0 Å were grouped as one cluster.

Acknowledgements This research was funded by the Ministry of Higher Education (MOHE) Malaysia under Grant ERGS/1-2013/5527134. TA was awarded a Graduate Research Fellowship by the Universiti Putra Malaysia and received a scholarship from the Ministry

of Higher Education, Malaysia, under the MyMaster program when working on this research.

Author contributions TA and TCL designed the experiment and drafted the manuscript. TCL, ABS, and YMN reviewed and edited the manuscript. TCL and TA designed the experiments in coordination with ABS and NMY. TA carried out the experiments.

Compliance with ethical standards

Conflict of interest We declare no competing financial interests.

References

- Andreini C, Bertini I, Cavallaro G et al (2008) Metal ions in biological catalysis: from enzyme databases to general principles. *JBIC J Biol Inorg Chem* 13:1205–1218. <https://doi.org/10.1007/s00775-008-0404-5>
- ATSDR US (1989) 1,2-dibromoethane—production, import, use and disposal. pp 81–84
- ATSDR US (2001) Toxicological profile for 1,2-dichloroethane. pp 1–297
- Auffinger P, Bielecki L, Westhof E (2003) The Mg^{2+} binding sites of the 5S rRNA loop E motif as investigated by molecular dynamics simulations. *Chem Biol* 10:551–561. [https://doi.org/10.1016/S1074-5521\(03\)00121-2](https://doi.org/10.1016/S1074-5521(03)00121-2)
- Barkigia KM, Berber MD, Fajer J et al (1990) Nonplanar porphyrins. X-ray structures of (2,3,7,8,12,13,17,18-octaethyl-and-octamethyl-5,10,15,20-tetraphenylporphyrinato)zinc(II). *J Am Chem Soc* 112:8851–8857. <https://doi.org/10.1021/ja00180a029>
- Belkin S (1992) Biodegradation of haloalkanes. *Biodegradation* 3:299–313. <https://doi.org/10.1007/BF00129090>
- Bendl J, Stourac J, Sebestova E et al (2016) HotSpot Wizard 2.0: automated design of site-specific mutations and smart libraries in protein engineering. *Nucleic Acids Res* 44:1–9. <https://doi.org/10.1093/nar/gkw416>
- Bidmanova S, Chaloupkova R, Damborsky J, Prokop Z (2010) Development of an enzymatic fiber-optic biosensor for detection of halogenated hydrocarbons. *Anal Bioanal Chem* 398:1891–1898. <https://doi.org/10.1007/s00216-010-4083-z>
- Chaudhry GR, Chapalamadugu S (1991) Biodegradation of halogenated organic compounds. *Microbiol Rev* 55:59–79
- Chovancová E, Kosinski J, Bujnicki JM, Damborský J (2007) Phylogenetic analysis of haloalkane dehalogenases. *Proteins* 67:305–316. <https://doi.org/10.1002/prot.21313>
- Chovancová E, Pavelka A, Benes P et al (2012) CAVER 3.0: a tool for the analysis of transport pathways in dynamic protein structures. *PLoS Comput Biol* 8:e1002708. <https://doi.org/10.1371/journal.pcbi.1002708>
- Clarke ND, Yuan SM (1995) Metal search: a computer program that helps design tetrahedral metal-binding sites. *Proteins* 23:256–263. <https://doi.org/10.1002/prot.340230214>
- Damborský J, Chaloupková R, Pavlová M et al (2010) Structure–function relationships and engineering of haloalkane dehalogenases. In: Timmis KN (ed) *Handbook of hydrocarbon and lipid microbiology*. Springer, Berlin, pp 1082–1098
- Dudev T, Lim C (2000) Tetrahedral vs octahedral zinc complexes with Ligands of biological interest: a DFT/CDM study. *J Am Chem Soc* 122:11146–11153. <https://doi.org/10.1021/ja0010296>
- Dudev T, Lim C (2008) Metal binding affinity and selectivity in metalloproteins: insights from computational studies. *Annu Rev Biophys* 37:97–116. <https://doi.org/10.1146/annurev.biophys.37.032807.125811a>
- Franken SM, Rozeboom HJ, Kalk KH, Dijkstra BW (1991) Crystal structure of haloalkane dehalogenase: an enzyme to detoxify halogenated alkanes. *EMBO J* 10:1297–1302
- Gehret JJ, Gu L, Geders TW et al (2012) Structure and activity of DmmA, a marine haloalkane dehalogenase. *Protein Sci* 21:239–248. <https://doi.org/10.1002/pro.2009>
- Goldman P (1965) The enzymatic cleavage of the carbon–fluoride bond in fluoroacetate. *J Biol Chem* 240:3434–3438
- Hellings HW, Richards FM (1991) Construction of new ligand binding sites in proteins of known structure. I. Computer-aided modeling of sites with pre-defined geometry. *J Mol Biol* 222:763–785
- Hellings HW, Caradonna JP, Richards FM (1991) Construction of new ligand binding sites in proteins of known structure. II. Grafting of a buried transition metal binding site into *Escherichia coli* thioredoxin. *J Mol Biol* 222:787–803
- Janssen DB (2004) Evolving haloalkane dehalogenases. *Curr Opin Chem Biol* 8:150–159. <https://doi.org/10.1016/j.cbpa.2004.02.012>
- Johnson R (2014) Artificial metalloenzymes: encoding copper catalysts. *Nat Chem* 6:1027–1027. <https://doi.org/10.1038/nchem.2127>
- Kamachi T, Nakayama T, Shitamichi O et al (2009) The catalytic mechanism of fluoroacetate dehalogenase: a computational exploration of biological dehalogenation. *Chemistry* 15:7394–7403. <https://doi.org/10.1002/chem.200801813>
- Kennes C, Pries F, Krooshof GH et al (1995) Replacement of tryptophan residues in haloalkane dehalogenase reduces halide binding and catalytic activity. *Eur J Biochem* 228:403–407. <https://doi.org/10.1111/j.1432-1033.1995.0403n.x>
- Khare SD, Kipnis Y, Greisen P et al (2012) Computational redesign of a mononuclear zinc metalloenzyme for organophosphate hydrolysis. *Nat Chem Biol* 8:294–300. <https://doi.org/10.1038/nchembio.777>
- Koike T, Inoue M, Kimura E, Shiro M (1996) Novel properties of cooperative dinuclear zinc(II) ions: the selective recognition of phosphomonoesters and their P–O ester bond cleavage by a new dinuclear zinc(II) cryptate. *J Am Chem Soc* 118:3091–3099. <https://doi.org/10.1021/ja953413m>
- Koudelakova T, Bidmanova S, Dvorak P et al (2013a) Haloalkane dehalogenases: biotechnological applications. *Biotechnol J* 8:32–45. <https://doi.org/10.1002/biot.201100486>
- Koudelakova T, Chaloupkova R, Brezovsky J et al (2013b) Engineering enzyme stability and resistance to an organic cosolvent by modification of residues in the access tunnel. *Angew Chem Int Ed Engl* 52:1959–1963. <https://doi.org/10.1002/anie.201206708>
- Krieger E, Koraimann G, Vriend G (2002) Increasing the precision of comparative models with YASARA NOVA—a self-parameterizing force field. *Proteins* 47:393–402. <https://doi.org/10.1002/prot.10104>
- Krooshof GH, Ridder IS, Tepper AWJW et al (1998) Kinetic analysis and X-ray structure of haloalkane dehalogenase with a modified halide-binding site. *Biochemistry* 37:15013–15023. <https://doi.org/10.1021/bi9815187>
- Kulakova AN, Larkin MJ, Kulakov LA (1997) The plasmid-located haloalkane dehalogenase gene from *Rhodococcus rhodochrous* NCIMB 13064. *Microbiology* 143:109–115. <https://doi.org/10.1099/00221287-143-1-109>
- Liu J-Q, Kurihara T, Ichihara S et al (1998) Reaction mechanism of fluoroacetate dehalogenase from *Moraxella* sp. B. *J Biol Chem* 273:30897–30902. <https://doi.org/10.1074/jbc.273.47.30897>
- Lovell T, Himo F, Han W-G, Noodleman L (2003) Density functional methods applied to metalloenzymes. *Coord Chem Rev* 238:211–232
- Makarewicz T, Kaźmierkiewicz R (2013) Molecular dynamics simulation by GROMACS using GUI plugin for PyMOL. *J Chem Inf Model* 53:1229–1234. <https://doi.org/10.1021/ci400071x>

- McCall KA, Huang C, Fierke CA (2000) Function and mechanism of zinc metalloenzymes. *J Nutr* 130:1437S–1446S
- Miner KD, Mukherjee A, Gao Y-G et al (2012) A designed functional metalloenzyme that reduces O₂ to H₂O with over one thousand turnovers. *Angew Chem Int Ed Engl* 51:5589–5592. <https://doi.org/10.1002/anie.201201981>
- Morris GM, Goodsell DS, Halliday RS et al (1998) Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J Comput Chem* 19:1639–1662
- Newman J, Peat TS, Richard R et al (1999) Haloalkane dehalogenases: structure of a *Rhodococcus* enzyme. *Biochemistry* 38:16105–16114. <https://doi.org/10.1021/bi9913855>
- Pavelka A, Chovancova E, Damborsky J (2009) HotSpot Wizard: a web server for identification of hot spots in protein engineering. *Nucleic Acids Res* 37:W376–W383. <https://doi.org/10.1093/nar/gkp410>
- Peat TS, Newman J, Balotra S et al (2015) The structure of the hexameric atrazine chlorohydrolase AtzA. *Acta Crystallogr Sect D Biol Crystallogr* 71:710–720. <https://doi.org/10.1107/S1399004715000619>
- Petrek M, Otyepka M, Banás P et al (2006) CAVER: a new tool to explore routes from protein clefts, pockets and cavities. *BMC Bioinform* 7:316. <https://doi.org/10.1186/1471-2105-7-316>
- Prokop Z, Monincová M, Chaloupková R et al (2003) Catalytic mechanism of the haloalkane dehalogenase LinB from *Sphingomonas paucimobilis* UT26. *J Biol Chem* 278:45094–45100. <https://doi.org/10.1074/jbc.M307056200>
- Schymkowitz JWH, Rousseau F, Martins IC et al (2005) Prediction of water and metal binding sites and their affinities by using the Fold-X force field. *Proc Natl Acad Sci USA* 102:10147–10152. <https://doi.org/10.1073/pnas.0501980102>
- Seffernick JL, McTavish H, Osborne JP et al (2002) Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP is a metalloenzyme. *Biochemistry* 41:14430–14437. <https://doi.org/10.1021/bi020415s>
- Seffernick JL, Reynolds E, Fedorov AA et al (2010) X-ray structure and mutational analysis of the atrazine chlorohydrolase TrzN. *J Biol Chem* 285:30606–30614. <https://doi.org/10.1074/jbc.M110.138677>
- Sigman JA, Kwok BC, Lu Y (2000) From myoglobin to heme-copper oxidase: design and engineering of a Cu B center into sperm whale myoglobin. *J Am Chem Soc* 122:8192–8196. <https://doi.org/10.1021/ja0015343>
- Stepankova V, Khabiri M, Brezovsky J et al (2013) Expansion of access tunnels and active-site cavities influence activity of haloalkane dehalogenases in organic cosolvents. *ChemBioChem* 14:890–897. <https://doi.org/10.1002/cbic.201200733>
- Stsiapanava A, Dohnalek J, Gavira J et al (2010) Atomic resolution studies of haloalkane dehalogenases DhaA04, DhaA14 and DhaA15 with engineered access tunnels. *Acta Crystallogr Sect D Biol Crystallogr* 66:962–969. <https://doi.org/10.1107/S0907444910027101>
- U.S. EPA (2004) Toxicological review of 1,2-dibromoethane (CAS No. 106-93-4). In: Support of summary information on the integrated risk information system (IRIS). EPA/635/R-04/067. 1–240
- U.S. EPA (2009) Toxicological review of 1,2,3-trichloropropane (CAS No. 96-18-4). In Support of summary information on the integrated risk information system (IRIS). EPA/635/R-08/010F. 1–247
- Ueno T, Koshiyama T, Abe S et al (2007) Design of artificial metalloenzymes using non-covalent insertion of a metal complex into a protein scaffold. *J Organomet Chem* 692:142–147. <https://doi.org/10.1016/j.jorganchem.2006.08.043>
- Valdez CE, Smith QA, Nechay MR, Alexandrova AN (2014) Mysteries of metals in metalloenzymes. *Acc Chem Res* 47:3110–3117. <https://doi.org/10.1021/ar500227u>
- Van Durme J, Delgado J, Stricher F et al (2011) A graphical interface for the FoldX forcefield. *Bioinformatics* 27:1711–1712. <https://doi.org/10.1093/bioinformatics/btr254>
- Verschueren KH, Seljée F, Rozeboom HJ et al (1993) Crystallographic analysis of the catalytic mechanism of haloalkane dehalogenase. *Nature* 363:693–698. <https://doi.org/10.1038/363693a0>
- Wu S, Zhang C, Xu D, Guo H (2010) Catalysis of carboxypeptidase A: promoted-water versus nucleophilic pathways. *J Phys Chem B* 114:9259–9267. <https://doi.org/10.1021/jp101448j>
- Yeung N, Lin Y-W, Gao Y-G et al (2009) Rational design of a structural and functional nitric oxide reductase. *Nature* 462:1079–1082. <https://doi.org/10.1038/nature08620>
- Zastrow ML, Peacock AFA, Stuckey JA, Pecoraro VL (2012) Hydrolytic catalysis and structural stabilization in a designed metalloprotein. *Nat Chem* 4:118–123. <https://doi.org/10.1038/nchem.1201>
- Zheng H, Chordia MD, Cooper DR et al (2014) Validation of metal-binding sites in macromolecular structures with the CheckMyMetal web server. *Nat Protoc* 9:156–170. <https://doi.org/10.1038/nprot.2013.172>

Affiliations

Thiau-Fu Ang^{1,3} · Abu Bakar Salleh^{2,3,4} · Yahaya M. Normi^{1,3} · Thean Chor Leow^{1,3,4}

¹ Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia

² Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia

³ Enzyme and Microbial Technology Research Centre (EMTech), Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia

⁴ Institute of Bioscience, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia