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Review

Evolution of the arginase fold and functional diversity

D. P. Dowling, L. Di Costanzo, H. A. Gennadios, and D. W. Christianson*

Roy and Diana Vagelos Laboratories, Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104 – 6323 (USA), Fax: +1-215-573-2201, e-mail: chris@sas.upenn.edu

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Abstract. Novel structural superfamilies can be identified among the large number of protein structures deposited in the Protein Data Bank based on conservation of fold in addition to conservation of amino acid sequence. Since sequence diverges more rapidly than fold in protein evolution, proteins with little or no significant sequence identity are occasionally observed to adopt similar folds, thereby reflecting unanticipated evolutionary relationships. Here, we review the unique α/β fold first observed in the manganese metalloenzyme rat liver arginase, consisting of a parallel eight-stranded β-sheet surrounded by several helices, and its evolutionary relationship with the zinc-requiring and/or iron-requiring histone deacetylases and acetylpolyamine amidohydrolases. Structural comparisons reveal key features of the core α/β fold that contribute to the divergent metal ion specificity and stoichiometry required for the chemical and biological functions of these enzymes.

Keywords. Metalloenzyme, protein structure, enzyme inhibitor, X-ray crystallography, cationic amino acid.

Introduction

The occurrence of specific proteins within the three domains of life (bacteria, archaea and eukarya) argues for their occurrence in the last common ancestor (LCA; also known as the last universal common ancestor, LUCA, or cenancestor) [1, 2]. Amino acid sequence identity is generally the primary determinant of genes conserved within the LCA, and the minimal gene content of the LCA has been proposed by Ouzounis and colleagues [3]. Interestingly, the minimal gene content of the LCA includes enzymes belonging to the arginase superfamily, such as histone deacetylase (HDAC) and agmatinase [3, 4], so members of this superfamily likely herald the very beginnings of life on the earth.

Sequence analysis alone, however, overlooks structural homologies between topologically identical enzymes that lack significant sequence identity, since sequence evolves more rapidly than structure in the divergent evolution of proteins [5]. For example, HDAC and arginase share the same α/β fold, yet these enzymes have experienced sufficient evolutionary drift that they exhibit insignificant amino acid sequence identity. In another example, it is likely that acetylpolyamine amidohydrolase (APAH) enzymes will share the arginase fold despite low sequence identity with arginase because they share ~20 – 25 % sequence identity with HDAC enzymes [6]. Ranea and colleagues describe 140 ancestral superfamilies consisting of 104 087 domains rooted in the LCA. These proteins include the greater family of Rossmannoid HAD-like hydrolases, which contain a signature parallel five-stranded β-sheet sandwiched between α-helices. Interestingly, the metalloenzymes arginase, HDAC, and APAH contain a Rossmannoid

* Corresponding author.

fold within a larger eight-stranded β-sheet, suggesting an evolutionary relationship with an ancestral HAD-like hydrolase [6–8].

The identification of arginase in the LCA reflects the importance of L-arginine homeostasis in metabolic functioning, and the recruitment of the arginase fold to serve other catalytic functions in various domains of life suggests that this particular α/β fold is robustly stable and readily evolvable. In the remainder of this review, we summarize structure-function relationships for arginase and compare these with other members of the arginase superfamily, including the HDACs and APAHs.

Arginase

Arginase is a binuclear manganese metalloenzyme that catalyzes the hydrolysis of L-arginine to form L-ornithine and urea, the final cystosolic reaction of the urea cycle [9–13]. In humans, hereditary defects in arginase compromise structure and catalysis, which results in an accumulation of arginine in the blood known as hyperarginemia [11, 14, 15]. Arginase deficiency can also result in the accumulation of nitrogen in the form of ammonia, which results in hyperammonemia [11, 16]. Two isozymes have been identified that share ~60% sequence identity: arginase I is the cystosolic enzyme in the liver that functions in the urea cycle [12, 13], but it is also found at low levels in nonhepatic tissues [11]; and arginase II is a mitochondrial enzyme expressed in nonhepatic tissues [10, 11]. Nonhepatic arginase isozymes function in L-arginine homeostasis [10, 11, 17, 18]. For example, arginases I and II are expressed in vascular smooth muscle cells, where both isozymes function in polyamine biosynthesis and endothelial cell proliferation [17, 19, 20].

Within the greater family of enzymes that metabolize L-arginine, nitric oxide synthase (NOS) is perhaps the most prominent [21–25]. Interestingly, NOS and arginase are reciprocally regulated at the level of transcription in murine macrophages and dendritic cells by Th1 and Th2 cytokines, respectively [26–29]. Furthermore, NOS and arginase are also reciprocally regulated at the level of enzyme activity: arginase activity functionally inhibits NOS by depleting the substrate pool of L-arginine, and NOS activity potentially inhibits arginase by generating and releasing small amounts of the intermediate *N*-hydroxy-L-arginine, a competitive inhibitor of arginase [30], and also by generating NO itself, which can nitrosylate cysteine residues of human arginase I to modulate its activity [31]. Since arginase inhibition can enhance NO biosynthesis and NO-dependent processes *in vivo*, arginase is a potential target for therapeutic intervention in a variety of diseases [9–11].

The first crystal structure of an arginase enzyme was that of rat liver arginase [32], also known as arginase I, which revealed a trimeric quaternary structure in which each monomer adopts an α/β fold consisting of a central parallel eight-stranded β-sheet (strand order 21387456) flanked on both sides by several α-helices (Figs. 1 and 2). Each monomer has approximate dimensions of 40×50×50 $Å^3$. A portion of the arginase fold partially resembles the Rossmann fold (α/β fold with strand order 321456) [8, 33, 34]. The subsequently determined crystal structures of trimeric human arginase I [35] and human arginase II [36], as well as the hexameric arginases from *Bacillus caldovelox* [37] and *Thermus thermophilus* (PDB accession codes 2EF4, 2EF5, and 2EIV), reveal identical tertiary structures for individual monomers from each species. Amino acid side chains that coordinate to the manganese ions in the arginase active site (Fig. 3) are located on the edge of the central β-sheet in loop segments immediately adjacent to strands 8, 7, and 4 (indicated schematically in Fig. 2). The binuclear cluster in the unliganded enzyme exhibits a Mn_A^{2+}-Mn_B^{2+} separation of ~3.3 Å, in agreement with the separation measured in an electron paramagnetic resonance spectroscopic study [38]. The crystal structure of unliganded rat arginase I reveals that Mn_A^{2+} is coordinated by H101, D128, D124, D232, and the metal-bridging hydroxide ion with square pyramidal geometry [32]. However, the crystal structure of unliganded human arginase I reveals an additional water molecule coordinated to Mn_A^{2+}, which completes a distorted octahedral metal coordination polyhedron [39]. The Mn_B^{2+} ion is coordinated by H126, D234, D124, D232, and a hydroxide ion with distorted octahedral geometry in rat arginase I and human arginase I. Importantly, the structure of unliganded human arginase I shows that the metal-bridging hydroxide ion and H141 interact with each other through a hydrogen-bonded water molecule [39]. These interactions are consistent with the proposed role of H141 as a proton shuttle in the regeneration of the nucleophilic metal-bridging hydroxide ion in catalysis [32]. The proposed mechanism is summarized in Figure 4 [35].

Further support for the proposed mechanism derives from numerous enzymological and X-ray crystallographic studies [40–42]. Specifically, the crystal structure of rat arginase I complexed with 2(*S*)-amino-6-boronohexanoic acid (ABH; Table 1) provides critical insights regarding the structural basis of catalysis [43]. This inhibitor is a reactive substrate analogue that is isosteric with the naturally occurring substrate, L-arginine. The crystal structure of the

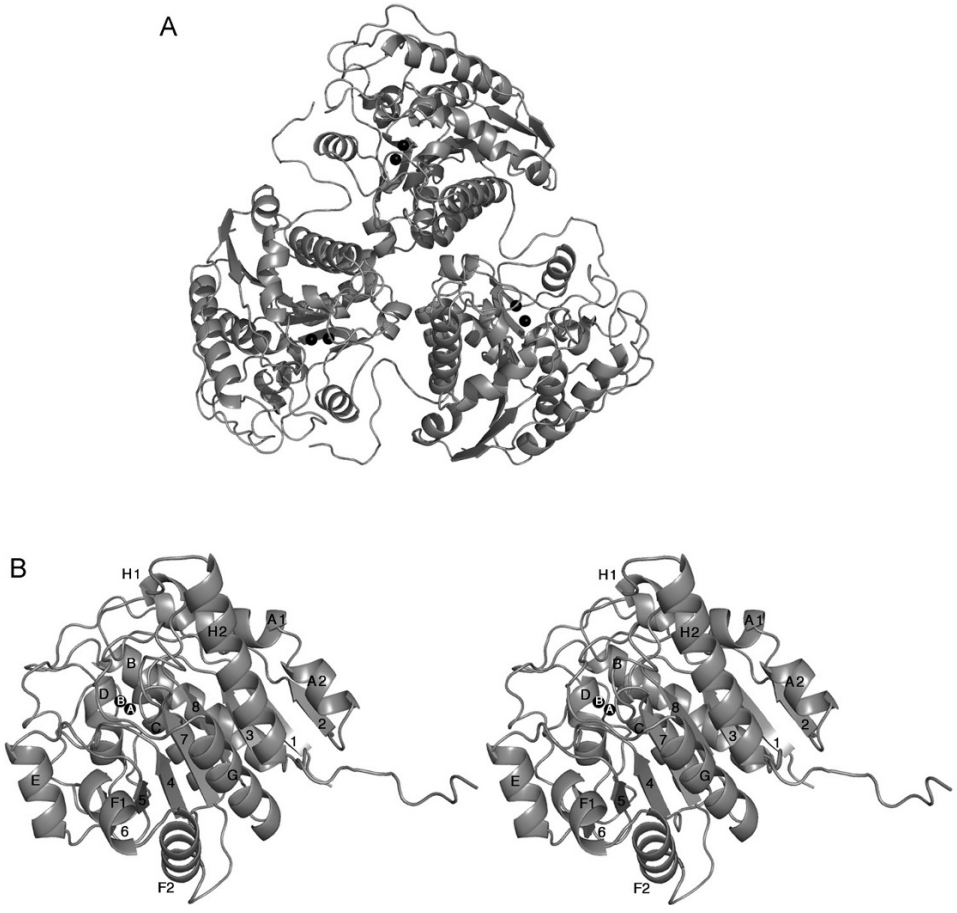


Figure 1. (*A*) Ribbon-plot of the rat arginase trimer; the Mn^{2+}-Mn^{2+} cluster in the active site of each monomer is represented by a pair of spheres. (*B*) Stereoview of a single rat arginase monomer with β-sheets and α-helices labeled.

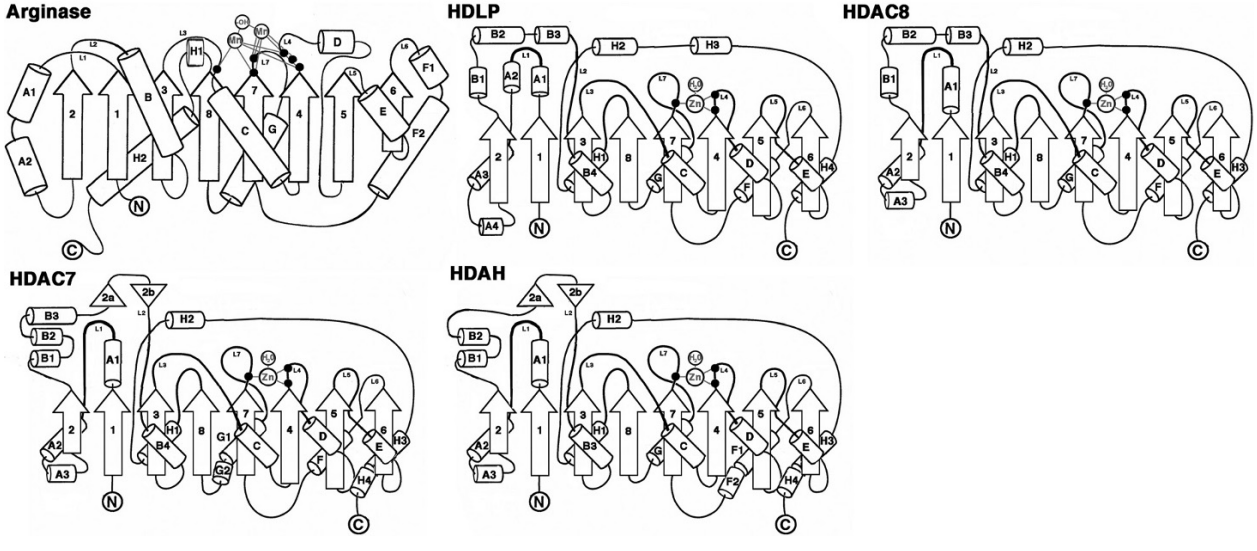


Figure 2. Secondary structure comparison of arginase [32], histone deacetylase-like protein (HDLP) [116], histone deacetylase (HDAC)8 [118, 119], HDAC7 [117], and histone deacetylase-like amidohydrolase (HDAH) [120]. The positions of the metal ions, liganded by residues at the C-terminal ends of β-strands 4 and 7, are indicated.

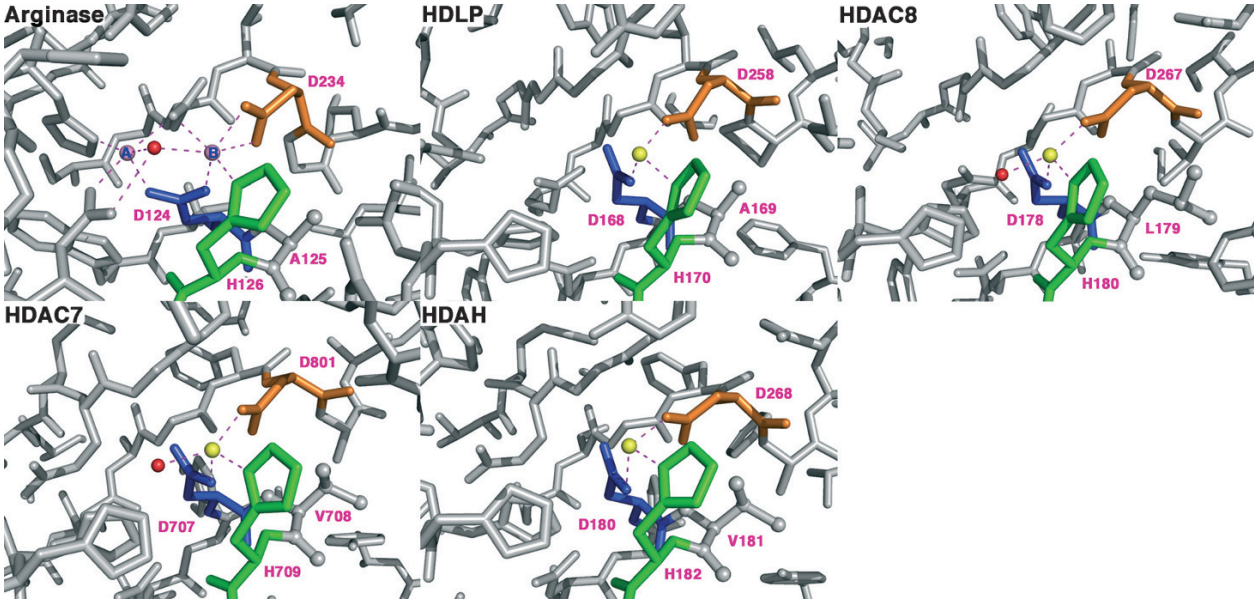


Figure 3. Metal-binding sites of rat arginase I (pdb accession code 1RLA), HDLP (pdb accession code 1C3P), HDAC8 (less the substrate molecule) (pdb accession code 2V5W), HDAC7 (pdb accession code 2NVR), and HDAH (less the acetate molecule) (pdb accession code 1ZZ0). Conserved metal ligands are shown with conserved colors in each enzyme active site. Zn^{2+} and Mn^{2+} ions appear as yellow and pink spheres, respectively; metal-bound solvent molecules are shown as red spheres. The D(A,V,L)H loop and an additional aspartate residue ~100 residues upstream in sequence are conserved for $Mn^{2+}{}_B$ binding in arginase and Zn^{2+} binding in the HDAC and HDAC-like enzymes.

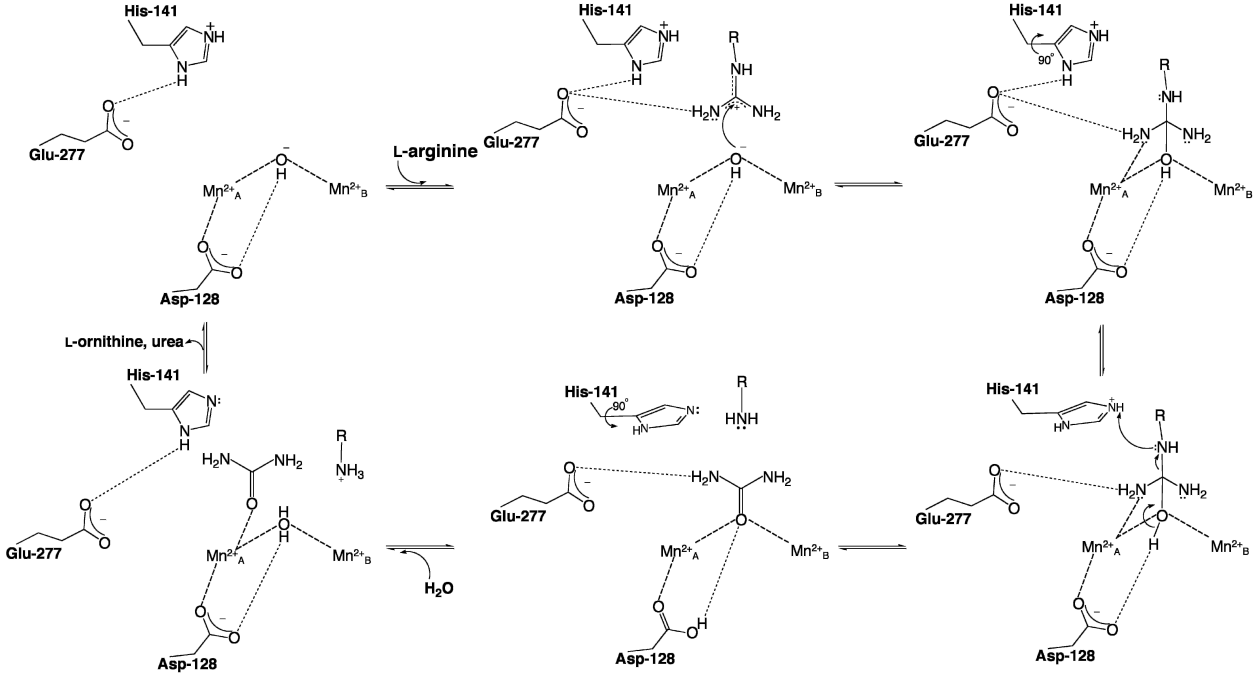
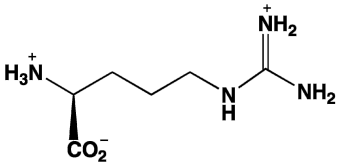
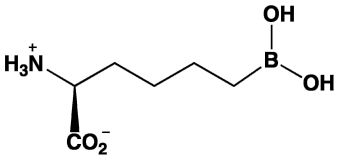
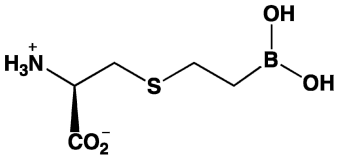
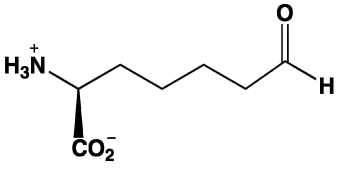
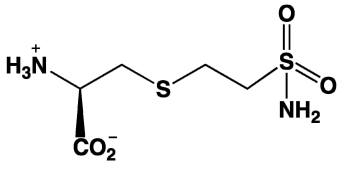


Figure 4. Proposed mechanism of human arginase I. Reprinted with permission from [35]. Copyright (2005) Proc. Natl. Acad. Sci. USA (http://www.pnas.org).

arginase-ABH complex reveals that the boronic acid moiety of ABH undergoes nucleophilic attack by the metal-bridging hydroxide to yield a tetrahedral boronate anion that mimics the tetrahedral intermediate in catalysis (Fig. 5) [35, 40]. The 1.29-Å resolution crystal structure of the human arginase I-ABH complex (K_d = 5 nM) reveals that high affinity is a consequence of strong metal-coordination and hydrogen bond interactions [35]. The same chemistry accompanies the binding of the related analogue (*S*)-

Table 1. Arginase I affinities at pH 8.5.

Compound	Abbreviation	Structure	Affinity
L-Arginine	Arg		$K_M = 1$ mM[a] [42]
2(*S*)-amino-6-boronohexanoic acid	ABH		$K_d = 5$ nM[b] [35]
(*S*)-(2-boronoethyl)-L-cysteine	BEC		$K_d = 270$ nM[b] [35]
(*S*)-2-amino-7-oxoheptanoic acid	AOH		$K_i = 60$ μM[a] [46]
S-(2-sulfonamidoethyl)-L-cysteine	SEC		$K_d = 52$ μM[a] [47]

[a] Rat arginase I, [b] human arginase.

(2-boronoethyl)-L-cysteine (BEC; Table 1) to rat arginase I [41], human arginase I [35], and human arginase II [36], although with slightly lower affinity compared with ABH [41, 43–45]. Analogously, the crystal structure of rat arginase I complexed with the inhibitor (*S*)-2-amino-7-oxoheptanoic acid (AOH; Table 1) reveals that the aldehyde moiety undergoes nucleophilic attack by the metal-bridging hydroxide ion to yield a tetrahedral gem-diol coordinated to the binuclear manganese cluster; however, the binding affinity of this reactive substrate analogue to rat arginase I is somewhat modest ($K_i = 60$ μM) [46].
Additional structural inferences regarding the binding of the tetrahedral intermediate and its flanking transition states emanate from the crystal structure of rat arginase I complexed with the sulfonamide inhibitor *S*-(2-sulfonamidoethyl)-L-cysteine (SEC; Table 1), which reveals that the tetrahedral sulfonamide group of the inhibitor is ionized with its NH group bridging the binuclear manganese cluster [47]. Finally, the recently reported [39] crystal structure of human arginase I complexed with thiosemicarbazide, an analogue of urea, reveals a metal-bridging binding mode for the C=S group that mimics the metal-bridging binding mode proposed for the urea C=O group in the penultimate product complex [32, 35].

Histone deacetylase

More than 40 years ago, Allfrey and colleagues [48] hypothesized that gene regulation could be achieved through the reversible acetylation of histone proteins. Subsequently, histone acetyl transferases and HDACs were discovered to covalently modify histone lysine residues for gene regulation [49–53]. The first human HDAC was discovered by Schreiber and colleagues [54] using a trapoxin-based affinity resin. Designated HDAC1 and categorized as a class I HDAC, this enzyme exhibited 60% amino acid sequence identity with the yeast deacetylase RPD3. Additional class I enzymes characterized to date include HDAC2–3 [55–58] and HDAC8 [59–61]. Database mining initially led to the discovery of class II HDAC

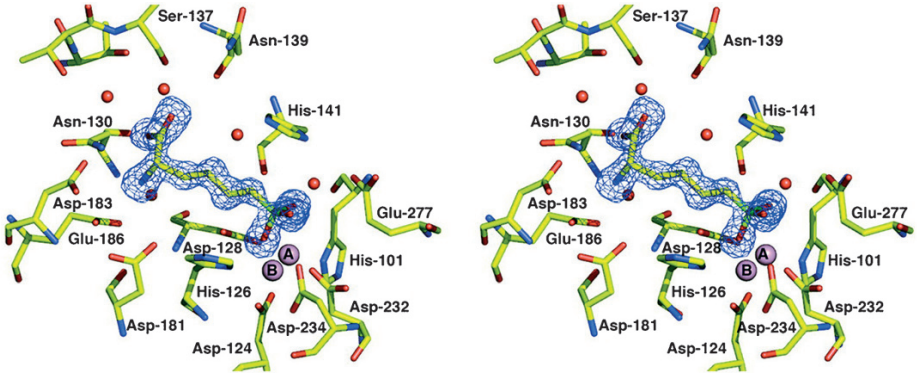


Figure 5. Omit electron density map of the wild-type human arginase I–2(*S*)-amino-6-boronohexanoic acid (ABH) complex showing that the metal-bridging hydroxide ion of the native enzyme attacks the boronic acid moiety of ABH to yield the tetrahedral boronate anion, thereby mimicking the first step of the catalytic mechanism outlined in Figure 4 (contoured at 2.5σ). Atoms are color-coded as: carbon (yellow), oxygen (red), nitrogen (blue), manganese (pink), and boron (light green). Water molecules appear as red spheres, and manganese ions A and B appear as larger pink spheres. Reprinted with permission from [35]. Copyright (2005) Proc. Natl. Acad. Sci. USA (http://www.pnas.org).

enzymes, which resemble yeast HdaI [62]; these enzymes include HDAC4–7 [63–66] and HDAC9–10 [67–69], and contain a catalytic core of ~420 residues similar to the catalytic core of the class I enzymes [70]. The class III enzymes are yeast Sir2 homologues, termed sirtuins, which contain a completely different NAD-binding Rossmann fold in comparison with other HDAC enzymes [52, 71, 72]. Deacetylation by the sirtuin proteins is NAD dependent [73–75]. Finally, HDAC11 is categorized as a class IV enzyme by phylogenetic analysis [76]. A summary of mammalian HDAC properties is found in Table 2.

Interestingly, while all enzymes in the greater HDAC family share a common substrate, an acetylated lysine residue contained within the context of a larger polypeptide or protein [77], the actual protein substrates and subcellular localizations differ from one HDAC to another. The class I enzymes HDAC1 and HDAC2 are primarily localized in the nucleus where they function in the nucleosome remodeling and histone deacetylation complex (NurD) as well as the SIN3 complex, which consists of a primary core of HDAC1, HDAC2, and the histone-binding proteins RbAp46 and RbAp48, in addition to several other proteins containing motifs involved in chromatin-mediated gene regulation (*e.g.*, helicase/ATPase, chromodomain, PHD finger, etc.) [78]. There are two co-repressors that rely on, at least in part, the catalytic mechanisms of HDAC enzymes through the interactions of NuRD, SIN3, and HDAC3, to functionally repress specific gene transcription. These co-repressors are the nuclear hormone receptor co-repressor N-CoR and the silencing mediator of retinoic and thyroid hormone receptors SMRT [78–81]. In contrast with HDAC1 and HDAC2, HDAC3 and HDAC8 are class I enzymes found in both the nucleus and the cytoplasm [82, 83]. Class II HDAC enzymes are subject to signal-dependent shuttling between the nucleus and cytoplasm [84, 85] and also interact with the N-CoR and/or SMRT co-repressors [86]. The class III enzymes (sirtuins) are nuclear, cytosolic, and mitochondrial proteins that catalyze the deacetylation of histones and non-histone substrates such as p53 and the FOXO transcription factor [87]. In addition to gene silencing, yeast Sir2 is involved in many cell functions (*e.g.*, DNA repair and chromosomal stability and longevity), including a role in the effects of caloric restriction on aging [88]. Finally, the class IV enzyme HDAC11 is localized in the nucleus where it is found in complex with HDAC6, and its expression is tissue specific for kidney, heart, brain, skeletal muscle, and testis [89, 90].

Aberrant recruitment of HDAC enzymes in tumor cells [91] has increased interest in this deacetylase family as a potential pharmaceutical target. The inhibition of HDAC is linked to both the up-regulation and down-regulation of only ~2–5 % of genes [92–95]. Although the gene set transcriptionally regulated by the HDAC inhibitors trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) vary from the gene set transcriptionally regulated by *N*-2-aminophenyl acylamide 3-pyridylmethyl-*N*-{4-[(2-aminophenyl)-carbamoyl]-benzyl}-carbamate (MS-275), the "core" set of genes regulated by all three HDAC inhibitors include such members as p21 (cell cycle regulation, significantly up-regulated), TRPM-2 (apoptosis, up-regulated), thymidylate synthetase and CTP synthase (DNA synthesis, both down-regulated), and TRP (transformation, down-regulated). These studies reveal HDAC-inhibited induction of p21, $p27^{Kip1}$, and/or *tob*1, three cell cycle regulating proteins, in p53-independent cell cycle arrest [92].

Table 2. Mammalian histone deacetylase (HDAC) properties.

	Enzyme	Primary localization	Structural fold and metal coordinating residues	pdb code	HDAC interacting partners[a]
Class I: Rpd3-like			Arginase/Rossmannoid		
	HDAC1	Nucleus [54]	D176,H178,D264	–	HDAC1,2,3 [128]
	HDAC2	Nucleus [54]	D177,H179,D265	–	HDAC1,3 [128, 153]
	HDAC3	Nucleus and cytoplasm [153]	D170,H172,D259	–	HDAC1–5,7 [65,66,153]
	HDAC8	Nucleus and cytoplasm [61, 83]	D178,H180,D267	1T64	–
Class II: Had1-like			Arginase/Rossmannoid		
	HDAC4	Nucleus and cytoplasm [85]	D840,H842,D934	–	HDAC3[d],SIRT1 [65, 88]
	HDAC5	Nucleus and cytoplasm [85]	D870,H872,D964	–	HDAC3[d] [65]
	HDAC6	Cytoplasm [159]	D649,H651,D742	–	SIRT2,HDAC11 [89, 90, 162]
	HDAC7	Nucleus and cytoplasm [160]	D707,H709,D801 C533,C535,H541,C618[b]	2NVR	HDAC3 [66]
	HDAC9	Nucleus and cytoplasm [161]	D820,H822,D914	–	–
	HDAC10	Nucleus and cytoplasm [67]	D172,H174,D265	–	–
Class III: sirtuins, NAD dependent			Rossmann[c]		
	SIRT1	Nucleus and cytoplasm [87, 163, 164]	–	–	HDAC4 [88]
	SIRT2	Cytoplasm [87, 162, 165, 166]	C195,C200,C221,C224	1J8F	HDAC6 [162]
	SIRT3	Mitochondria [87]	–	–	–
	SIRT4	Mitochondria [87]	–	–	–
	SIRT5	Mitochondria [87]	C166,C169,C207,C212	2B4Y 2NYR	–
	SIRT6	Nucleus [87]	–	–	–
	SIRT7	Nucleus [87]	–	–	–
Class IV: HDAC11-related	HDAC11	Nucleus [90]	Arginase/Rossmannoid D181,H183,D261	–	HDAC6 [89,90]

[a] Complex partners may involve additional protein subunits in the formation of complexes, such as RbAp46 and RbAp48 for HDAC1 and HDAC2, or C-terminal-binding protein (CtBP), heterochromatin P1 (HP1), MEF2 transcription factor, *etc.*, for various class II members.
[b] A structural zinc is seen in the HDAC7 crystal structure [117]. These residues are conserved in many of the class II members.
[c] In addition to a Rossmann domain, the sirtuin proteins contain a small domain consisting of a three-stranded zinc ribbon motif [167]. A structural zinc is seen in the crystal structures of both SIRT2 [71] and SIRT5 [168, 169]. These residues appear conserved throughout class III.
[d] Interaction in the nucleus.

Treatment of pancreatic adenocarcinoma cells with TSA implicates HDAC inhibition in signaling and transcriptional regulation, as various transcription factor-binding sites are more abundant in TSA-inhibited cells [94], correlating with a general increase in gene transcription [96]. The TSA-inhibited cells also exhibit an increased ratio of pro-apoptic BIM (a Bcl-2 family member containing the conserved BH3-only motif [97]) and anti-apoptotic Bcl-2 gene products [94], supporting the proposal that TSA promotes apoptosis by the induction and repression of pro- and anti-apoptosis factors, respectively. Thus, HDAC enzymes are validated as therapeutic drug targets for cancer chemotherapy, and several HDAC inhibitors have entered clinical trials [98, 99]. To date, Vorinostat (the trade name of SAHA) is approved for the treatment of cutaneous T cell lymphoma [100].

The catalytic activities of several HDAC enzymes are regulated by post-translational modifications. For instance, HDAC1 is a substrate for covalent attachment of SUMO-1 (small ubiquitin-related modifier), which reduces transcriptional repression by HDAC1 [101]. Covalent phosphorylation of HDAC1 is said to increase deacetylase activity yet decrease the ability to form complexes [102, 103], although conflicting data suggest that no change in catalytic activity results from phosphorylation [104]. While phosphorylation also activates HDAC2 and HDAC3 [105–107], phosphorylation inactivates HDAC8 [108]. Covalent phosphorylation of HDAC8 also decreases degradation of the human ever-shorter telomeres 1B protein (hEST1B/SMG5) through an unknown mechanism, and phosphorylated HDAC8 coimmunoprecipitates with hEST1B/SMG5. Seto and colleagues [108, 109] propose that additional cofactors may be necessary to probe the relationship between HDAC8 phosphorylation and the ubiquitin-mediated proteasome degradation of hEST1B, since the ubiquitination of hEST1B is not regulated by its acetylation. Class II HDAC enzymes are phosphorylated at two conserved serine residues under stress conditions. This allows HDAC4 and HDAC5 to interact with chaperone protein 14-3-3, which shuttles these proteins to the cytoplasm, resulting in gene derepression [85]. This dynamic transcriptional regulation within class II HDAC enzymes is implicated in cardiac hypertrophy and heart failure [110, 111]. Further studies have identified several kinases, such as Ca^{2+}/calmodulin-depend-

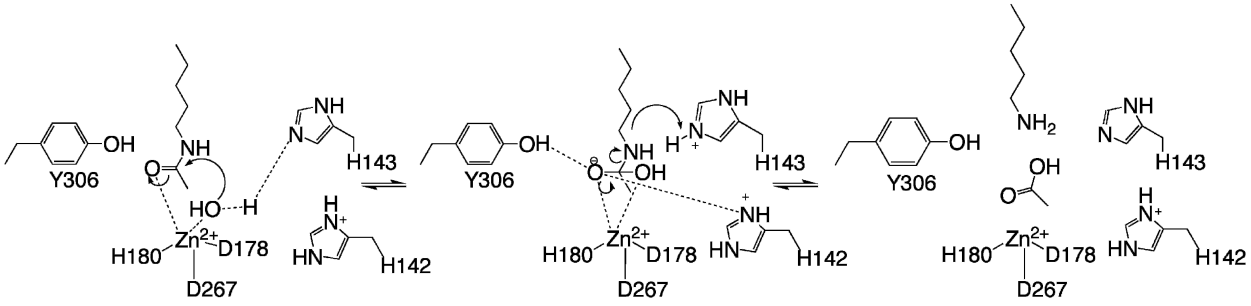


Figure 6. Proposed mechanism of HDAC8 [125]. Classically, H142 is suggested to be the general base and H143 the general acid [116]. However, more recent results suggest that H143 is a general base and general acid in the catalytic cycle as shown [125].

ent kinases and protein kinase D, which modify this class of enzymes and/or their recognition proteins, such as the myocyte enhancer factor-2 (MEF2) family, usually resulting in HDAC translocation to the cytoplasm [112–115].

The first crystal structure of a member of the HDAC family was that of a monomeric bacterial homologue from *A. aeolicus*, histone deacetylase-like protein (HDLP) [116]. Remarkably, despite sharing only ~13% sequence identity to arginase, HDLP was found to adopt an identical α/β fold to that of the previously determined rat arginase I structure [32], consisting of a central eight-stranded β-sheet (strand order 21387456) sandwiched between several α-helices (Fig. 2). The subsequently determined crystal structures of HDAC7 [117] and HDAC8 [118, 119], as well as the crystal structure of the bacterial class IIa homologue histone deacetylase-like amidohydrolase (HDAH) [120], revealed identical arginase topologies and monomeric structures (Fig. 2); HDAC8 and HDAH dimerize in the presence of certain inhibitors [118–120].

The structure of HDLP revealed the presence of a single metal ion interpreted as Zn^{2+} at the base of a ~12-Å-deep hydrophobic active site tunnel [116], and this arrangement was also subsequently observed in the crystal structures of HDAC8 [118, 119], HDAH [120], and HDAC7 [117] (Fig. 6). Thus, although these enzymes share the arginase topology, indicating evolutionary divergence from a common primordial ancestor, their metal binding specificity and stoichiometry have substantially diverged. Strikingly, a question still remains as to the identity of the metal ion required for HDAC8 catalysis *in vivo*, since catalytic activity is greatest with Co^{2+} or Fe^{2+} [121]. Unpublished work cited by Finnin and colleagues [116] indicates comparable activity for HDLP with Co^{2+} and Zn^{2+}, but no significant activity was detected using Fe^{2+} or Mn^{2+}.

In HDAC8, the active site Zn^{2+} ion is coordinated by H180, D178, D267, and a water molecule. These residues are conserved in arginase as H126, D124, and D234; thus, the Zn^{2+} binding site of HDAC enzymes corresponds to the $Mn^{2+}{}_{B}$ site of arginase [10]. The crystal structures of the Y306F HDAC8-substrate complex and native HDAC7 reveal a zinc-bound water molecule within hydrogen bonding distance to both H142 and H143 (HDAC8 numbering), the proposed general acid and general base catalytic pair [116–120, 122]. Vannini and colleagues [122] note that the zinc-bound water molecule is not within hydrogen bonding distance of Y306, suggesting that this residue does not serve as a general base in the catalytic mechanism [123, 124]. Although H142 had been previously considered the general base in the proposed catalytic mechanism [116, 118–120, 122], recent biochemical data implicate H143 as the general base because H143A HDAC8 is catalytically inactive [125]. Weak activity measured for H141A HDAC1 [126] (HDAC1 H141 corresponds to HDAC8 H143) is most likely due to copurification of other endogenous HDAC enzymes with recombinant HDAC1 in these experiments [126–128]. Instead of serving as a general base in catalysis, the protonated imidazolium group of H142 may play a role in stabilizing the tetrahedral intermediate during catalysis [125]. The C=O of the substrate acetyl moiety likely coordinates to the Zn^{2+} ion and accepts a hydrogen bond from Y306, thereby polarizing the C=O bond for nucleophilic attack by a water molecule promoted by Zn^{2+} and H143 [116–120, 122]. A mechanistic scheme consistent with the most current structural and functional data for HDAC8 is shown in Figure 6.

Acetylpolyamine amidohydrolases

APAHs catalyze the deacetylation of polyamines such as putrescine, spermidine, and spermine by cleavage of a non-peptide amide bond. These enzymes are found in both mammalian and bacterial species and function in the polyamine biosynthetic pathway to

degrade acetylated polyamines [129, 130]. In eukaryotes, the degradation of spermine is achieved by the sequential activities of spermidine/spermine-N^1-acetyltransferase (SSAT) and polyamine oxidase (PAO) to generate putrescine as the final product. The eukaryotic APAH homolog N^8-acetylspermidine deacetylase is a cytoplasmic enzyme that rapidly deacetylates N^8-acetylspermidine generated by the nuclear enzyme N^8-acetylspermidine transferase [129]. In contrast with mammalian polyamine catabolism, bacteria utilize polyamine oxidase (PAO) and APAH for polyamine degradation [130]. Arginase also plays a key role in this pathway by generating ornithine for the first and rate-limiting step of polyamine biosynthesis [131, 132].

Polyamines are vital for cell growth and proliferation and along with their biosynthetic enzymes are expressed at higher levels in tumor cells compared to normal tissues [133–137]. The accumulation of N^8-acetylspermidine due to the inhibition of N^8-acetylspermidine deacetylase induces differentiation in HL60 human leukemia cells [138] and stimulates the growth of L1210 murine lymphocytic leukemia cells [139]. Although the biological function of APAH has not been fully elucidated, polyamine deacetylation is proposed to modulate DNA structure and function in a manner similar to that proposed for histone deacetylation [139]. Spermidine stabilizes DNA and chromosomal structure *in vitro*, and acetylation reduces spermidine-binding affinity. Spermidine enters the cell nucleus where it binds to DNA primarily through charge-charge interactions. N^8-Acetylspermidine transferase acetylates spermidine and reduces spermidine-DNA affinity, which leads to polyamine dissociation. N^8-acetylated spermidine diffuses to the cytoplasm where it is rapidly deacetylated by N^8-acetylspermidine deacetylase. Spermidine can then be recycled as necessary [140].

APAH from *Mycoplana ramosa* exhibits broad substrate specificity and catalyzes the deacetylation of acetylputrescine, N^1-acetylspermidine, N^8-acetylspermidine, and N^1-acetylspermine [141, 142]. In contrast, human N^8-acetylspermidine deacetylase catalyzes the deacetylation of only N^8-acetylspermidine [141, 142]. Other prokaryotic APAHs, including those from *Streptomyces avellaneus* [143], *Arthrobacter* sp. [144], and *Micrococcus rubens* [145], are similarly specific and catalyze the deacetylation of only acetylputrescine.

The APAH enzyme from *M. ramosa* is a homodimer of 36.3-kDa subunits that requires one Zn^{2+} ion per subunit for catalytic activity; excess Zn^{2+} is inhibitory [142, 146]. Currently, no crystal structure is available for APAH. Based on sequence gazing and comparisons with the Zn^{2+}-binding site of carboxypeptidase A, Sakurada and colleagues [142] propose a Zn^{2+}-binding site in APAH with a HEXXH+E motif. However, this proposal is likely to be incorrect since APAH shares higher sequence identity with HDAC enzymes (20–25%) and histone deacetylase-like proteins (24–26%) than with carboxypeptidase A (15%). Specifically, the metal-binding ligands of HDLP (D168, H170, and D258), HDAH (D180, H182, and D268), and HDAC8 (D178, H180, and D267), which correspond to the Mn_B^{2+} site of arginase (D124, H126, and D234), are conserved in APAH as D195, H197, and D284 (Fig. 7). In support of the proposed Zn^{2+}-binding site of APAH, the D284A mutant shows diminished catalytic activity in comparison with the wild-type enzyme that is partially rescued by excess Zn^{2+} (H. A. Gennadios and D. W. Christianson, unpublished results). In addition to conserved metal-binding ligands, APAH also shares several hydrophobic core regions with arginase and HDAC enzymes (Fig. 7).

The APAH and HDAC enzymes share remarkable similarities and are proposed to be evolutionarily related [6]. Interestingly, when proteins with significant amino acid sequence similarity to human HDAC1 are compared, some of the eukaryotic histone deacetylases (*e.g.*, *Saccharomyces cerevisiae* HDA1) are found to be more similar to the bacterial APAH enzymes than to other histone deacetylases [6]. Furthermore, HDAC enzymes and APAH enzymes are functionally similar in that both families of enzymes recognize and cleave the acetyl group from an acetylaminopropyl-containing substrate, and both families of enzymes reconstitute the positive charge of the substrate to increase its affinity for negatively charged molecules (*e.g.*, DNA) [6]. Accordingly, the catalytic mechanism and three-dimensional structure of *M. ramosa* APAH are predicted to be very similar to those of HDAC enzymes.

Divergent evolution of the arginase fold

The crystal structures of HDLP, HDAH, HDAC8, and the catalytic domain of HDAC7 all reveal the arginase topology: an α/β fold consisting of a central parallel eight-stranded β-sheet (strand order 21387456) sandwiched between α-helices [116–118]. Closer inspection reveals that structural variations among the enzymes are found in their α-helical content, as well as in the lengths of corresponding β-strands (Fig. 2). That these HDAC enzymes adopt the arginase fold despite sharing <15% amino acid sequence identity indicates substantial evolutionary divergence that has influenced metal specificity, stoichiometry, and quaternary structure.

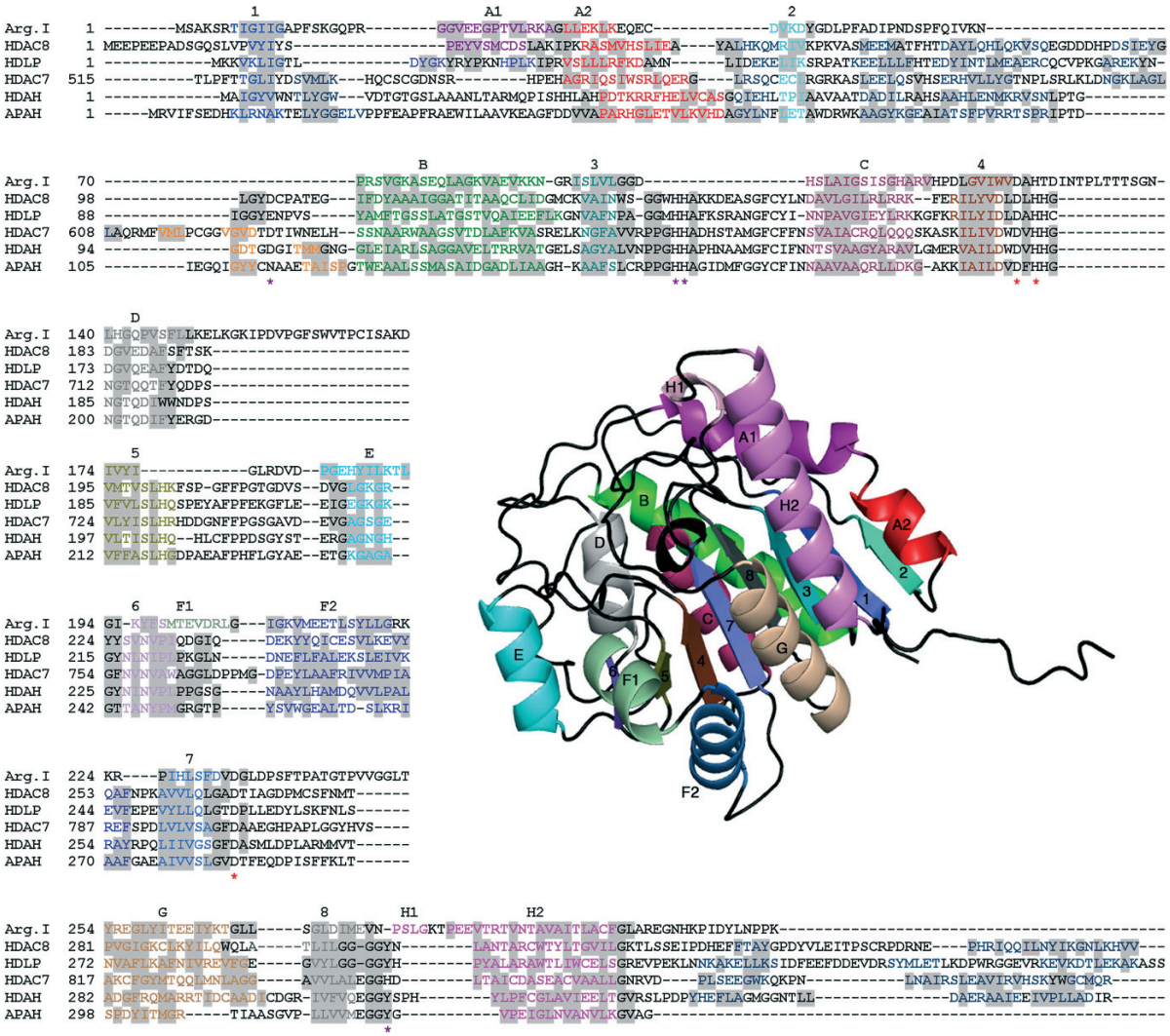


Figure 7. Amino acid sequences of human arginase I (Arg.I), human HDAC7, human HDAC8, HDAH from *Bordetella sp.*, and HDLP from *A. aeolicus* aligned by secondary structure homology. Sequences were first aligned using TOP3D and then loop regions were manually adjusted with appropriate gaps to optimize the alignment of each sequence with arginase and with its class partners (HDAC8 and HDLP are class I deacetylases, while HDAC7 and HDAH are class II deacetylases). The sequence of APAH was first aligned with HDAH by optimization of amino acid sequence identity, and then by manual optimization of predicted secondary structure with the observed secondary structure of arginase. Hydrophobic regions within areas of defined secondary structure and catalytic residues are highlighted in gray. The human arginase I structure is shown with secondary structural elements labeled and color-coordinated. The predicted secondary structure of APAH is indicated by the same color scheme. Additional β-strand and α-helix secondary structural elements observed in HDAC enzymes but not observed in arginase are colored orange and navy blue, respectively. A purple asterisk indicates conserved catalytic residues in deacetylases, and a red asterisk indicates conserved metal ligands.

Metal specificity

The location of the metal binding site at the edge of the central β-sheet near the C-terminal ends of strands 4 and 7 is conserved in arginase, HDAC enzymes, and HDAC-like enzymes. Specifically, the D(A,V,L)H + D motif (Fig. 3) is conserved for metal binding in these enzymes despite their significant evolutionary divergence. However, arginase binds two Mn^{2+} ions, whereas HDAC enzymes and HDAC-like enzymes bind a single transition metal ion (typically Zn^{2+}) as a consequence of substantial amino acid sequence divergence, including the substitution of residues that coordinate to $Mn^{2+}{}_A$ in arginase. Interestingly, while HDAC and HDAC-like enzymes were initially presumed to be zinc enzymes, Gantt and colleagues [121] suggest that HDAC8 is a possible Fe^{2+}-requiring enzyme *in vivo* since catalytic activity diminishes when the enzyme is substituted with other metal ions in the order $Co^{2+} > Fe^{2+} > Zn^{2+} > Ni^{2+}$. However, all crystal structures of HDAC and HDAC-like enzymes contain predominantly Zn^{2+}, which is normally added in the protein expression and purification steps.

Zinc coordination polyhedra in proteins are usually observed with a coordination number (CN) of 4 and a

slightly distorted tetrahedral geometry, with the possibility of an increased CN of 5 allowing for the electrostatic stabilization of a charged intermediate, as seen in the HDAC mechanism. The Zn^{2+} ion is considered a "borderline" metal in hardness with an atomic radius of 0.74 Å compared to Mn^{2+}, a "hard" metal with an atomic radius of 0.80 Å that favors octahedral coordination (CN = 6) [147, 148]. The protein ligands to the active site Zn^{2+} ion in HDAC and HDAC-like enzymes, two aspartate residues and one histidine residue, are somewhat atypical for mononuclear Zn^{2+} enzymes, which predominantly favor histidine residues in catalytic sites [121, 149]. In HDAC8, two of the Zn^{2+} ligands, D178 and H180, are located in a bend at the end of strand 4 and are separated by an aliphatic residue (L179 in HDAC8; Fig. 3). A search of the Protein Data Bank (pdb.org) using EMBL-EDI (http://www.ebi.ac.uk/msd-srv/msdsite/getEntry) for Zn^{2+}-containing proteins with a ligand set of Asp_2His yields 68 structures, of which only HDLP, HDAC8, HDAH, and HDAC7 contain Asp_2His ligand sets for mononuclear Zn^{2+} coordination in a catalytic site. Other pdb entries include: alkaline phosphatase [*e.g.*, 1HQA ($Zn^{2+}{}_3$) and 2GA3 ($Zn^{2+}{}_2$-Mg^{2+}) clusters with $AspHis_2$, $Asp_2HisSer$, and Asp_2(Glu)Thr ligand sets], aminopeptidase (*e.g.*, 1RTQ, $Zn^{2+}{}_2$ cluster with Asp_2His and AspHisGlu ligand sets), glutamate carboxypeptidase II (*e.g.*, 2OR4, $Zn^{2+}{}_2$ cluster with Asp_2His and AspHisGlu ligand sets), epimerase (1TQX, mononuclear Zn^{2+} site with Asp_2His_2 ligand set), inorganic pyrophosphatase (1WPP, $Zn^{2+}{}_2$ cluster with Asp_2His and Asp_3 His ligand sets), isomerase (1DE6, Zn^{2+}-Mn^{2+} cluster with $Asp_2HisGlu$ and Asp_2His ligand sets, respectively), lectin (2OX8, several mononuclear Zn^{2+} sites, one with $Asp_2HisGlu$ ligand set), phosphodiesterase (1XN0, Zn^{2+}-Mg^{2+} cluster with Asp_2His_2 ligand set), pyrophosphatase-phosphodiesterase (2GSU, $Zn^{2+}{}_2$ cluster with $Asp_2HisThr$ and $AspHis_2$ ligand sets), and a superantigen (1EU4, $Zn^{2+}{}_2$ cluster with Asp_2His and Asp ligand sets).

The same search performed on proteins containing Fe^{2+}, Co^{2+}, or Mn^{2+} yields 3, 1, and 55 hits, respectively. The Fe^{2+} ions coordinated by Asp_2His ligand sets are all contained in binuclear metal clusters: human calcineurin heterodimer (1AUI and 2P6B, Fe^{2+}-Zn^{2+} cluster with Asp_2His and $AspHis_2$ ligand sets, respectively) and inorganic pyrophosphatase mutant (2IW4, $Fe^{2+}{}_2$ cluster with Asp_2His and Asp_3His ligand sets). The single Co^{2+}-containing protein is alkaline phosphatase from *E. coli* (1Y6V, $Co^{2+}{}_3$ cluster with Asp_2 HisSer, $AspHis_2$, and AspGluThr ligand sets). The 55 Mn^{2+}-containing proteins consist primarily of arginase and arginase-like protein structures, such as amidinohydrolase (1GQ6 and 1GQ7) and agmatinase (1WOG and 1WOI). A few enzymes share the Asp_2His ligand set of the arginase $Mn_B{}^{2+}$ binding site: binuclear manganese-binding bacteriophage λ Ser/Thr protein phosphatase (1G5B), mononuclear manganese-binding galactosyltransferase LgtC from *Neisseria meningitides* (1GA8); binuclear manganese-binding inorganic pyrophosphatase (Family II) (1K23 and 2ENX), binuclear manganese-binding protein phosphatase-1 (1U32, 2BCD and 2IAE), and a putative diadenosine tetraphosphatase (2QJC). Binuclear lectin proteins ($Asp_2GluHis$) also appear in this search. Analysis of the above-referenced proteins reveals that 1G5B, 1U32, 2BCD, and 2QJC share a similar D(I,L)H + D motif for Mn^{2+} binding despite varying protein folds.

Quaternary structure

Crystal structure data show that the arginase fold has evolved to accommodate various quaternary structures, ranging from monomers and dimers observed for HDAC and HDAC-like enzymes to trimers and hexamers for arginases. While all mammalian arginases are heterologous trimers with C_3 symmetry (rat arginase I [32], human arginase I [35], human arginase II [36]), bacterial arginases are hexameric and contain two C_3 trimers that associate in face-to-face fashion with overall D_3 symmetry [37]. An important feature of trimer stabilization in rat arginase I is a ~20-residue "S"-shaped tail at the C terminus that accounts for 54 % of intersubunit contacts (Fig. 8). However, Mora and colleagues [150] indicate that this tail is not necessary for trimerization of human arginase I because its deletion does not disrupt trimerization. Additionally, conserved R255-E256 and R308-D204 intermolecular salt links stabilize quaternary structure in rat arginase I and human arginase I [32, 35]. The E256Q, R308A, R308E, and R308K variants of arginase I behave as monomers in solution. All show lower thermal stability and R308 variants show k_{cat} and catalytic efficiency values between 33–41 % and 13–17 % of those reported for the wild-type enzyme. However, Sabio and colleagues [151, 152] report comparable kinetics to wild-type arginase for the E256Q variant. In human arginase II, additional salt links are observed: the side chain of R201 forms an intermonomer salt link with E263, and the side chain of R205 forms an intermonomer hydrogen bond with N209 [36].

HDAC8 functions as a monomer in solution [119], while HDAC1–3 enzymes function in multiprotein complexes that are involved in transcriptional regulation [78, 79]. These other class I enzymes contain N-terminal segments important for oligomerization [76, 128, 153], and recombinant mouse HDAC1 and recombinant human HDAC3 expressed in eukaryotic cells purify as active enzymes only in multi-protein/

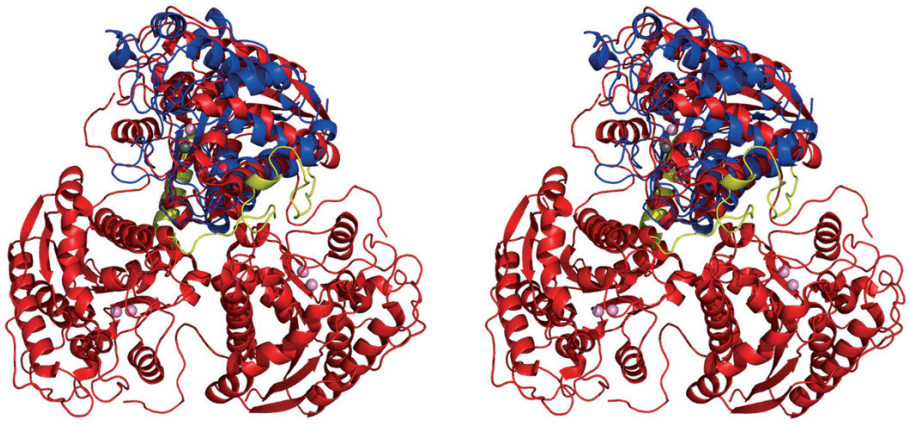


Figure 8. Stereoview of the superposition of human arginase I (red) and HDAC8 (blue). The two manganese ions are indicated as pink spheres, while the zinc ion is gray. The HDAC8 C-terminal segment that would block intersubunit interactions comparable to those observed in the arginase trimer is indicated in yellow.

multi-HDAC complexes [127]. Specifically, recombinant HDAC3 is capable of forming dimers and trimers [153], and SMRT and N-CoR both serve as HDAC3 activating cofactors [154–156]. In contrast with the ~20-residue "S" shaped tail at the C terminus of arginase that facilitates trimerization, HDAC8 contains a ~40-residue C-terminal tail with a loop-3_{10}-helix-loop segment that would destabilize arginase-like trimer assembly (Fig. 8). Other class I HDAC enzymes including HDAC1 and HDAC3 have additional C-terminal domains containing a nuclear localization signal (NLS) [82, 128], and HDAC2 has a C-terminal basic amino acid stretch similar to that of HDAC1 [128], demonstrating that the C-terminal segments of proteins with arginase folds are "evolvable" for stabilizing homomeric or heteromeric protein-protein interactions. The exception among the class I enzymes is HDAC8, which contains an apparent NLS domain in its central catalytic domain [60].

The HDAC1–3 enzymes also contain N-terminal domains that govern oligomerization, as well as interactions with other HDAC enzymes and/or proteins such as Sin3 and RbAp48 [76, 128, 153]. Additionally, various class IIa HDAC enzymes are known to interact with other proteins, *e.g.*, HDAC4 and HDAC5 interact with 14-3-3 proteins when localized in the cytoplasm and HDAC3 when localized in the nucleus [85, 157], and HDAC4–5, HDAC7 and HDAC9 interact with C-terminal-binding protein (CtBP), heterochromatin P1 (HP1), MEF2 transcription factor and many additional transcriptional corepressors, kinases, and DNA-anchoring transcriptional factors [157]. The requirement of multiprotein complexes for recombinant class I HDAC deacetylase activity is somewhat reminiscent of the fact that arginase evolved to increase enzyme stability and activity through trimer formation; arginase is less stable as a monomer [151, 152], and except for data published by Sabio and colleagues [151], it also is less active as a monomer [152].

Concluding remarks

In summary, arginase, HDAC enzymes, and APAH all represent examples of divergent evolution from a primordial ancestral metal-requiring hydrolase. Clearly, the arginase fold is readily evolvable in catalyzing guanidinium and amide hydrolysis reactions using two or one transition metal ions, respectively. Strikingly, the metal ion stoichiometry and specificity of these reactions also appear to be readily evolvable, in that maximal arginase activity requires a Mn^{2+}-Mn^{2+} cluster [158], but HDAC enzymes and related APAHs are more promiscuous as they function with a single Fe^{2+}, Zn^{2+}, or Co^{2+} ion [121]. Future studies of these fascinating enzymes promise to reveal new insights regarding myriad biological functions such as arginine homeostasis, nucleosome remodeling, transcription regulation, cell growth, and proliferation.

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