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Structure, Mechanism, and Inhibition of Histone Deacetylases and Related Metalloenzymes

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Abstract

Metal-dependent histone deacetylases (HDACs) catalyze the hydrolysis of acetyl-L-lysine side chains in histone and non-histone proteins to yield L-lysine and acetate. This chemistry plays a critical role in the regulation of numerous biological processes. Aberrant HDAC activity is implicated in various diseases, and HDACs are validated targets for drug design. Two HDAC inhibitors are currently approved for cancer chemotherapy, and other inhibitors are in clinical trials. To date, X-ray crystal structures are available for four human HDACs (2, 4, 7, 8) and three HDAC-related deacetylases from bacteria (histone deacetylase-like protein, HDLP; histone deacetylase-like amidohydrolase, HDAH; acetylpolyamine amidohydrolase, APAH). Structural comparisons among these enzymes reveal a conserved constellation of active site residues, suggesting a common mechanism for the metal-dependent hydrolysis of acetylated substrates. Structural analyses of HDACs and HDAC-related deacetylases guide the design of tight-binding inhibitors, and future prospects for developing isozyme-specific inhibitors are quite promising.

Introduction

Histone deacetylases (HDACs) function in transcriptional corepressor complexes where they catalyze the deacetylation of acetyl-L-lysine side chains in histone proteins, which typically alters chromatin structure and represses transcription. Since HDAC1 was first isolated [1], 18 HDACs have been identified: class I HDACs 1, 2, 3, and 8; class IIa HDACs 4, 5, 7, and 9; class IIb HDACs 6 and 10; class III enzymes, designated sirtuins 1–7; and the sole class IV enzyme, HDAC11 [2]. The metal-dependent class I, II, and IV HDACs are related to acetylpolyamine amidohydrolases and acetoin utilization proteins [3]; the class III enzymes, sirtuins 1–7, are evolutionarily and mechanistically distinct and are not discussed in this review. Intriguingly, many HDACs exhibit activity against non-histone substrates [4, 5]. Accordingly, these enzymes are sometimes more generally designated as "lysine deacetylases". The HDACs are being studied as drug targets for certain cancers [6–8], fibrotic diseases [9], cardiorenal disorders [10], neurodegeneration [11], and psychiatric disorders [12].

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Arginase-deacetylase fold

The first crystal structure of an HDAC was actually that of an HDAC-related deacetylase, the histone deacetylase-like protein (HDLP) from *Aquifex aeolicus*, related to HDAC1 by ~35% sequence identity [13]. Surprisingly, however, the α/β deacetylase fold seen in HDLP was first observed three years earlier in arginase, a binuclear manganese metalloenzyme [14]. Low sequence identities of ~15% between arginase and HDLP, and ~12% between arginase and HDACs, precluded homology prediction prior to the crystal structure determinations. It is now clear that the arginases, arginase-related enzymes (e.g., agmatinase, formiminoglutamate hydrolase [15]), HDACs, and HDAC-related deacetylases evolved from a common metalloprotein ancestor.

The central feature of the arginase-deacetylase fold is an 8-stranded parallel β -sheet (Figure 1a). In arginase, residues from loops L3, L4, and L7 coordinate to 2 Mn^{2+} ions. Metal binding is partially retained in HDACs, where conserved residues in loops L4 and L7 coordinate to a single Zn^{2+} ion [16, 17]. To date, this α/β fold has been observed in structures of the catalytic domains of HDAC2 [18•], HDAC4 [19], and HDAC7 [20], and full-length HDAC8 [21, 22]. This α/β fold has also been observed in three HDAC-related deacetylases: HDLP [13], HDAC-like amidohydrolase (HDAH) from *Bordetella/Alcaligenes* strain FB188 [23], and acetylpolyamine amidohydrolase (APAH) from *Mycoplana ramosa* and *Burkholderia pseudomallei* [24•, 25].

Metal ion function

Catalysis by HDACs and HDAC-related deacetylases requires a single transition metal ion. The catalytic metal ion binding site corresponds to the Mn^{2+}_B binding site in arginase and shares a common sequence motif (Figure 1b) [17]. Although arginase and the HDACs share no significant overall sequence identity, the conservation of metal ligands in the face of substantial evolutionary drift is consistent with divergence from a common metalloprotein ancestor.

While the HDACs and HDAC-related deacetylases are typically studied *in vitro* as Zn^{2+} -containing enzymes, the metal ion preference *in vivo* may differ. HDAC8 exhibits increased activity when substituted with Fe^{2+} , suggesting that it could function as a ferrous enzyme *in vivo* [26]. Crystal structures of HDAC8 substituted with Zn^{2+} or Fe^{2+} in complex with a hydroxamate inhibitor reveal similar metal coordination geometries [27•]. In contrast, APAH exhibits optimal activity with Mn^{2+} , followed closely by Zn^{2+} [28]. Arginase requires two Mn^{2+} ions for maximal activity [29], so the apparent preference of APAH for Mn^{2+} may be an evolutionary remnant.

Among the HDACs, HDAC8 is the most studied in terms of structure-function relationships. Enzymological studies confirm that a 1:1 metal ion stoichiometry is required for catalysis; 1:2 stoichiometry is inhibitory for Zn^{2+} but not for Fe^{2+} [26]. Interestingly, the X-ray crystal structure of HDAC8 complexed with the hydroxamate inhibitor 3-(1-methyl-4-phenylacetyl-1H-2-pyrrolyl)-N-hydroxy-2-propenamide reveals that H78, D87, H90, and D92 in the L2 loop of monomer A chelate a second metal ion, Zn^{2+}_B (Figure 2) [30]. Since the L2 loop contains D101, a conserved residue that is critical for substrate binding [30, 31], it is possible that the binding of Zn^{2+}_B inhibits activity by disrupting D101-substrate recognition. It should be noted that the Zn^{2+}_B site in HDAC8 does not correspond to the structural Zn^{2+}_B site in the class IIa deacetylases HDAC4 [19] and HDAC7 [20] (Figure 2), which is not inhibitory and is believed to play a role in mediating protein-protein interactions.

Two monovalent cations, typically K^+ but occasionally Na^+ , have been identified in most crystal structures of HDACs and HDAC-related deacetylases (Figure 2). Based on binding affinities and cellular concentrations, K^+ is the preferred monovalent cation *in vivo* [32••]. The weaker affinity site 1 (K^+_A) is formed in part by D176, which also accepts a hydrogen bond from active site residue H142. Coordination of K^+_A by D176 lowers the pK_a of H142, which is inhibitory; this suggests that H142 requires a higher pK_a for optimal catalytic activity, i.e., it must be protonated [32••]. Monovalent cation site 2 is ~21 Å away from the active site and exhibits higher affinity; the binding of K^+_B to this site activates catalysis.

A third monovalent cation site is observed in loop L7 of the HDAC-related deacetylase APAH, where K^+_C is liganded by the backbone C=O groups of F286, D289 and S292, the side chain of S292, and two water molecules (Figure 3) [24••]. The occupancy of K^+_C is higher when Y323 in the adjacent loop L8 is in the "out" conformation, which does not support catalysis. Thus, the binding of K^+_C may contribute to the inhibition of APAH observed at elevated K^+ concentrations [24••]. Although the binding of K^+_C has not yet been observed in HDAC8, we speculate that a third K^+ binding site could similarly contribute to the inhibition of HDAC8 observed at elevated K^+ concentrations [32••].

Catalytic mechanism

Catalysis by the HDACs and HDAC-related deacetylases proceeds through a promoted-water mechanism similar to that advanced for the prototypical Zn^{2+} -metalloenzymes thermolysin [33] and carboxypeptidase A [34]: the substrate carbonyl is polarized by Zn^{2+} coordination and/or hydrogen bonding, which facilitates the general base-promoted nucleophilic attack of a Zn^{2+} -bound water molecule (Figure 4) [13, 21, 22, 30, 32••]. In carboxypeptidase A, the relative invariance of K_M when the catalytic Zn^{2+} ion is substituted with Co^{2+} , Mn^{2+} , or Cd^{2+} suggests that the substrate carbonyl does not coordinate to the metal ion in the precatalytic enzyme-substrate complex [35]. In contrast, K_M varies significantly when the catalytic Zn^{2+} ion of HDAC8 is substituted with Co^{2+} and Fe^{2+} [26], so the substrate carbonyl likely coordinates to the active site metal ion in the precatalytic HDAC8-substrate complex. Consistent with these results, crystal structures of complexes with inactive HDAC8 mutants H143A and Y306F show that the substrate carbonyl coordinates to the catalytic Zn^{2+} ion [30, 31] and accepts a hydrogen bond from Y306 in H143A HDAC8 [30]. Thus, both Zn^{2+} and Y306 polarize the substrate carbonyl for nucleophilic attack. The Zn^{2+} coordination geometry in native HDAC8 is 5-coordinate square pyramidal and includes two water molecules [27•], one of which must be displaced by the substrate carbonyl [31].

Nucleophilic attack at the substrate carbonyl is believed to be the rate-determining step in catalysis by HDAC8 due in part to the enhanced hydrolytic activity of a substrate containing an activated trifluoroacetyl-L-lysine moiety [36]. One of the tandem histidine residues, H142-H143, serves as a general base in this step. The catalytic functions of these histidine residues are influenced by hydrogen bonds with D176 and D183, respectively. While H142 was initially proposed to be the general base [13, 31], this proposal is not supported by the residual activity and pH-rate dependence of H142A HDAC8 [37]. Furthermore, the K^+ -dependence of catalytic activity in H142A, D176A, and D176N HDAC8 mutants suggests that H142 serves as a general electrostatic catalyst [32••]. Therefore, H143 is the likely general base, consistent with the dramatic 10^5 -fold loss of catalytic activity measured for H143A HDAC8 [30]. The second histidine in the H158-H159 tandem pair of APAH is similarly critical for catalysis: H159A APAH exhibits no detectable catalytic activity, consistent with the loss of a general base [24••].

The tetrahedral intermediate and its flanking transition states are stabilized by coordination to Zn^{2+} and hydrogen bonds with Y306, H142, and H143 in HDAC8. While no structure is currently available for the binding of a transition state analogue to HDAC8, the binding of the buffer molecule *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) to APAH mimics the tetrahedral intermediate shown in Figure 4 and its flanking transition states: one Zn^{2+} -bound sulfonate oxygen is stabilized by hydrogen bonds with H158 and H159, and the other Zn^{2+} -bound sulfonate oxygen is stabilized by a hydrogen bond with Y323 [24••]. Since H143 of HDAC8 is closer to the leaving amino group, it is the likely proton donor that facilitates collapse of the tetrahedral intermediate. Thus, H143 serves as a general base-general acid catalyst (SL Gantt, PhD thesis, University of Michigan, 2006). Product acetate is initially coordinated to Zn^{2+} , as exemplified by the structure of the HDAH-acetate complex [23], and may subsequently exit through an internal channel [37].

Substrate specificity

Although the chemistry of catalysis is shared between HDAC8 and APAH, substrate specificity is markedly different. The dimer interface of APAH forms a constricted, "L"-shaped tunnel leading to the catalytic Zn^{2+} ion, which is accessible only to narrow, flexible substrates [24••]. Accordingly, APAH exhibits optimal activity against acetylpolymers and the small HDAC8 substrate L-Lys(ϵ -acetyl)coumarin, but not larger polypeptide substrates. In comparison, the active site of monomeric HDAC8 is relatively broad to better accommodate large protein substrates.

In the L2 loop of all class I and class II HDACs, D101 is strictly conserved to enforce substrate specificity: crystal structures of the inactive H143A and Y306F HDAC8 mutants complexed with a tetrapeptide substrate show that the side chain of D101 accepts hydrogen bonds from the backbone NH group of the scissile acetyl-L-lysine residue at position n and the backbone NH group of the adjacent residue at position $n + 1$ (Figure 5a) [30, 31]. Mutagenesis studies demonstrate that D101E HDAC8 exhibits 15% residual catalytic activity, but D101N HDAC8 exhibits only 0.003% residual activity, comparable to that of D101A HDAC8 [30]. Thus, residue 101 must have a branched side chain capable of simultaneously accepting two hydrogen bonds from a polypeptide substrate. Crystal structures also show that the NH group of the scissile acetyl-L-lysine side chain donates a hydrogen bond to the backbone carbonyl of G151, an interaction that presumably helps to orient the substrate amide group for catalysis [30, 31]. Additionally, there is evidence for the molecular recognition of a KRHR segment starting at position $n + 4$ of longer peptide substrates by an exosite on the protein surface [38].

The molecular determinants of substrate recognition by APAH differ from those of HDAC8 (Figure 5b). The crystal structures of the H159A APAH- N^8 -acetylspermidine and H159A APAH-acetylspermine complexes show that the positively charged substrate amino groups engage in hydrogen bond and cation- π interactions with residues defining the narrow active site cleft from both monomers of the APAH dimer. Similar to HDAC8, however, is a hydrogen bond between the backbone carbonyl of G167 and the amide NH group of the substrate [24••].

Inhibitor binding

HDAC inhibitors often exploit chelate interactions with the catalytic Zn^{2+} ion (Figure 6a). For example, hydroxamic acid inhibitors ionize to form exceedingly stable 5-membered ring chelates with Zn^{2+} [39]. Significantly, suberoylanilide hydroxamic acid (SAHA) was the first HDAC inhibitor approved for cancer chemotherapy [40, 41], and the crystal structure of the HDLP-SAHA complex was the first to reveal a hydroxamate- Zn^{2+} chelate complex in an HDAC-related deacetylase [13]. Interestingly, oxime amides, which partially mimic

hydroxamic acids, are effective Zn^{2+} ligands, and density functional theory calculations predict both 5- and 6-membered ring chelate complexes in HDAC active sites [42]. However, these binding modes remain to be proven in X-ray crystal structure determinations.

The first crystal structure of a benzamide inhibitor complexed with an HDAC was that of the HDAC2-*N*-(2-aminophenyl)-(4-phenyl)benzamide complex [18•]. This inhibitor class is becoming increasingly important in light of clinical trials with benzamides Entinostat/MS-275 and Mocetinostat/MGCD0103 in cancer chemotherapy. The crystal structure of the HDAC2-benzamide complex reveals that the inhibitor coordinates to the catalytic Zn^{2+} ion through both the carbonyl and amino groups to form an unusual 7-membered ring chelate complex.

Intriguingly, the structure of the HDAC4-5-(trifluoroacetyl)thiopene-2-carboxamide complex shows that the highly electrophilic carbonyl of the trifluoromethylketone is hydrated as the gem-diol to form a 4-membered ring chelate complex with the catalytic Zn^{2+} ion [19]. However, HDAC4 is a low-activity isozyme and its active site tyrosine is replaced by a histidine residue, H976; although the H976Y mutant exhibits increased activity, the side chain of residue 976 points away from the active site in wild-type HDAC4 and H976Y HDAC4. Thus, structures of HDAC4-trifluoromethyl ketone complexes do not fully mimic transition state binding by HDACs. Notably, the binding of a ketonic gem-diol in a metalloenzyme active site was first observed in a carboxypeptidase A-trifluoromethylketone complex [43].

Cyclic peptides and depsipeptides comprise a particularly fascinating class of HDAC inhibitors [44], especially since the bicyclic depsipeptide romidepsin (Istodax) (Figure 6b) was recently approved for the treatment of cutaneous T-cell lymphoma [45]. In the absence of an X-ray crystal structure, modeling studies suggest that upon disulfide bond reduction *in vivo*, the thiol side chain of romidepsin coordinates to the active site Zn^{2+} ion [46].

The mode of action of the marine natural product largazole is similar to that of romidepsin. Largazole is a cyclic depsipeptide isolated from the cyanobacterium *Symploca* sp. in Key Largo, Florida (whence it is named), that exhibits remarkable antiproliferative activity [47]. The generation of the inhibitory thiol side chain of largazole requires hydrolysis of the thioester moiety *in vivo* (Figure 6b). The resulting largazole thiol is a potent inhibitor of several HDAC isozymes [48, 49], and modification of the depsipeptide skeleton yields analogues that are even more potent inhibitors than largazole itself [50•]. The recently-determined structure of HDAC8 complexed with largazole thiol reveals that the inhibitor binds in the active site with its side chain thiolate coordinated to the catalytic Zn^{2+} ion [51•].

Finally, a new class of chiral α -aminoketone inhibitors has been reported recently [52]. A novel feature of these compounds is that they interact with the internal acetate release channel, which is believed to contribute to remarkable selectivity among class I HDACs. X-ray crystal structures of two HDAC8- α -aminoketone complexes reveal that only the inhibitor α -amino group coordinates to the active site Zn^{2+} ion. The ketone carbonyl oxygen atom of each inhibitor is too far ($\geq 2.8 \text{ \AA}$) from the Zn^{2+} ion to make an inner-sphere coordination interaction, so this interaction is instead described as a weakened electrostatic interaction.

Conclusions

Rapidly growing interest in HDAC metalloenzymes as validated targets for therapeutic intervention has led to a wealth of recent structural and functional data on HDACs, HDAC-related deacetylases, and their inhibitor complexes. While tight-binding inhibitors can mimic

the 4-membered ring Zn^{2+} chelate interactions of the transition state in catalysis, excellent inhibitors also result from the use of different functional groups capable of forming 5-, 6-, and 7-membered ring Zn^{2+} chelates. Alternatively, as exemplified by macrocyclic depsipeptides, an inhibitor can effectively utilize a monodentate Zn^{2+} ligand, such as a thiolate group, to achieve affinities in the nanomolar range or better. Macrocyclic inhibitors are capable of interactions with the mouth of the active site cleft, and other examples include macrolides [53•] and ketolides [54•]. Given that the mouth of the active site is structurally divergent among HDAC isozymes, inhibitors that interact with this region of the protein surface promise to illuminate new approaches toward isozyme specificity. This is a prominent goal of current research efforts [55], and macrocyclic inhibitors appear to be perfectly suited for such molecular customization. Clearly, the structural biology of HDACs and HDAC-related deacetylases will continue to provide exciting new opportunities for the study of enzyme structure-mechanism and structure-inhibition relationships for many years to come.

Research Highlights

- Metal-dependent histone deacetylases (HDACs) catalyze the hydrolysis of acetyl-L-lysine side chains in histone and non-histone proteins to regulate important biological processes.
- HDACs and HDAC-related deacetylases such as acetylpolyamine amidohydrolase adopt the α/β fold first observed in the binuclear manganese metalloenzyme arginase.
- HDACs employ a promoted-water mechanism for catalysis, in which the active site metal ion (Zn^{2+} or Fe^{2+} *in vivo*) and a histidine general base activate a metal-bound water molecule for nucleophilic attack at the substrate.
- Potent HDAC inhibitors chelate the active site metal ion.
- Macrocyclic HDAC inhibitors interact with the mouth of the active site, highlighting strategies for the design of isozyme-specific inhibitors that may be therapeutically useful.

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Figure 1. Arginase-deacetylase fold

(a) Topology diagrams of arginase, HDAC8, and APAH reveal a common α/β fold with a central, 8-stranded parallel β -sheet (strand order 21387456). The relative positions of metal ligands are indicated on arginase (loops L3, L4, and L7), and HDAC8 and APAH (loops L4 and L7) (each loop is numbered after its preceding β -strand). Green circles indicate residues conserved in arginase, HDAC, APAH, and all related enzymes; yellow circles indicate residues conserved only in arginase and arginase-related metalloenzymes. (b) The $\text{Mn}^{2+}_{\text{B}}$ site of arginase is conserved in HDAC8, APAH, and related metalloenzymes as **D(A,V,L,F)HX_{~100}D** (boldface indicates metal ligands). The $\text{Mn}^{2+}_{\text{A}}$ site of arginase is not conserved in HDACs or HDAC-related deacetylases. Non-protein metal ligands (red spheres) are solvent molecules in arginase and HDAC8, and the oxygen atoms of a hydroxamate inhibitor in APAH.

Figure 2. Metal binding sites

Structures of HDAC8, HDAC7, and APAH illustrate the binding sites of catalytic metal ion Zn^{2+}_A and structural metal ions K^+_A and K^+_B (note that the K^+_B site is occupied by Na^+ in APAH). Additionally shown are putative inhibitory metal ions Zn^{2+}_B in HDAC8 and K^+_C in APAH, as well as structural metal ion $\text{Zn}^{2+}_{B'}$ in HDAC7.

Figure 3. Mode of inhibition by excess K^+

The "out" conformation of Y323 in the L8 loop of APAH is facilitated by the binding of K^+_C (magenta sphere) to the adjacent L7 loop. Ligands to K^+_C include the backbone C=O groups of F286, D289, and S292; the side chain of S292; and two water molecules (red spheres). This third monovalent cation binding site may contribute to the inhibition of enzyme activity at elevated K^+ concentrations by destabilizing the "in" conformation of Y323 required for catalysis. Although the binding of a third monovalent cation to HDACs has not yet been observed, such a binding mode could similarly account for the inhibitory effects of excess K^+ . Reprinted with permission from reference [24••]. Copyright 2011 American Chemical Society.

Figure 4. Proposed mechanism of HDACs and HDAC-related enzymes

The active site transition metal ion of HDAC8 (Zn^{2+} or Fe^{2+}) and general base H143 promote the nucleophilic attack of a metal-bound water molecule at the metal-coordinated $\text{C}=\text{O}$ group of the acetyl-L-lysine substrate (for clarity, only the side chain of acetyl-L-lysine is shown). As drawn, the nucleophilic lone electron pair on the metal-bound water molecule becomes available only upon proton abstraction, e.g., the electron pair of the breaking O-H bond could add to the π^* orbital of the substrate carbonyl, although other molecular orbital explanations are possible. The oxyanion of the tetrahedral intermediate and its flanking transition states are stabilized by metal coordination as well as hydrogen bond interactions with Y306, H143, and H142. H143 serves as a general acid catalyst to facilitate the collapse of the tetrahedral intermediate to form acetate and L-lysine after an intervening proton transfer (possibly mediated by H143). We speculate that the side chain of Y306 may undergo a conformational transition from an "out" conformation to an "in" conformation to accommodate substrate binding and catalysis, based on the conformational mobility of the corresponding residue in related enzymes (Y976 in H976Y HDAC4 [19] and Y323 in APAH [24•]). If so, this could suggest an induced-fit substrate binding mechanism reminiscent of that described for Y248 in carboxypeptidase A [34].

Figure 5. Stereoviews of enzyme-substrate recognition

(a) Molecular recognition of a polypeptide substrate in the active site of Y306F HDAC8 is governed by the side chain of D101, which accepts hydrogen bonds from the backbone NH group of the scissile acetyl-L-lysine residue at position n and the adjacent residue at position $n + 1$. Residue 101 must be capable of making these dual hydrogen bond interactions for optimal function [30, 31]. Selected active site residues are indicated. Atoms are color-coded as follows: C = green (protein) or yellow (substrate), N = blue, O = red. Metal coordination and hydrogen bond interactions are indicated by black and orange dashed lines, respectively; the catalytic Zn²⁺ ion is a lavender sphere, and the Zn²⁺-bound water molecule is shown as a smaller red sphere. (b) Molecular recognition of a polyamine substrate as observed in the H159A APAH-acetylspermine complex [24••]. The positively-charged amino groups of the substrate make hydrogen bonded salt links with the negatively-charged side chains of E117 and E106 from the adjacent monomer; one secondary amino group also makes an electrostatic interaction with Y168 (3.3 Å, red dashed line) and a cation- π interaction with F225 (green dashed line). The side chain of Y323 may undergo a conformational change to donate a hydrogen bond to the scissile carbonyl linkage. Atoms are color-coded as follows: C = yellow (protein, Y323 "in" conformation), gray (protein, Y323 "out" conformation), olive (substrate), or light brown (E106 from the adjacent monomer in the background, which is not labeled), N = blue, O = red. Metal coordination and hydrogen bond interactions are

indicated by black and orange dashed lines, respectively; the catalytic Zn^{2+} ion is a lavender sphere, and water molecules are smaller red spheres.

Figure 6. Zn^{2+} coordination by HDAC inhibitors

(a) Examples of inhibitors capable of forming 4-, 5-, 6-, and 7-membered ring chelates with the catalytic Zn^{2+} ion are shown. It is likely that some of these inhibitors, e.g., the hydroxamic acids, ionize once they are fully bound to the metal ion. (b) Romidepsin and largazole are depsipeptide prodrug HDAC inhibitors that convert into active inhibitory thiols by *in vivo* reduction and hydrolysis reactions, respectively. The crystal structure of the HDAC8-largazole thiol complex reveals that the thiol side chain of the active inhibitory form of largazole coordinates to the active site Zn^{2+} ion, presumably as the negatively-charged thiolate anion [51•].