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General Base-General Acid Catalysis in Human Histone Deacetylase 8

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Abstract

Histone deacetylases (HDACs) regulate cellular processes such as differentiation and apoptosis, and are targeted by anti-cancer therapeutics in development and in the clinic. HDAC8 is a metal-dependent class I HDAC and is proposed to use a general acid-base catalytic pair in the mechanism of amide bond hydrolysis. Here, we report site-directed mutagenesis and enzymological measurements to elucidate the catalytic mechanism of HDAC8. Specifically, we focus on the catalytic function of Y306 and the histidine-aspartate dyads H142-D176 and H143-D183. Additionally, we report X-ray crystal structures of four representative HDAC8 mutants: D176N, D176N-Y306F, D176A-Y306F, and H142A-Y306F. These structures provide a useful framework for understanding enzymological measurements. The pH dependence of k_{cat}/K_M for wild-type Co(II)-HDAC8 is bell-shaped with two pK_a values of 7.4 and 10.0. The upper pK_a reflects the ionization of the metal-bound water molecule and shifts to 9.1 in Zn(II)-HDAC8. The H142A mutant has 230-fold lower activity than wild-type HDAC8, but the pK_{a1} value is not altered. Y306F HDAC8 is 150-fold less active than the wild-type enzyme; crystal structures show that Y306 hydrogen bonds with the zinc-bound substrate carbonyl, poised for transition state stabilization. The H143A and H142A/H143A mutants exhibit activity that is over 80,000-fold lower than wild-type HDAC8; the buried D176N and D176A mutants have significant catalytic

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ACCESSION CODES

The atomic coordinates and crystallographic structure factors of HDAC8 mutants D176N, D176N-Y306F, D176A-Y306F, and H142A-Y306F have been deposited in the Protein Data Bank (www.rcsb.org) with accession codes 5DC5, 5DC6, 5DC7, and 5DC8, respectively.

Notes

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effects, with more subtle effects from D183N and D183A. These enzymological and structural studies strongly suggest that H143 functions as a single general base-general acid catalyst, while H142 remains positively charged and serves as an electrostatic catalyst for transition state stabilization.

The reversible acetylation of lysine residues is an important regulatory mechanism that controls many processes, including gene expression, cellular differentiation, cancer progression, and metabolic reprogramming.¹⁻⁴ Lysine acetylation was first found in histones,⁵ but many additional target proteins are also regulated by lysine acetylation.^{6,7} The enzymes responsible for the acetylation and deacetylation of lysine residues are the histone acetyltransferases and the histone deacetylases (HDACs), respectively. HDACs are classified into four groups based on sequence identity. The class III HDACs (SIRT1-7) consume NAD⁺ as a co-substrate during the deacetylation reaction, and will not be further discussed here.⁸ A divalent metal ion cofactor is required by the enzymes in classes I (HDACs 1-3 and HDAC8), II (HDACs 4-6 and HDACs 9,10) and IV (HDAC11).⁹ Although the HDACs were originally proposed to be zinc-dependent enzymes, Fe(II)- and Co(II)-HDAC8 display higher catalytic activity and lower K_M values than Zn(II)-HDAC8, with the more prevalent Fe(II) possibly serving as the *in vivo* metal cofactor under certain conditions.¹⁰

The metal-dependent HDACs are targets for cancer chemotherapy, and four inhibitors to date have been approved by the FDA for clinical use: Vorinostat,¹¹⁻¹³ Istodax,^{14,15} Belinostat,^{16,17} and Panobinostat.¹⁸ Other HDAC inhibitors are currently in clinical trials targeting various types of cancer. Intriguingly, HDAC inhibitors also show promise for treating neurological diseases such as Huntington's disease, Parkinson's disease, spinal muscular atrophy, and multiple sclerosis.^{19,20} A better understanding of the HDAC catalytic mechanism will inform the design of new inhibitors, particularly with regard to hydrogen bond interactions that can target active site residues depending on their protonation states.

HDAC8 was the first human HDAC to yield an X-ray crystal structure,^{21,22} and this isozyme has been highly amenable to *in vitro* characterization in solution and in the crystal.²³⁻³⁰ Indeed, HDAC8 is an excellent model system for understanding catalysis by all metal-dependent HDACs. Based on the crystal structure of an HDAC-like protein from *Aquifex aeolicus* reported five years prior to the structure determination of HDAC8, a catalytic mechanism was proposed for the metal-dependent HDACs in which tandem histidine residues H142 and H143 serve as a general base-general acid catalyst pair.³¹ Specifically, H142 is suggested to be the general base catalyst, deprotonating the metal-bound water molecule to activate it for attack on the substrate carbonyl. The resulting oxyanion of the tetrahedral intermediate is stabilized by the divalent metal ion and by a hydrogen bond from Y306. H143 is proposed to serve as the general acid catalyst that protonates the amino leaving group to facilitate the collapse of the tetrahedral intermediate to form products lysine and acetate.

In HDAC8, H142 and H143 each donate a hydrogen bond to an aspartate side chain so as to form D176-H142 and D183-H143 dyads (Figure 1). Such histidine-aspartate dyads most often occur with syn-oriented carboxylates that serve to orient the histidine imidazole group,

stabilize the proper tautomeric form required for catalysis, and/or enhance the basicity of the imidazole group.^{32–34} For example, H36 in myoglobin exhibits a relatively high pK_a of approximately 8 due to a hydrogen bond with the negatively charged carboxylate group of E38.^{35,36} If the carboxylate-histidine dyad is buried or partially buried, the pK_a will be greater, and if the histidine side chain is hydrogen bonded to an uncharged carbonyl instead of a negatively charged carboxylate, the pK_a will be about half as much.³⁷

In the H143-D183 pair of HDAC8, D183 is solvent-exposed; this residue is conserved as aspartate, asparagine, or glutamine in other HDAC isozymes. The mutation of the corresponding asparagine residue in the histone deacetylase-like amidohydrolase from *Bordetella/Alcaligenes* strain FB188 (a class II HDAC homologue) has a relatively minor effect on catalysis, reducing activity to 25% of that measured for the wild-type enzyme,³⁸ suggesting that the identity of the amino acid at this position is not critical for catalysis. In contrast, the H143A mutant of HDAC8 shows a substantial, 80,000-fold loss of activity (Table 2), consistent with a proposed role as a general base and/or acid catalyst.²⁵ The H142-D176 pair of HDAC8 is more buried, and D176 is strictly conserved among metal-dependent HDACs. In addition to forming a hydrogen bond with H142, the D176 carboxylate coordinates to a monovalent cation (typically K^+) that inhibits catalysis by 10-fold.²⁶ Finally, Y306 is conserved in class I, IIb, and IV HDACs, and is a histidine in the class IIa HDACs. Mutation of this histidine to a tyrosine in HDACs 4 and 7 increases the deacetylase activity by 1000- to 6000-fold, highlighting the importance of a tyrosine at this position for maximal catalytic activity.^{39,40}

The crystal structure of Y306F HDAC8 complexed with an intact tetrapeptide assay substrate²³ reveals that H142 is 0.5 Å closer to the catalytic Zn(II) ion than H143, and H143 is 1.9 Å closer to the acetyllysine NH group than H142 (Figure 1). Accordingly, the proposed roles of H142 and H143 as general base and general acid catalysts, respectively, would seem reasonable.³¹ However, it was later discovered that, of the two monovalent cations observed in the structures of HDAC8, the monovalent cation coordinated by D176 is inhibitory, suggesting that H142 is protonated in the active form of the enzyme.²⁶ This would not be expected if H142 were the general base catalyst. One alternative mechanism that has been proposed is the use of a single residue as a general base-general base catalyst,²⁶ as found for metalloproteases such as thermolysin and carboxypeptidase A.^{24,41,42} To determine whether HDAC8 uses a general base-general acid pair or a single general base-general acid in catalysis, we now report structural and functional studies of HDAC8 mutants focusing on the histidine-aspartate dyads H142-D176 and H143-D183 as well as Y306. The resulting data are most consistent with a mechanism in which H143 functions as a single general base-acid and H142 remains protonated throughout the catalytic cycle to serve as a general electrostatic catalyst, while Y306 stabilizes the oxyanion of the tetrahedral intermediate and its flanking transition states through hydrogen bonding.

MATERIALS AND METHODS

Materials

Unless specified, chemicals and supplies were purchased from Fisher or Sigma. All chemicals were of the highest quality available. Chromatography resins were purchased from GE Healthcare, AMPSO and CAPSO from SigmaUltra.

Expression and purification of wild-type HDAC8 and HDAC8 mutants

Site-directed mutagenesis of the pHD2-TEV-His plasmid was carried out using a QuikChange kit (Stratagene). For enzymological studies, recombinant HDAC8 was expressed and purified as previously described, and the His₆-tag was cleaved with TEV protease.¹⁰ The co-purifying metals were removed as previously described to make apo-HDAC8, which was then reconstituted with stoichiometric Co(II) or Zn(II) before measuring catalytic activity.¹⁰ Because Co(II)-HDAC8 has higher catalytic activity than Zn(II)-HDAC8,¹⁰ HDAC8 mutants were assayed with Co(II) for increased sensitivity. For X-ray crystallographic studies, site-directed mutagenesis of an alternative construct optimized for crystallization, HDAC8-6His-pET20b, was similarly performed using a QuikChange kit, and mutants were expressed and purified as described.^{25,29}

HDAC8 activity assay

The catalytic activity of wild-type and mutant HDAC8 was measured using a commercially available fluorescent assay (Enzo Life Sciences) with either the p53-based Fluor de Lys HDAC8 substrate Ac-Arg-His-Lys(Ac)-Lys(Ac)-AMC (AMC = aminomethylcoumarin) or the histone H4-based Fluor de Lys H4-AcK16 substrate Ac-Lys-Gly-Gly-Ala-Lys(Ac)-AMC,¹⁰ as indicated. The steady-state kinetic parameters, k_{cat} , K_{M} and $k_{\text{cat}}/K_{\text{M}}$, were determined from initial reaction rates using 1.2–50 μM HDAC8 and 10–2000 μM substrate in assay buffer-1 [25 mM Tris (pH 8.0), 137 mM NaCl, and 2.7 mM KCl]. All assay buffers were pre-treated with Chelex resin (Bio-Rad) to remove trace divalent metal ions, which can alter the catalytic activity of HDAC8.¹⁰

The pH dependence of $k_{\text{cat}}/K_{\text{M}}$ was determined for mutant and wild-type HDAC8 using the following series of non-chelating buffers:^{43,44} MOPS (pH 6.5 and 7.0), Hepes (pH 7.1, 7.3, 7.6, 7.8, and 8.1), AMPSO (pH 8.4, 8.7, and 8.8), CAPSO (pH 9.2, 9.4, and 9.5) and CAPS (pH 10.0) in assay buffer-2 [50 mM buffer, 140 mM NaCl and 3 mM KCl]. Control experiments demonstrated that the enzyme activity is not affected by altering the identity of the buffer, only the pH. The concentration of substrate used was kept below the experimentally determined K_{M} value for each mutant. The pH dependence of $k_{\text{cat}}/K_{\text{M}}$ for the HDAC8 mutants was measured following pre-incubation of the mutants (15 – 125 μM Co(II)-enzyme) on ice for 0–15 minutes at the assay pH; the enzyme concentration for pre-incubation depended on mutant activity. To determine the pH dependence of wild-type HDAC8 (0.4 μM enzyme), assays were initiated by addition of the HDAC8 without pre-incubation at the assay pH, due to the observed instability of the enzyme at elevated pH and low enzyme concentrations. The pH of each assay was determined by combining the reaction components and measuring the pH of the final solution at 25° C, substituting the appropriate buffers in place of the enzyme and substrate solutions. Because the substrates

used have relatively low k_{cat}/K_M and high K_M values, the reported $\text{p}K_a$ values are assumed to be thermodynamic $\text{p}K_a$ values and not perturbed by kinetic effects.

The majority of the pH dependence data are bell-shaped, reflecting two ionizations, and are fit to Equation 1.

$$V = \frac{k_{\text{obs}} \cdot E_{\text{tot}}}{\left(\frac{[H^+]}{K_{a1}} + 1 + \frac{K_{a2}}{[H^+]}\right)} \quad \text{Eq. (1)}$$

For any bell-shaped pH dependence, it is possible that the ascending and descending limbs may be crossed, with the $\text{p}K_a$ for the group titrated in the acidic limb being greater than the $\text{p}K_a$ for the group titrated in the basic limb. However, in the data analyzed here, there is no evidence that the $\text{p}K_a$ s are crossed. The pH dependence of the H142A mutant differs from wild-type HDAC8, and appears to have a third $\text{p}K_a$ in its pH dependence profile. The data for H142A HDAC8 can be fit to Eq. 2, which is derived from a model in which three ionizations determine the observed catalytic rate.

$$V = \frac{k_{\text{obs1}} \cdot E_{\text{tot}}}{\left(\frac{[H^+]}{K_{a1}} + 1 + \frac{K_{a2}}{[H^+]} + \frac{K_{a2} \cdot K_{a3}}{[H^+]^2}\right)} + \frac{k_{\text{obs2}} \cdot E_{\text{tot}}}{\left(\frac{[H^+]^2}{K_{a1} \cdot K_{a2}} + \frac{[H^+]}{K_{a2}} + 1 + \frac{K_{a3}}{[H^+]}\right)} \quad \text{Eq. (2)}$$

In this model, the unprotonated (E) and triply protonated ($\text{E}^{3\text{H}}$) forms of the enzyme are inactive, while E^{H} and $\text{E}^{2\text{H}}$ are catalytically active, with rate constants of k_{obs1} and k_{obs2} , respectively.

pH stability measurements

To measure the pH stability of the enzyme, 5 μM Co(II)-HDAC8 was incubated for 20 min at 25 °C in assay buffer-2 at pH values between 5.5 and 11.0. The enzyme was then diluted to 0.44 μM in assay buffer-1 at pH 8.0 and allowed to re-equilibrate for 15 min before measuring catalytic activity at pH 8.0 with 0.4 μM HDAC8 and 50 μM substrate. The pH stabilities of the mutants were similarly determined, but with higher enzyme concentrations to allow for measurable catalytic activity. The pH stability of the substrate was demonstrated by preincubating the Fluor de Lys HDAC8 substrate for 20 min at pH 5.5 to 11.0 before reactions were initiated by dilution of the substrate into the pH 8.0 assay. The time-dependent effects of alkaline pH on wild-type HDAC8 were determined by pre-incubating 0.44 μM Co(II)-HDAC8 in assay buffer-2 at elevated pH at 25 °C for 1–60 min before measuring the catalytic activity at the same pH as used for pre-incubation.

Crystallization and data collection

Crystals of the D176N HDAC8–M344, D176N–Y306F HDAC8–substrate, D176A–Y306F HDAC8–substrate, and H142A–Y306F HDAC8–substrate complexes were prepared by cocrystallization in sitting drops using the vapor diffusion method at 16 °C (D176N–Y306F HDAC8 and H142A–Y306F HDAC8) or 21 °C (D176N HDAC8 and D176A–Y306F HDAC8).

D176N HDAC8 was cocrystallized with M344 by adding a 1.5 μ L drop containing 5.0 mg/mL protein, 50 mM Tris (pH 8.0), 150 mM KCl, 5% (v/v) glycerol, 5% (v/v) DMSO, 1 mM DTT, 2 mM M344, and 30 mM glycylglycylglycine to a 1.5 μ L drop of precipitant solution and equilibrating against a 150 μ L reservoir of precipitant solution [100 mM imidazole (pH 7.0), 15% (w/v) PEG 1500 (Hampton Research), and 4 mM TCEP].

D176N-Y306F HDAC8 was cocrystallized with the tetrapeptide assay substrate Ac-Arg-His-Lys(Ac)-Lys(Ac)-aminomethylcoumarin (Enzo Life Sciences, BML-KI178) by adding a 600 nL drop containing 3.5 mg/mL protein, 2 mM substrate, 30 mM glycylglycylglycine, 50 mM Tris (pH 8.0), 91.1 mM KCl, 54.8 mM NaCl, 3% (v/v) glycerol, 0.6 mM DTT, and 0.4 mM MgCl_2 to a 600 nL drop of precipitant solution and equilibrating against a 100 μ L reservoir of precipitant solution [100 mM Tris (pH 8.0), 13% (w/v) PEG 8000 (Hampton Research), and 4 mM TCEP].

D176A-Y306F HDAC8 was cocrystallized with the tetrapeptide assay substrate by adding a 500 nL drop containing 4.5 mg/mL protein, 2.5 mM substrate, 30 mM glycylglycylglycine, 50 mM Tris (pH 8.0), 76.4 mM KCl, 68.5 mM NaCl, 2.5% (v/v) glycerol, 0.5 mM DTT, and 0.5 mM MgCl_2 to a 500 nL drop of precipitant solution and equilibrating against a 100 μ L reservoir of precipitant solution [100 mM Tris (pH 8.0), 15% (w/v) PEG 10000 (Hampton Research), and 4 mM TCEP].

H142A-Y306F HDAC8 was cocrystallized with the tetrapeptide assay substrate by adding a 600 nL drop containing 3.5 mg/mL protein, 2 mM substrate, 30 mM glycylglycylglycine, 50 mM Tris (pH 8.0), 91.1 mM KCl, 54.8 mM NaCl, 3% (v/v) glycerol, 0.6 mM DTT, and 0.4 mM MgCl_2 to a 600 nL drop of precipitant solution and equilibrating against a 100 μ L reservoir of precipitant solution [100 mM Tris (pH 8.0), 10% (w/v) PEG 35000 (Fluka), and 4 mM TCEP].

Crystals of each mutant typically appeared within 1 day. Crystals were flash-cooled in liquid nitrogen after transfer to a cryoprotectant solution consisting of precipitant solution supplemented with 20–25% glycerol. X-ray diffraction data were collected on beamline X29 at the National Synchrotron Light Source (NSLS, Brookhaven National Laboratory, Upton, New York) for the D176N HDAC8–M344 and D176A-Y306F HDAC8–substrate complexes, and on beamline NE-CAT 24-ID-C at the Advanced Photon Source (APS, Argonne National Laboratory, Argonne, Illinois) for the D176N-Y306F HDAC8–substrate and H142A-Y306F HDAC8–substrate complexes. Data were indexed, integrated and scaled using HKL2000.⁴⁵ Data collection statistics are recorded in Table 1.

Phasing, model building, and structure refinement

Crystals belonged to space group $P2_1$ with 2 molecules in the asymmetric unit for D176N HDAC8, or space group $P2_12_12_1$ for D176N-Y306F HDAC8, D176A-Y306F HDAC8, and H142A-Y306F HDAC8. Crystal structures were solved by molecular replacement using PHENIX⁴⁶ with the atomic coordinates of the H143A HDAC8–tetrapeptide substrate complex (PDB accession code 3EWF)²⁵ less substrate, metal ions, and solvent molecules used as a search probe for rotation and translation function calculations. The models were refined with iterative cycles of refinement in PHENIX⁴⁶ and manual model rebuilding in

COOT.⁴⁷ Solvent molecules and inhibitors were added after initial rounds of refinement. Translation Libration Screw (TLS) refinement was performed in the late stages of refinement for the D176N HDAC8–M344 and D176N–Y306F HDAC8–substrate and complexes. TLS groups were automatically determined using PHENIX. For the H142A–Y306F HDAC8–substrate complex, anisotropic temperature factor refinement was performed at the last stage of refinement for all atoms except water molecules. Final refinement statistics are recorded in Table 1.

Portions of the N-terminus, the C-terminus, L2 loops and individual side chains that were characterized by missing or broken electron density were excluded from the final model. Occasional ambiguous electron density peaks were observed in the structures. These peaks were usually elongated and potentially corresponded to disordered PEG fragments or other molecules present in the crystallization buffer. However, since these electron density peaks were not confidently interpretable, they were left unmodeled.

RESULTS

Catalytic activities of HDAC8 mutants

The activity of HDAC8 was measured using two commercially available substrates to determine their suitability for characterizing the steady-state kinetic parameters and pH dependence of the HDAC8 mutants. The Fluor de Lys HDAC8 substrate has a peptide sequence of Arg-His-Lys(Ac)-Lys(Ac), and is deacetylated by wild-type Co(II)-HDAC8 with a $k_{\text{cat}}/K_{\text{M}}$ value that is 2.6-fold higher than that of the Fluor de Lys H4-AcK16 substrate (Lys-Gly-Gly-Ala-Lys(Ac)) (Table 2). However, the Fluor de Lys HDAC8 substrate contains histidine, which can ionize within the physiological pH range. Therefore, the Fluor de Lys H4-AcK16 substrate was used for the pH-dependence studies except when the catalytic activity of an HDAC8 mutant was too low to accurately measure with this substrate. Parenthetically, we note that $k_{\text{cat}}/K_{\text{M}}$ values have previously been determined for the H142A, D176A, and D176N mutants with K^+ as the only monovalent ion present, but no other kinetic parameters were determined.²⁶ Additionally, kinetic parameters for H143A HDAC8 were also previously reported.²⁵

To determine the relative catalytic contributions of H142, the proposed³¹ general base catalyst, and H143, the proposed³¹ general acid catalyst, these residues were each mutated to alanine. The H142A mutation causes a 230-fold decrease in the value of $k_{\text{cat}}/K_{\text{M}}$ relative to wild-type HDAC8, which is entirely due to a decrease in k_{cat} (Table 2 and Figure 2). An even more dramatic change is observed for the H143A and H142A/H143A mutants, both of which decrease the value of $k_{\text{cat}}/K_{\text{M}}$ by more than 80,000-fold, with a 12-fold increase in K_{M} for H143A. These results indicate that both H142 and H143 are essential for optimal catalytic efficiency, but that H143 is significantly more important for accelerating the rate-determining step(s). The H143A substitution sufficiently compromises hydrolytic activity to enable cocrystallization of the enzyme complexed with an intact tetrapeptide substrate.²⁵

In the proposed HDAC8 mechanism, Y306 hydrogen bonds with the scissile substrate carbonyl²⁵ and maintains this hydrogen bond to stabilize the oxyanion of the tetrahedral intermediate and its flanking transition states. The Y306F substitution reduces the value of

$k_{\text{cat}}/K_{\text{M}}$ by 150-fold (Table 2). This is largely due to a decrease in k_{cat} , consistent with a role for Y306 in transition state stabilization. As was observed for the H143A mutant, the catalytic activity of Y306F HDAC8 is sufficiently compromised to enable cocrystallization of enzyme complexes with an intact tetrapeptide substrate (see below).^{23,29}

The side chains of D176 and D183 are hypothesized to orient and polarize H142 and H143, respectively. These proposed functions were tested by measuring the effect of mutating each aspartate side chain to either asparagine, which removes the negative charge, or alanine, which removes both the negative charge and the ability to accept a hydrogen bond. The D176N mutation decreases the value of $k_{\text{cat}}/K_{\text{M}}$ by 380-fold, while the less conservative D176A mutation decreases $k_{\text{cat}}/K_{\text{M}}$ by 10,000-fold (Figure 2). These decreases are comparable to or larger than the loss of activity observed for the H142A mutant. In contrast, the D183A and D183N mutations have only a minimal impact on catalytic efficiency (5.1-fold and 3.6-fold lower values of $k_{\text{cat}}/K_{\text{M}}$, respectively) (Table 2).

pH Dependence of wild-type HDAC8

To gain insight regarding the ionizations of catalytically important groups in the free enzyme or substrate, the pH dependence of $k_{\text{cat}}/K_{\text{M}}$ was determined for wild-type HDAC8 and the active site mutants. Control experiments demonstrated that the Fluor de Lys substrates are stable within the range of pH 6.0 to 10.5. At relatively high enzyme concentrations, the activity of wild-type Co(II)-HDAC8 (5 μM) or mutant Co(II)-HDAC8 is not affected by pre-incubating the enzyme for 15 min between pH 6.0 and 10.5. However, at high pH (≥ 9) and low concentrations of HDAC8 ($\leq 0.4 \mu\text{M}$), a time-dependent loss in activity is observed with a $t_{1/2}$ of 15 min at pH 9.5 and 7 min at pH 10.0 (data not shown). Therefore, assays to measure the pH dependence of wild-type HDAC8, which used lower enzyme concentrations than the mutant HDAC8 assays, were initiated by addition of HDAC8 without pre-incubation at the assay pH.

For wild-type Co(II)-HDAC8, $k_{\text{cat}}/K_{\text{M}}$ values for deacetylation of the Fluor de Lys H4-AcK16 substrate display a bell-shaped pH dependence, with a slope indicative of one proton transfer for each limb. The $\text{p}K_{\text{a}}$ values for wild-type Co(II)-HDAC8 are 7.4 ± 0.1 and 10.0 ± 0.2 (Figure 3 and Table 3). The upper $\text{p}K_{\text{a}}$ is at the limit of the pH range that can be measured for HDAC8; however, a single- $\text{p}K_{\text{a}}$ fit of these data for wild-type Co(II)-HDAC8 ($\text{p}K_{\text{a}} = 7.2 \pm 0.1$) is significantly worse than the two- $\text{p}K_{\text{a}}$ fit ($R = 0.970$ compared with 0.997 for a fit with two $\text{p}K_{\text{a}}$ s). As the Fluor de Lys HDAC8 substrate contains a histidine residue, it is possible that $\text{p}K_{\text{a}1}$ could reflect ionization of the substrate. However, the Fluor de Lys H4-AcK16 substrate lacks a histidine residue yet exhibits the same pH dependence for deacetylation by wild-type Co(II)-HDAC8 (data not shown), indicating that ionization of the histidine in the Fluor de Lys HDAC8 substrate does not affect the pH dependence of the wild-type catalytic activity. The two $\text{p}K_{\text{a}}$ s observed in Figure 3 are therefore interpreted as reflecting two ionizations in wild-type Co(II)-HDAC8.

Metal substitution experiments were next performed to examine whether either of the observed $\text{p}K_{\text{a}}$ values are due to the ionization of the metal-bound water molecule. When Co(II) is replaced with Zn(II), $\text{p}K_{\text{a}1}$ is essentially unchanged, while the value of $\text{p}K_{\text{a}2}$ decreases by 0.9 pH units for wild-type Zn(II)-HDAC8 (Figure 3 and Table 3). This

suggests that pK_{a2} reflects the ionization of the metal-bound water molecule, since Zn(II)-H₂O has a lower pK_a than Co(II)-H₂O.⁴⁸ Previously reported pK_a values for recombinant wild-type HDAC8, $pK_{a1} = 7.6 \pm 0.1$ and $pK_{a2} = 8.9 \pm 0.1$,⁴⁴ are within experimental error of those reported here for wild-type Zn(II)-HDAC8.

pH Dependence of HDAC8 mutants

Further information about the mechanism was sought by measuring the effects of HDAC8 mutants on the observed pK_a values. The pH dependence of the H142A mutant is not well-described by the typical bell-shaped curve that is indicative of two ionizations affecting catalysis (Figure 3): above pH 9.0, the values of k_{cat}/K_M for deacetylation are higher than predicted by the two- pK_a fit of Eq. 1 (dashed line), and a better fit to the data results when a third ionization is added (solid line, Eq. 2). The three- pK_a fit of Eq. 2 indicates that the acidic and basic pK_a values (pK_{a1} and pK_{a3}) are unchanged relative to wild-type Co(II)-HDAC8, with an additional ionization with $pK_{a2} = 8.0 \pm 0.1$ for a group whose protonation enhances activity by 8-fold ($k_{obs1} = 13 \text{ M}^{-1}\text{s}^{-1}$ and $k_{obs2} = 1.6 \text{ M}^{-1}\text{s}^{-1}$) (Table 3). Importantly, for either fit of the H142A HDAC8 pH dependence data, the value of pK_{a1} is unchanged, within experimental error, from that of wild-type Co(II)-HDAC8. These data suggest that neither pK_{a1} nor pK_{a2} in wild-type HDAC8 reflect the ionization of H142.

The mutational effects of Y306, D176 or D183 on the ionizations important for catalysis were also determined. The D176A, D183A, D183N, and Y306F mutations have little effect on the value of pK_{a1} (7.4–7.6), while all mutants show a 1–2 log unit decrease in the value of pK_{a2} relative to wild-type Co(II)-HDAC8 (Table 3). This could reflect a direct change in the pK_a in an enzyme group, such as the metal-water ligand, or be an indirect effect due to diminished stability and/or decreased affinity for monovalent cations in the HDAC8 mutants at elevated pH; the D176N and D176A mutations decrease cation affinity for both activating and inhibitory monovalent cations.²⁶ Although the fitted value of pK_{a1} increases to 8.0 for the D176N mutant, the similarity in the values of pK_{a1} and pK_{a2} (within 0.1 pH units of each other) makes it difficult to accurately determine the pK_a values, so this difference may not be significant. The contributions of H143 to the pH dependence could not be directly determined, as the catalytic activities of the H143A and H142A/H143A mutants were too low to be accurately measured at non-optimal pH, even with high enzyme concentrations (50 μM). These results suggest that the observed pH dependence of k_{cat}/K_M for wild-type HDAC8 does not reflect the ionization state of Y306, D176, or D183.

Crystal structure of the D176N HDAC8–M344 complex

In wild-type HDAC8, the carboxylate side chain of D176 coordinates to the inhibitory monovalent cation (typically K⁺) and also accepts hydrogen bonds from H142 and Y174. The 1.94 Å-resolution crystal structure of the D176N HDAC8-M344 complex reveals that the overall structure is generally similar to that of the wild-type HDAC8-M344 complex, with a root mean square (rms) deviation of 0.45 Å for 357 C α atoms and 354 C α atoms for monomers A and B, respectively.

The side chain of N176 adopts a slightly different conformation compared to that of D176 in the wild-type enzyme, and this results in an altered hydrogen bond network (Figure 4). The

side chain NH₂ group of N176 donates hydrogen bonds to the backbone carbonyl group of H180 and the side chain hydroxyl group of S199, each of which coordinate to K⁺ in wild-type HDAC8. Consequently, the inhibitory K⁺ site is empty in D176N HDAC8. While the side chain carbonyl group of N176 is still within hydrogen bonding distance to H142, this hydrogen bond is poorly oriented and approximately 0.5 Å longer than that between D176 and H142 in the wild-type enzyme. The hydrogen bond between H142 and the hydroxamate Zn(II)-binding group of the inhibitor M344 is not affected by the D176N mutation. Furthermore, Y174 no longer interacts with N176, but instead donates a hydrogen bond to the side chain carbonyl of nearby residue Q263, which in turn undergoes a conformational change to break a hydrogen bond with Zn(II) ligand D178. Notably, in this structure and all other mutant structures, the previously identified activating monovalent site,²⁶ 10 Å from the active site, is still fully occupied by K⁺.

Crystal structure of the D176N-Y306F HDAC8–substrate complex

The Y306F mutation significantly reduces HDAC8 activity (Table 2) and accordingly enables cocrystallization of the enzyme with an intact peptide substrate.^{23,29} The 1.55 Å-resolution crystal structure of D176N-Y306F HDAC8 complexed with the tetrapeptide assay substrate Ac-Arg-His-Lys(Ac)-Lys(Ac)-aminomethylcoumarin reveals an overall structure quite similar to that of the corresponding Y306F HDAC8-substrate complex,²³ with an rms deviation of 0.28 Å for 362 Cα atoms and 0.29 Å for 363 Cα atoms for monomers A and B, respectively.

The substrate binding mode is essentially identical in the active sites of D176N-Y306F HDAC8 and Y306F HDAC8 (Figure 5), consistent with the relatively minor increase in K_M measured for D176N HDAC8 (Table 2). However, as observed in the D176N HDAC8-M344 complex, the D176N mutation influences hydrogen bond networks in the active site; the binding of K⁺ to the monovalent cation site is blocked due to the hydrogen bonds between the side chain NH₂ group of N176, the backbone carbonyl group of H180, and the side chain hydroxyl group of S199. Additionally, N176 is no longer within hydrogen bond distance to H142. Instead, Y174 donates a hydrogen bond to H142, which causes the side chain of H142 to shift 1.1 Å away from its original position in the Y306F HDAC8 structure. As a result, H142 does not stabilize the catalytic water molecule that weakly interacts with the active site Zn(II) ion (occupancy = 0.2–0.4, Zn(II)—O separation = 2.7–2.8 Å). Finally, Q263 adopts two conformations in both monomers: a primary conformation similar to that observed in wild-type HDAC8 and Y306F HDAC8, in which the carboxamide side chain hydrogen bonds with the backbone amide NH group and the side chain carboxylate group of Zn(II) ligand D178; and an alternative, minor conformation, similar to that observed in D176N HDAC8.

Crystal structure of the D176A-Y306F HDAC8–substrate complex

Crystals of D176A HDAC8 could not be prepared with typical hydroxamate inhibitors such as M344, SAHA, or TSA. However, crystals could be prepared by cocrystallization of the inactive double mutant D176A-Y306F HDAC8 with the tetrapeptide assay substrate. The 2.30 Å-resolution crystal structure of the D176A-Y306F HDAC8-substrate complex reveals an overall structure quite similar to that of the Y306F HDAC8-substrate complex,²³ with an

rms deviation of 0.27 Å for 362 Cα atoms and 0.34 Å for 363 Cα atoms for monomers A and B, respectively.

The substrate binds in a similar manner to D176A-Y306F HDAC8 and Y306F HDAC8, consistent with the relatively minor change in K_M measured for D176A HDAC8 (Table 2). However, the D176A mutation compromises the K^+ site and significantly influences the chemistry of catalysis in the active site. In contrast with the structure of D176N HDAC8, a residual electron density peak is observed in the K^+ binding site. However, a Bijvoet difference Fourier map calculated with X-ray diffraction data collected near the K^+ edge indicates that this electron density peak is not residual K^+ . Therefore, this peak is fit with a water molecule, which is within hydrogen bonding distance (2.9–3.3 Å) to residues formerly involved in K^+ coordination: the backbone carbonyl groups of D178, H180, L200, and A176, and the side chain hydroxyl group of S199. As in D176N-Y306F HDAC8, the lack of interaction with the side chain of residue 176 causes the side chain imidazole group of H142 to shift 1.4 Å toward Y174 to form a hydrogen bond. As a result, H142 is neither stabilized as the positively charged imidazolium cation, nor is it in a position to stabilize the Zn(II)-bound transition state. These electrostatic and structural features are likely responsible for the 10,000-fold reduction in k_{cat}/K_M measured for D176A HDAC8 (Table 2). A stereoview of the D176A-Y306F HDAC8-substrate complex is found in Figure 6.

Crystal structure of the H142A-Y306F HDAC8–substrate complex

Crystals of H142A HDAC8 could not be prepared with typical hydroxamate inhibitors such as M344, SAHA, or TSA. However, crystals could be prepared by cocrystallization of the inactive double mutant H142A-Y306F HDAC8 with the tetrapeptide assay substrate, in similar manner as for D176A-Y306F HDAC8. The 1.30 Å-resolution crystal structure of the H142A-Y306F HDAC8-substrate complex is quite similar overall to that of the Y306F HDAC8-substrate complex,²³ with rms deviations of 0.26 Å for 362 Cα atoms and 0.29 Å for 362 Cα atoms for monomers A and B, respectively.

The tetrapeptide substrate binds in a similar manner to H142A-Y306F HDAC8 and Y306F HDAC8 (Figure 7), consistent with the essentially unchanged K_M value measured for H142A HDAC8 (Table 2). The K^+ binding site is not disrupted by the H142A mutation, and a fully-occupied K^+ ion is coordinated in identical fashion to that observed in the Y306F HDAC8-substrate complex²³ (that being said, it should be noted that the K^+ concentration in the crystallization buffer (150 mM) is significantly higher than that in the assay buffer (3 mM)). However, the H142A mutation significantly affects the active site structure in the vicinity of the Zn(II)-bound water molecule. Having lost its hydrogen bond with H142, this water molecule is more weakly bound and is likely more mobile (the weaker electron density peak was modeled with occupancy = 0.5) and its coordination interaction with the Zn(II) ion lengthens from 2.1 Å in Y306F HDAC8 to 2.4 Å in H142A-Y306F HDAC8. To compensate, the side chain of H143 tilts slightly to make a stronger hydrogen bond with this water molecule (the N—O distance shortens from 2.9 Å to 2.6 Å), without disrupting the H143-D183 dyad. Additionally, two water molecules fill the void created by the deletion of the H142 side chain.

DISCUSSION

In the years since HDAC inhibitors were discovered to be potential anticancer therapeutics, intense study has focused on the cellular functions of HDACs and the downstream effects of their inhibition.² Significantly less is known about the catalytic mechanism by which these metal-dependent HDACs function.^{24,49,50} A key feature of the originally proposed mechanism,³¹ tested here, is a general base-general acid pair in which H142 is the general base catalyst and H143 is the general acid catalyst.

H143 is a dual general base-general acid catalyst

The relative activities of the H142A, H143A and H142A-H143A mutants reveal that H143 is much more important for catalytic activity than H142 (Table 2). The X-ray crystal structure of H143A HDAC8 complexed with the tetrapeptide substrate reveals that this mutation does not perturb the remaining active site residues, which indicates that the 80,000-fold loss in activity relative to the wild-type enzyme is not due to protein misfolding²⁵ but rather reflects a critical role for H143 in catalysis. The structure of the Y306F HDAC8–substrate complex shows that H143 hydrogen bonds with the Zn(II)-bound solvent molecule (Figure 1).²³ The rate-limiting steps in the catalytic mechanism of wild-type HDAC8 are proposed to be those mediated by the general base catalyst and/or the general acid catalyst. This is based on the faster deacetylation of substrates containing trifluoroacetyllysine than those with acetyllysine,^{51,52} as the electron-withdrawing fluorine atoms should accelerate both the formation and the breakdown of the tetrahedral intermediate. Analysis of inhibition of HDAC8 by K⁺ suggested that H142 is protonated in the form of the enzyme with the highest catalytic activity,²⁶ which would argue against a general base function for H142. The lack of any shift in pK_{a1} for the H142A mutant (Table 3) also supports the proposal that the general base catalyst is not H142. Thus, the model that is most consistent with the available data is one in which H143 is the general base catalyst.

Which residue, then, functions as a general acid catalyst? The two obvious candidates are H143, which would be protonated after functioning as the general base catalyst, and the positively charged H142 imidazolium group. In the structure of the Y306F HDAC8–substrate complex,²³ H143 is closer to the substrate amide NH group (3.8 Å) and is in a much more favorable position to serve as the general acid catalyst than H142, which is 5.0 Å away from the amide NH group (this nitrogen atom must be protonated to facilitate collapse of the tetrahedral intermediate). Additionally, the steric bulk of the H143 side chain and the tetrahedral intermediate appear to block H142 from access to the leaving group. Therefore, we conclude that H143 serves as both the general acid and the general base catalyst.

H142 is an electrostatic catalyst

While our data suggest that H142 is neither the general base catalyst nor the general acid catalyst, the question remains as to what role it plays in the deacetylation reaction. Significant transition state stabilization is indeed lost in H142A HDAC8, with a 230-fold reduction in k_{cat}/K_M in comparison with the wild-type enzyme and no change in K_M (Table 2). Previous studies demonstrate that the K⁺ inhibition is eliminated in the H142A but not the D176A/N mutants.²⁶ In HDAC8, H142 forms a hydrogen bond with D176, which is a

K^+ ligand, thus linking H142 to the K^+ in site 1. The findings here show that the HDAC8 mutants D176A and D176N, which weaken the binding affinity of the inhibitory K^+ ion,²⁶ also decrease the occupancy of the K^+ ion near the active site, while site 2, the distant K^+ site, remains fully occupied in these mutants. In the H142A-Y306F mutant, both K^+ binding sites are still intact and occupied. These results provide further evidence that the K^+ ion in site 1, which is near the divalent metal ion, is indeed the inhibitory ion. The inhibition by K^+ was previously proposed to diminish catalytic activity by decreasing the fraction of protonated H142.²⁶ Therefore, we hypothesize that the positively charged H142 imidazolium group serves as an electrostatic catalyst, stabilizing the negative charge of the tetrahedral intermediate and its flanking transition states.²⁶ This is somewhat similar to the mechanism of thermolysin, which also utilizes a positively charged histidine for transition state stabilization.⁴¹ However, a key difference is that in thermolysin, the histidine is positioned to hydrogen bond with the substrate carbonyl and incipient oxyanion of the tetrahedral intermediate, whereas in HDAC8, both H142 and H143 are positioned to hydrogen bond with the metal-bound water molecule.²³ Even so, the electrostatic interaction between the positively charged imidazole group of H142 and the developing negative charge of the transition state for amide hydrolysis could be significant. The H142 imidazole group is also important for positioning and stabilizing the Zn(II)-bound water molecule, as demonstrated by the lower occupancy/increased mobility of that particular water molecule in the H142A-Y306F HDAC8-substrate complex.

Ionizations reflected in the pH-dependence profile

The value of pK_{a2} in wild-type HDAC8 shifts from 10.0 to 9.1 upon metal substitution from Co(II) to Zn(II), while pK_{a1} is essentially unchanged at 7.4 or 7.6, respectively (Table 3). The pH dependence of the H143A mutant could not be measured, due to its extremely low activity. However, no shift in pK_{a1} was observed for H142A or the other mutants, within error. The ionization of pK_{a1} is therefore tentatively assigned to the general base catalyst, H143. Based on the shift in pK_{a2} with metal substitution, the basic limb of the pH-dependence profile is interpreted as reflecting the deprotonation of the metal-bound water molecule. This same trend in pK_a values for Co(II) and Zn(II) substitution is observed for the bacterial deacetylase LpxC, which functions in lipid A biosynthesis.^{53,54} There are multiple possible causes of the loss in HDAC8 activity at high pH upon the formation of metal-bound hydroxide ion, including tighter binding of hydroxide than water to the catalytic metal ion,⁵⁵ and the need for H143 to acquire a proton from the metal-bound water molecule in the first step of the reaction so that it can subsequently donate this proton to the leaving group.

The unusual pH dependence observed for H142A HDAC8 seen in Figure 3 is likely related to the role of H142 as an electrostatic catalyst. In this pH profile, the lower and upper pK_a values are unchanged relative to wild-type HDAC8, while a new pK_a is introduced at 8.0, reflecting the ionization of a group whose deprotonation decreases the rate of catalysis. The source of this newly unmasked pK_a is not known.

Potential roles of histidine-aspartate dyads

H143 donates a hydrogen bond to D183, and the relatively minor effects on catalysis and pH dependence caused by the D183N and D183A substitutions are consistent with the partially solvent-exposed environment of D183 (Figure 1). While only the D183N mutant can form hydrogen bonds, there is no significant difference in $k_{\text{cat}}/K_{\text{M}}$ and only a 3-fold difference in K_{M} between the D183N and D183A mutants, suggesting that D183 is not the sole residue responsible for orienting H143. Rather, crystal structure analysis reveals that H143 is surrounded by a network of steric contacts, including close packing with H142 and F208.

More deleterious than the mutation of D183 is the mutation of D176 (Table 1), a buried residue that accepts a hydrogen bond from H142. Since all of the mutants except for H142A show a decrease in $\text{p}K_{\text{a}2}$, and even wild-type HDAC8 is somewhat unstable at high pH and low protein concentrations, the shift in $\text{p}K_{\text{a}2}$ for the mutants may reflect changes in protein stability. However, no clues regarding this aspect of the pH dependence are evident from the crystal structures. Catalytic efficiency, $k_{\text{cat}}/K_{\text{M}}$, is reduced 380-fold in D176N HDAC8 compared with the wild-type enzyme (Table 2), mainly due to a 170-fold reduction in k_{cat} . This activity loss is consistent with a significant reduction in transition state stabilization due to the destabilization of the positively charged imidazolium form of H142, resulting from the loss of its hydrogen bond with the negatively charged carboxylate of D176 and replacement with the poorly oriented, weaker hydrogen bond with the neutral carboxamide group of N176. Changes in the hydrogen bond network within the active site and weaker binding of the catalytic water molecule to Zn(II), as reflected by significantly lower occupancy/increased mobility of this water molecule in the active site of D176N HDAC8, would also be likely to contribute to the effects observed for D176N HDAC8, which is slightly less active than the H142A mutant. Even more dramatic is the 10,000-fold reduction in the value of $k_{\text{cat}}/K_{\text{M}}$ measured for D176A HDAC8, which is 40-fold less active than the H142A mutant, in which the imidazole/imidazolium side chain is completely deleted (Table 2). In the D176A mutant, the H142 side chain is retained but its hydrogen bond partner is compromised. Comparison of the X-ray crystal structures of H142A-Y306F HDAC8 and D176A-Y306F HDAC8 suggests a possible explanation. In H142A-Y306F HDAC8, K^+ binding to the nearby inhibitory site is preserved whereas K^+ is absent in the structure of D176A-Y306F HDAC8. Even though K^+ decreases catalytic activity when it binds to this site in wild-type HDAC8, this inhibitory effect requires the side chain of H142.²⁶ In the absence of H142, the bound K^+ ion either might have no effect on the rate of catalysis, or it might be beneficial, serving as a weak electrostatic catalyst in place of the imidazolium ion, providing moderate enhancement of transition state stabilization in H142A HDAC8 relative to D176A HDAC8. This possibility might help account for at least a portion of the greater loss of activity in D176A HDAC8.

Y306 stabilizes the tetrahedral intermediate and its flanking transition states

The phenolic side chain of Y306 is proposed to stabilize the tetrahedral intermediate by donation of a hydrogen bond to the developing oxyanion of the tetrahedral intermediate,³¹ and this is experimentally supported by the 150-fold decrease in $k_{\text{cat}}/K_{\text{M}}$ for the Y306F mutant. Similar decreases in $k_{\text{cat}}/K_{\text{M}}$ (~200-fold) are observed upon the mutation of the tyrosine or threonine that stabilizes the tetrahedral intermediate in thermolysin and

LpxC.^{56,57} Since the formation of the tetrahedral intermediate is an endothermic reaction, the transition states flanking this intermediate closely resemble this intermediate in terms of their structure and free energy based on Hammond's Postulate.⁵⁸ Therefore, since Y306 is positioned to hydrogen bond with the oxyanion of the tetrahedral intermediate and the Y306F substitution decreases $k_{\text{cat}}/K_{\text{M}}$ while not increasing the value of K_{M} , the primary role of Y306 in HDAC8 is transition state stabilization.

The crystal structure of H143A HDAC8 complexed with an intact tetrapeptide substrate was the first to reveal that Y306 also donates a hydrogen bond to the zinc-bound carbonyl group of the scissile amide linkage of acetyllysine, thereby suggesting an additional role for Y306 in substrate binding and orientation.²⁵ However, the substrate K_{M} value actually decreases 2-fold in Y306F HDAC8 (Table 2), indicating slightly enhanced enzyme-substrate affinity. Regardless, since the substrate K_{M} value does not increase for Y306F HDAC8, we conclude that the active site metal ion alone is mainly responsible for polarization and activation of the scissile carbonyl for the chemistry of hydrolysis, and the hydrogen bond with Y306 becomes increasingly important as the tetrahedral transition state structure is approached.

CONCLUSIONS

The results of this study provide compelling biochemical evidence that HDAC8 employs a catalytic mechanism in which H143 is a single general base-general acid catalyst, while H142 is an electrostatic catalyst that may also assist in properly positioning H143 through steric interactions. A revised HDAC8 mechanism based on these findings is presented in Figure 8. This mechanism differs from that originally proposed based on the crystal structure of the histone deacetylase-like protein from *Aquifex aeolicus*, in which H142 serves as a general base catalyst and H143 serves as a general acid catalyst.³¹ Importantly, the revised mechanism predicts different protonation states of H142 and H143 in the native enzyme than originally proposed, which may be targeted by effective HDAC inhibitors. The revised mechanism is consistent with the results of QM/MM studies reported by Zhang and colleagues suggesting that H143 serves as a general base-general acid,^{60,61} but it contrasts with the conclusions of QM/MM studies reported by Wiest and colleagues suggesting that H142 serves as a general base and H143 serves as a general acid.⁶² Future studies will probe and further clarify the details of acid-base chemistry for catalysis and inhibitor binding to HDAC8 as well as other members of the HDAC family of enzymes.

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Figure 1. Active Site of Y306F HDAC8 (PDB accession code 2V5W)

Residues proposed to be important for the catalytic activity are shown (H142, H143, D176, D183 and the mutant Y306F), with an acetyllysine-containing substrate bound (Lys(Ac)). The D176 side chain coordinates to a K^+ ion and hydrogen bonds with H142; the D183 side chain hydrogen bonds with H143. D178, H180, D267, and a solvent molecule (red sphere) coordinate to Zn(II); the Zn(II)-bound solvent molecule hydrogen bonds with H142 and H143.

Figure 2. Substrate-dependence of activity for wild-type and mutant HDAC8s

Initial rates for substrate deacetylation were measured for 1.2 μM Co(II)-substituted wild-type HDAC8 (●, Fluor de Lys H4-AcK16 substrate), 10 μM H142A (□, Fluor de Lys H4-AcK16), 20 μM D176A (△, Fluor de Lys HDAC8) and 50 μM H143A (■, Fluor de Lys HDAC8); the k_{obs} value is $v_0/[E]$. Assays were performed at 25 °C and included the indicated substrate concentrations in 25 mM Tris (pH 8.0), 140 mM NaCl, and 2.7 mM KCl. The Michaelis-Menten equation was fit to the resulting initial rates, corrected for the enzyme concentration ($k_{\text{obs}} = v_0/[E]$), to calculate values for k_{cat} , K_{M} and $k_{\text{cat}}/K_{\text{M}}$, which are listed in Table 2. Note that the x-axis is shown as a log scale.

Figure 3. pH Dependence of wild-type and mutant HDAC8s

The pH dependence of $k_{\text{cat}}/K_{\text{M}}$ was determined from initial rates using 50 μM Fluor de Lys H4-AcK16 at 25 $^{\circ}\text{C}$ in assay buffer-2 with 0.4 μM wild-type Co(II)-HDAC8 (●), 0.4 μM wild-type Zn(II)-HDAC8 (□) or 5 μM Co(II)-H142A (△). The pK_{a} values, listed in Table 3, were determined from a fit of Eq. 1 to the data. For H142A HDAC8, the data are better described by 3 ionizations (Eq. 2, solid grey line) than by 2 ionizations (Eq. 1, dotted black line).

Figure 4.

Comparison of the D176N HDAC8–M344 complex (monomer B) with the wild-type HDAC8–M344 complex (monomer B; PDB accession code 1T67). Atomic color codes are as follows: C = yellow (protein, D176N HDAC8), tan (ligand, D176N HDAC8), blue (protein, wild-type HDAC8), or light grey (ligand, wild-type HDAC8); N = blue; O = red; Zn(II) = yellow (D176N HDAC8) or blue (wild-type HDAC8) sphere; and Na⁺ = blue sphere (wild-type HDAC8; this site is empty in the mutant enzyme). Metal coordination and hydrogen bond interactions are shown as solid and dashed lines, respectively (black for D176N HDAC8 and purple for wild-type HDAC8). The simulated annealing omit map (contoured at 4.0σ) shows that the side chain of N176 hydrogen bonds with the side chain hydroxyl group of S199 and the backbone carbonyl group of H180, thus preventing Na⁺ or K⁺ ions from binding in the monovalent cation site of the mutant enzyme.

Figure 5.

Comparison of the D176N-Y306F HDAC8–substrate complex (monomer B) with the Y306F HDAC8–substrate complex (monomer A; PDB accession code 2V5W). Atomic color codes are as follows: C = yellow (protein, D176N-Y306F HDAC8), tan (substrate, D176N-Y306F HDAC8), blue (protein, Y306F HDAC8), or light grey (substrate, Y306F HDAC8); N = blue; O = red; Zn(II) = yellow (D176N-Y306F HDAC8) or blue (Y306F HDAC8) sphere; and K⁺ = blue sphere (Y306F HDAC8; this site is empty in D176N-Y306F HDAC8). Water molecules are indicated as red and orange spheres for D176N-Y306F HDAC8 and Y306F HDAC8, respectively. Metal coordination and hydrogen bond interactions are shown as solid and dashed lines, respectively (black for D176N-Y306F HDAC8 and purple for Y306F HDAC8). The Zn(II)-associated water molecule in D176N-Y306F is weakly bound with occupancy 0.2 and is too far from the metal ion for inner sphere coordination with Zn(II)–O separation = 2.7 Å (solid green line). The simulated annealing omit map (contoured at 4.0 σ) shows that the side chain of N176 hydrogen bonds with the side chain hydroxyl group of S199 and the backbone carbonyl group of H180, thus preventing metal ion binding in the monovalent cation site of D176N-Y306F HDAC8.

Figure 6.

Active site of the D176A-Y306F HDAC8-substrate complex (monomer A). Atomic color codes are as follows: C = yellow (protein), tan (substrate), Zn(II) = yellow sphere. The simulated annealing omit map (contoured at 4.0σ) shows the side chain of A176 that disrupts the monovalent cation binding site. Instead of a K^+ ion, a water molecule (red sphere) is bound in this site. Metal coordination and hydrogen bond interactions are shown as solid and dashed black lines, respectively. No Zn(II)-bound water molecule is observed in this structure.

Figure 7.

Comparison of the H142A-Y306F HDAC8–substrate complex (monomer B) with the Y306F HDAC8–substrate complex (monomer A; PDB accession code 2V5W). Atomic color codes are as follows: C = yellow (protein, H142A-Y306F HDAC8), tan (substrate, H142A-Y306F HDAC8), blue (protein, Y306F HDAC8), or light grey (substrate, Y306F HDAC8); N = blue; O = red; and Zn(II) and K⁺ = yellow (H142-Y306F HDAC8) or blue (Y306F HDAC8) sphere. Water molecules are indicated as red and orange spheres for D176N-Y306F HDAC8 and Y306F HDAC8, respectively. Metal coordination and hydrogen bond interactions are shown as solid and dashed lines, respectively (black for D176N-Y306F HDAC8 and purple for Y306F HDAC8). The simulated annealing omit map (contoured at 5.0σ) shows the side chain of A142.

Figure 8. Modified HDAC8 catalytic mechanism

The data reported herein support a new mechanism in which H143 is both the general base and the general acid catalyst, and H142 is protonated and serves as an electrostatic catalyst. The catalytic metal ion and Y306 orient the substrate carbonyl and stabilize the tetrahedral intermediate and its flanking transition states.

Table 1

Data collection and refinement statistics

	D176N HDAC8-M344 complex	D176N-Y306F HDAC8-substrate complex	D176A-Y306F HDAC8-substrate complex	H142A-Y306F HDAC8-substrate complex
<i>Unit cell</i>				
space group symmetry	$P2_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
a, b, c (Å)	51.5, 83.0, 94.4	82.2, 98.2, 104.3	81.8, 97.7, 103.6	82.8, 97.9, 104.6
α, β, γ (deg)	90, 97.1, 90	90, 90, 90	90, 90, 90	90, 90, 90
<i>Data collection</i>				
wavelength (Å)	1.075	0.979	1.075	0.979
resolution limits (Å)	43.5–1.94	49.2–1.55	44.3–2.30	49.0–1.30
total/unique reflections	217033/58414	866657/122036	364707/37434	1455775/208317
$R_{\text{merge}}^{a,b}$	0.098 (0.574)	0.110 (1.039) ^c	0.141 (0.906) ^c	0.090 (0.855) ^c
$I/\sigma(I)^a$	10.3 (2.2)	15.6 (2.0)	15.0 (2.0)	17.8 (2.7)
redundancy ^a	3.7 (3.6)	7.1 (6.6)	9.7 (8.0)	7.0 (6.1)
completeness (%) ^a	99.9 (99.7)	99.9 (99.9)	99.1 (97.2)	100 (100)
<i>Refinement</i>				
reflections used in refinement/test set	58391/2960	121931/6116	37384/1872	208196/10474
R_{cyst}^d	0.182	0.147	0.170	0.128
R_{free}^e	0.210	0.166	0.216	0.149
protein atoms ^f	5601	6159	5695	6472
water molecules ^f	274	610	253	872
ligand molecules ^f	2	2	2	2
Zn(II) ions ^f	2	2	2	2
K ⁺ ions ^f	2	2	2	4
glycerol molecules ^f	–	3	4	2
<i>Rms deviations from ideal geometry</i>				
bonds (Å)	0.006	0.010	0.003	0.008

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	D176N HDAC8-M344 complex	D176N-Y306F HDAC8-substrate complex	D176A-Y306F HDAC8-substrate complex	H142A-Y306F HDAC8-substrate complex
angles (°)	1.0	1.2	0.7	1.3
dihedral angles (°)	12	13	13	12
<i>Ramachandran plot (%)</i> ^g				
allowed	90.7	90.2	89.9	91.0
additionally allowed	9.3	9.6	10.1	9.0
generously allowed	–	0.2	–	–
PDB accession code	5DC5	5DC6	5DC7	5DC8

^aValues in parentheses refer to the highest shell of data.

^b $R_{merge} = \Sigma |I_h - \langle I \rangle| / \Sigma I_h$, where $\langle I \rangle$ is the average intensity for reflection h calculated from replicate reflections.

^c $R_{crys} = \Sigma ||F_o| - |F_c|| / \Sigma |F_o|$ for reflections contained in the working set. $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively.

^dGiven the high redundancy for the outer shells of these data sets, R_{pim} is a more appropriate measure of the data quality than R_{merge} . $R_{pim} = 0.044$ (0.442), 0.047 (0.331), and 0.035 (0.375) for D176N-Y306F HDAC8, D176A-Y306F HDAC8, and H142A-Y306F HDAC8, respectively.

^e $R_{free} = \Sigma ||F_o| - |F_c|| / \Sigma |F_o|$ for reflections contained in the test set held aside during refinement.

^fPer asymmetric unit.

^gCalculated with PROCHECK version 3.4.4.

Table 2

Catalytic activity of HDAC8 mutants^a

HDAC8	Fluor de Lys Substrate	k_{cat} (s ⁻¹)	K_M (μM)	k_{cat}/K_M (M ⁻¹ s ⁻¹)	Fold Decrease in k_{cat}/K_M
wild-type	H4-AcK16	0.90 ± 0.06	320 ± 20	2800 ± 100	–
H142A	H4-AcK16	0.0032 ± 0.0004	310 ± 40	12 ± 1	230
D176N	H4-AcK16	0.0052 ± 0.0004	700 ± 90	7.4 ± 0.5	380
D183A	H4-AcK16	0.43 ± 0.05	780 ± 70	550 ± 20	5.1
D183N	H4-AcK16	0.18 ± 0.02	230 ± 20	770 ± 20	3.6
Y306F	H4-AcK16	0.0031 ± 0.0002	160 ± 30	19 ± 3	150
wild-type	HDAC8	1.2 ± 0.1	160 ± 6	7500 ± 200	–
H143A	HDAC8	0.0002 ± 0.0001	2000 ± 1000	0.09 ± 0.02	80,000
H142A-H143A	HDAC8	n.d. ^b	> 600	~0.05	~150,000
D176A	HDAC8	0.00011 ± 0.00007	150 ± 20	0.75 ± 0.04	10,000

^a Co(II)-substituted HDAC8 mutants were assayed at 1.2 – 50 μM HDAC8 with 5 – 2000 μM substrate at 25 °C in 25 mM Tris, pH 8.0, 140 mM NaCl and 2.7 mM KCl. Initial velocities were determined based on changes in fluorescence, and the Michaelis-Menten equation fit to the resulting initial velocities rates.

^b k_{cat} could not be determined for this mutant because the reaction was not saturated at 1 mM substrate.

Table 3p*K*_a values for wild-type HDAC8 and mutants ^a

Enzyme	p <i>K</i> _{a1}	p <i>K</i> _{a2}	p <i>K</i> _{a3}
Co(II)-wild-type	7.4 ± 0.1	10.0 ± 0.2	
Zn(II)-wild-type	7.6 ± 0.1	9.1 ± 0.3	
Co(II)-H142A	7.4 ± 0.1 ^b (7.2 ± 0.1)	8.0 ± 0.1 ^b (8.1 ± 0.2)	10.0 ± 0.2 ^b
Co(II)-D176A	7.4 ± 0.3	7.8 ± 0.2	
Co(II)-D176N	8.0 ± 1.0	8.1 ± 1.0	
Co(II)-D183A	7.6 ± 0.2	8.6 ± 0.2	
Co(II)-D183N	7.4 ± 0.1	8.9 ± 0.2	
Co(II)-Y306F	7.1 ± 0.1	9.2 ± 0.1	

^a Apo-HDAC8 mutants were incubated with stoichiometric Co(II) at 4 °C, and assayed at 0.4 to 50 μM HDAC8 with 50 – 250 μM Fluor de Lys H4-AcK16 substrate, at 25 °C in 50 mM buffer, 140 mM NaCl and 2.7 mM KCl. Initial velocities were determined based on changes in fluorescence, and Eq. 1, derived for two ionizations, was fit to the resulting initial rates.

^b Eq. 2, derived for 3 ionizations, was fit to the pH dependence of the initial rates for H142A.