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Chelatable trace zinc causes low, irreproducible KDAC8 activity

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Abstract

Acetylation is an important regulatory mechanism in cells, and emphasis is being placed on identifying substrates and small molecule modulators of this post-translational modification. However, the reported *in vitro* activity of the lysine deacetylase KDAC8 is inconsistent across experimental setups, even with the same substrate, complicating progress in the field. We detected trace levels of zinc, a known inhibitor of KDAC8 when present in excess, even in high-quality buffer reagents, at concentrations that are sufficient to significantly inhibit the enzyme under common reaction conditions. We hypothesized that trace zinc in solution could account for the observed variability in KDAC8 activity. We demonstrate that addition of chelators, including BSA, EDTA, and citrate, and/or the use of a phosphate-based buffer instead of the more common tris-based buffer, eliminates the inhibition from low levels of zinc as well as the dependence of specific activity on enzyme concentration. This results in high KDAC8 activity that is consistent across buffer systems, even using low concentrations of enzyme. We report conditions that are suitable for several assays to increase both enzyme activity and reproducibility. Our results have significant implications for approaches used to identify substrates and small molecule modulators of KDAC8 and interpretation of existing data.

Keywords

hdac; histone deacetylase; lysine deacetylase; zinc

1. Introduction

Acetylation of lysine is a reversible post-translational modification. Thousands of unique mammalian protein sequences have been reported to contain acetylated lysine residues *in vivo*.^{1–5} The lysine deacetylase family of enzymes (KDACs, also known as histone deacetylases, EC 3.5.1.98) is responsible for catalyzing the hydrolysis of ϵ -N-acetyllysine residues, thereby returning lysine to its unmodified state.^{6,7} KDAC1-11 are metal-dependent enzymes which require a single divalent metal ion in the active site to perform catalysis.^{8–11} The catalytic metal ion usually reported for these enzymes is Zn^{2+} . KDAC8 is inhibited by

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excess Zn^{2+} , but not by other transition metal ions, such that activity begins to decrease as soon as Zn^{2+} is in stoichiometric excess compared to the enzyme.⁸ A specific binding site for the inhibitory Zn^{2+} has been identified by crystallography.¹² Inhibition by Zn^{2+} has not been reported for other KDACs.

Proper regulation of lysine acetylation, and therefore of KDACs, is important for a variety of biological processes, including development and growth, memory formation, and regulation of metabolism.^{13–17} Conversely, aberrant KDAC activity has also been linked to numerous diseases, in particular chronic diseases such as asthma, cancers, muscular disorders, and diabetes.^{13,18–20} KDAC8 in particular has been linked to several different types of cancer, although the underlying mechanism by which KDAC8 contributes to these disease states is unclear.²¹

To better understand how KDAC8 contributes to various disease states, it is necessary to have a sensitive and reproducible technique for identifying biologically relevant substrates of KDAC8. Similarly, development of potential therapeutic interventions targeting KDAC8 requires robust assay conditions to determine whether potential small molecule modulators of KDAC8 can affect deacetylation of these substrates. Thus, there is a need for sensitive, robust, and reproducible *in vitro* lysine deacetylase assays which meet these requirements. Typical methods for detection of deacetylation include fluorophore-conjugated substrates, coupled detection of produced free lysine, coupled detection of produced acetate, and HPLC or mass spectrometric detection of the peptide product.^{22–27} Ideal reaction conditions should permit detection with any of these approaches while ensuring consistent results that are not method-dependent.

A significant issue with all existing KDAC *in vitro* assays is that KDAC8 activity is partially dependent on the buffer used. We have previously observed that changing the buffering system results in significantly different activity.²⁸ Furthermore, literature reports from various groups often report inconsistent activity measurements for KDAC8, with up to nearly 100-fold variation even with the same substrate.^{8,28–31} Here we demonstrate that much of that variation can be attributed to metal content of the reaction solution (i.e., reduced activity due to Zn^{2+} contamination), which is rarely reported as a control. Furthermore, we have identified buffer conditions that can be used across assays to minimize the effect of variations in Zn^{2+} content and support high KDAC8 activity at enzyme concentrations much lower than has typically been utilized.

2. Materials and Methods

2.1 Protein expression and purification

KDAC8 was expressed in both *E. coli* and insect cells, and then purified as described previously.^{22,32} Briefly, KDAC8-His₆ is affinity purified using Talon Co²⁺ resin (Clontech). The His₆ tag is then cleaved using tobacco etch virus protease during dialysis, followed by a second affinity column to obtain highly purified enzyme with identical activity from either expression system.³² The resulting protein contains cobalt as the active site metal, which has previously been demonstrated to have comparable activity to the zinc enzyme.⁸ The expected metal content of 1:1 cobalt:KDAC8 was verified by ICPMS, and saturation

confirmed by activity assays in the presence of 100-fold excess Co^{2+} as previously described.³²

2.2 Activity assays

Deacetylation was monitored and specific activity was calculated as previously described.²² Briefly, purified KDAC8 (200 nM unless otherwise noted) was incubated with 100 μM peptide substrate (Figure S1), either ac-{Kac}-AMC (Enzo Life Sciences) or ac-FR{Kac}RW-am (custom synthesis by Genscript), for 60 min at 25 °C in either Buffer T (50 mM tris pH 8.0, 2.7 mM KCl, 137 mM NaCl) or Buffer Px (potassium phosphate pH 7.6 where x indicates the concentration in mM, 100 mM KCl, 5% glycerol). Where noted, buffers were supplemented with BSA and/or pH-adjusted stock solutions of the sodium salts of citrate or EDTA. Reactions were stopped by addition of suberoylanilide hydroxamic acid (SAHA) to a final concentration of 100 μM , mixed 1:1 with 0.5 M NaCl, and added in triplicate to black polypropylene 96 well plates (Corning). An equal volume of 0.1 mg mL^{-1} fluorescamine (Acros Organics) in dimethyl sulfoxide was added to each well, allowed to react for 20 minutes, and then samples were excited at 390 nm and fluorescence measured at 485 nm. Fluorescence from each sample, minus the fluorescence of an inhibited mixture, was converted to pmol product using a standard curve of N- α -acetyl-L-lysine amide hydrochloride (Chem-Impex International). Specific activity was determined by dividing pmol product by reaction time and by μg of enzyme. All data is the average of two or more independent replicates, and error bars represent the standard deviation. Specific activity was then normalized to either the specific activity in Buffer T for the ac-{Kac}-AMC substrate or in Buffer P30 for ac-FR{Kac}RW-am to obtain the relative activity. To determine steady-state kinetic parameters, endpoint experiments were performed as described above with 5 concentrations of ac-FR{Kac}RW-am, ranging from 100 μM to 2000 μM . The Michaelis constant (K_M , an approximation of binding affinity) and maximum velocity (V_{max}) were calculated by nonlinear fitting to the data as described previously.²² V_{max} was normalized to activity in Buffer P30.

2.3 Mass spectrometry

Detection of deacetylation by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) on an Autoflex Speed MALDI TOF/TOF (Bruker Daltonics) was performed on ac-FR{Kac}RW-am reacted overnight with KDAC8 at 25 °C as described previously.³¹ Briefly, reactions were initiated as described above for fluorescence assays. After addition of SAHA, samples were diluted 1:100 in TA85 (85% acetonitrile, 15% water, and 0.1% trifluoroacetic acid). 0.5 μL was spotted onto a MTP Anchorchip target plate (Bruker Daltonics) and allowed to dry, and 0.5 μL of matrix (1.4 mg mL^{-1} α -cyano-4-hydroxycinnamic acid in TA85) was spotted on each sample.

2.4 ICPMS

Metal content in buffers and purified enzymes was measured by inductively coupled plasma mass spectrometry (ICPMS) by the Elemental Analysis core facility at Oregon Health & Science University. 200 μL samples of solutions were diluted 5-fold with ICP grade nitric acid, all handling being done with acid-rinsed glassware. NIST water (SRM 1643f) was used as a standard. Samples were analyzed on an Agilent Technologies 7700 series ICPMS.

Samples run included water and known standards of zinc interspersed with unknowns, blinded to the instrument operator. ICPMS results are reported with coefficient of variance errors.

3. Results

We previously reported that KDAC8 activity with a commercially-available fluorescently labeled substrate is dependent on the buffer conditions during the deacetylation reaction, with significant enhancement in Buffer T supplemented with BSA at the concentration recommended by the substrate manufacturer.²⁸ Adding BSA to Buffer T to 1 mg mL^{-1} increased KDAC8 activity almost 10-fold over the basal activity level of $1.3 \pm 0.6 \text{ pmol min}^{-1} \mu\text{g}^{-1}$ (normalized to 1; Figure 1A). We hypothesized that BSA could be exerting its effect due to a macromolecular crowding effect caused by the high concentration of BSA.^{33–35} We therefore tested activity of other agents that modulate viscosity and mimic macromolecular crowding, but these agents did not enhance activity (Figure S2). These results suggest that BSA was not increasing activity by acting as a macromolecular crowding mimetic.

As BSA has been previously reported to bind Zn^{2+} ,³⁶ and KDAC8 has previously been demonstrated to bind and be inhibited by excess free Zn^{2+} in solution,⁸ we next hypothesized that the BSA may be increasing activity by chelating trace zinc found in the reaction solutions. Thus, addition of chelating molecules that bind excess Zn^{2+} should increase KDAC8 activity in a similar manner to BSA. We supplemented Buffer T with two other chelating agents, EDTA and citrate, instead of BSA (Figure 1A). Similar to BSA, both of these chelators were able to increase KDAC8 activity by several-fold. Simultaneous supplementation of Buffer T with both BSA and EDTA resulted in the same activity as BSA alone, suggesting that BSA alone chelated all available Zn^{2+} . Furthermore, citrate, EDTA, and BSA were each able to successfully increase KDAC8 activity in reactions even when $0.5 \mu\text{M}$ additional Zn^{2+} (2.5-fold molar excess compared to KDAC8) was added, albeit still with slightly lower activity than in the absence of the additional Zn^{2+} . We detected trace amounts of zinc in nearly all of our stock solutions by ICPMS, and eliminated use of low-purity reagents or stocks that were clearly contaminated (such as a tris(2-carboxyethyl)phosphine stock with 7000 ppm zinc). The zinc concentrations in the final stocks used for the assays were $108 \pm 1 \text{ nM}$ (phosphate) and $130 \pm 1 \text{ nM}$ (tris), with background levels from our water supply of $27.0 \pm 0.3 \text{ nM}$. In contrast, other transition metals (cobalt, iron, nickel, copper) were present at concentrations at least 1 order of magnitude lower, and no patterns indicative of significant presence in stock solutions were observed. The measured zinc concentrations in the stock solutions approach those of the enzyme used in the reaction, and so would be sufficient to dramatically reduce activity. Note that even several hundred nM zinc in the final reaction mixture corresponds to no more than 10 ppm in the reagent stocks (and usually far less), and so are well below the maximum amount permitted by quality controls for nearly all commercial reagents. Consistent with the hypothesis that trace Zn^{2+} affects KDAC8 activity, specific activity of KDAC8 increased dramatically when we increased the concentration of enzyme in the tris-based buffer (Figure S3A). Furthermore, addition of BSA almost entirely negated this effect (Figure 1B). Interestingly, the rate enhancement effect of BSA is not apparent in the phosphate-based Buffer P30 (Figure S3B). Addition of

BSA to Buffer P30 does not increase activity with the fluorescently labeled substrate, nor does addition of a 2.5-fold molar excess Zn^{2+} decrease activity in this buffer (Figure 1C), indicating that 30 mM phosphate is sufficient to eliminate the effect of trace zinc.

We next wanted to demonstrate that the lack of sensitivity to excess Zn^{2+} in phosphate-based buffer was neither substrate-specific nor subject to known artifacts of using a fluorophore-conjugated substrate.³¹ We previously published an alternate assay which allows characterization of unlabeled potential KDAC substrates by relying on the reaction of free amines (a product of the deacetylation reaction) with fluorescamine; however, the assay is not compatible with tris nor BSA because the free amines in both of these substances result in high background.²² As this assay is performed in Buffer P30, we examined whether a known chelating agent would affect KDAC8 activity in this assay. Consistent with our observations with BSA in Buffer P30, adding even a very high concentration of EDTA (500 μM) or a 2.5-fold molar excess Zn^{2+} did not significantly affect the KDAC8 deacetylation rate ($12 \pm 4 \text{ pmol min}^{-1} \mu\text{g}^{-1}$) in Buffer P30 against an unlabeled peptide substrate (ac-FR{K-ac}RW-am from human ADAP1; Figure 2A). In addition, unlike in Buffer T, there was no clear trend between KDAC8 concentration and specific activity in phosphate-based buffer (Figure S3B).

To probe the relationship between phosphate concentration and KDAC8 activity, we compared KDAC8 activity in reaction solutions containing a range of potassium phosphate concentrations and found that activity decreased linearly as phosphate concentration increased over a range of 10–30 mM (Figure 2B). Concentrations below 10 mM potassium phosphate are likely too low for effective buffering, as the reaction consistency was substantially reduced. Surprisingly, under conditions where no excess Zn^{2+} was added, KDAC8 appeared to be at least two-fold more active in Buffer P10 compared to Buffer P30. To verify that the phosphate itself was capable of binding free zinc in this context, we examined the effect of increasing amounts of zinc in both Buffer P10 and Buffer P30 (Figure 2C). We hypothesized that the enzyme would be more sensitive to excess Zn^{2+} in Buffer P10 due to the lower phosphate concentration. Consistent with this hypothesis, KDAC8 activity remained stable until Zn^{2+} was added in greater than 10-fold molar excess over the enzyme in Buffer P30, but KDAC8 was sensitive to the addition of an equimolar amount of Zn^{2+} in Buffer P10. This effect was Zn^{2+} specific, as titration with Co^{2+} did not change activity in either buffer (data not shown).

Next, we were interested in whether we could combine the effect of chelators with the 10 mM phosphate buffer to create a buffer system that promoted high KDAC8 activity while protecting the enzyme from excess Zn^{2+} . To address this possibility, we added 2.0 μM Zn^{2+} to reactions containing KDAC8 and ac-FR{Kac}RW-am in Buffer P10 and tested the effect of adding either citrate or EDTA to chelate the additional Zn^{2+} (Figure 3). We determined that 2.5 mM citrate and 5.0 μM EDTA were the lowest concentrations of chelator that were able to completely chelate 2.0 μM Zn^{2+} , resulting in maximal deacetylase activity even in the presence of significant levels of Zn^{2+} . We also tested whether KDAC8 concentration affected specific activity in Buffer P10 and found only a small effect (Figure 3C), analogous to what was observed in Buffer P30 (Figure S3B). Inclusion of EDTA did not affect the activity at either enzyme concentration. The increase in activity in the chelator-containing

Buffer P10 compared to Buffer P30 is due entirely to an increase in $V_{\max}$ (Table S1), consistent with the expected allosteric Zn^{2+} inhibition.⁸

To determine whether we had identified buffer conditions that were both compatible with multiple assays and as robust as the BSA-containing Buffer T, we directly compared activity of KDAC8 against the ac-{Kac}-AMC substrate in Buffer T and Buffer P10 supplemented with the various chelating agents (Figure 4). While this reduced concentration of citrate (2.5 mM compared to 5 mM in Figure 1) was not sufficient to restore maximal activity in Buffer T, there were no other significant differences in activity for any of the other conditions tested. Thus, we determined that using Buffer P10 as well as either 2.5 mM citrate or 5 μM EDTA supported similar activity to BSA-containing Buffer T. We were also able to detect deacetylation of the unlabeled peptide substrate using MALDI-TOF mass spectrometry using Buffer P10 with either 2.5 mM citrate or 5.0 μM EDTA (Figure S4).

4. Discussion

Basal activity levels of KDAC8 vary widely between reports in the literature, in some cases by almost two orders of magnitude.^{8,28–31} This has made it difficult to understand how active KDAC8 is with potential substrates and to compare the effectiveness of potential inhibitor or activator molecules. Our data indicate that a major contributor to the variability is Zn^{2+} levels in the reaction. This is a delicate balance, as KDACs require metal in equivalent stoichiometric ratio for catalytic activity, but any excess Zn^{2+} inhibits KDAC8.⁸ Our detection of trace levels of zinc in virtually all commonly used buffer reagents is consistent with prior reports that utilized chelator treatments of reagents and plasticware when measuring activity of KDAC8, and which also reported relatively high activity compared to other literature reports with the same substrates.^{8,9,29–31} As addition of three very different metal chelating reagents were all able to increase KDAC8 activity similarly in a commonly utilized tris-based buffer, we conclude that background Zn^{2+} in the buffer is leading to reduced KDAC8 activity (Figure 1A). In further support of this, reducing the citrate concentration from 5.0 mM to 2.5 mM led to reduced activity in the tris-based buffer (Figures 1A and 4), but not the phosphate-based buffer (Figure 3A). As even a slight amount of excess Zn^{2+} affects KDAC8 activity, the level of metal contamination found in commonly purchased reagents could account for differences in KDAC8 activity across the literature. The need to overcome this trace zinc may also account for why most prior studies of KDAC8 utilized enzyme concentrations much greater than required under our conditions.^{8,9,23–25,29,30,37–39} Indeed, in tris-based buffer (but not in phosphate-based buffer), the specific activity of KDAC8 was much lower at low enzyme concentrations; however, this effect could be eliminated by addition of BSA (Figures S3 and 1C). Critically, even a 500-fold excess of EDTA did not impair enzyme activity during the course of the reaction (Figure 2A), indicating that the chelator is not sufficient to strip the catalytic metal from the enzyme under reaction conditions.

While simply including BSA as has been previously done can compensate for some amount of Zn^{2+} contamination, it is not compatible with all assays. We recently published an assay which has significant advantages over other published deacetylase assays; however, it is not compatible with tris or BSA, due to interference from free amines, and instead relies on a

phosphate-based buffer system.²² This assay, initially developed using Buffer P30, inherently shields KDAC8 from Zn^{2+} contamination, as phosphate itself acts to reduce the free zinc in solution (Figure 3), presumably due to non-specific charge interactions and the low solubility of zinc phosphate. The affinity of tris for metal ions, including Zn^{2+} , is insufficient to significantly occupy the zinc ions under these conditions,⁴⁰ and so all or nearly all of the zinc in solution will be available to bind the enzyme when in tris-based buffers. Here, we unexpectedly determined that using a lower phosphate concentration (10 mM) resulted in increased KDAC8 activity, which corresponds to the difference in activity we previously reported when comparing our assay and buffer system to an acetate-based detection system in a tris-based buffer (pre-treated to eliminate trace metals).^{22,24,37} However, this increase came at the cost of reduced protection against free Zn^{2+} (Figure 2C). Fortunately, adding a chelating agent (either citrate or EDTA) to the low concentration phosphate-based buffer supported both high activity and protection from excess Zn^{2+} (Figure 3).

The buffer conditions presented here (10 mM potassium phosphate and either 2.5 mM citrate or 5.0 μM EDTA) are compatible across assays and eliminated variability in activity measurements due to variations in Zn^{2+} levels present in our buffer components (Figures 4 and S4). While it is possible that some commercially-obtained buffer components may require a higher concentration of chelator to suppress Zn^{2+} inhibition, an EDTA concentration as high as 500 μM is tolerated by the enzyme (Figure 2A). While inclusion of even these low levels of chelators is not a reasonable approach when the metal binding properties of KDACs are being investigated, this approach offers an inexpensive, cross-platform method for ensuring that measured enzyme activity is reproducible and unaffected by background levels of Zn^{2+} for other types of KDAC8 studies. Additionally, inclusion of a chelator allows high KDAC8 activity even at low enzyme concentrations, which greatly reduces the amount of enzyme needed to assess KDAC8 activity. This is especially advantageous for studies which involve screening KDAC8 activity with a large number of substrates or small-molecules. Although excess Zn^{2+} has not been reported to inhibit other KDACs in a similar manner, performing deacetylation reactions under the conditions proposed here should result in activity at least equivalent to the previously used buffer conditions. The robust activity observed under these conditions make the fluorescamine-based assay a powerful quantitative tool for screening potential substrates, as it will be very sensitive even for relatively weak substrates *in vitro*. Additionally, this assay is robust enough to easily use for small molecule screening. Several unlabeled peptide substrates derived from human proteins have been identified, such as ac-FR{Kac}RW-am, and can be used instead of AMC-labeled substrates to avoid artifacts caused by the fluorophore.^{22,31}

We have determined that screening for substrates and small molecule modulators of KDAC8 without controlling for trace Zn^{2+} levels in reaction solutions does not allow for direct comparisons between research groups, reagent lots, or assays. Furthermore, we provide a single set of optimized conditions to avoid this issue, and to easily and reproducibly quantify KDAC8 activity across substrates and in the presence of small molecule modulators of KDAC8 *in vitro*. Minimizing the effect of trace zinc will improve reproducibility and reliability of KDAC8 assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A

Supplementary data related to this article can be found at

Abbreviations

AMC	7-amino-4-methylcoumarin
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetic acid
HPLC	high performance liquid chromatography
KDAC	lysine deacetylase
ICPMS	inductively coupled plasma mass spectrometry
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
ppm	parts per million
SAHA	suberoylanilide hydroxamic acid
TOF	time-of-flight

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- Inconsistency in reported KDAC8 activity is likely due to trace Zn^{2+} .
- Chelating agents or phosphate buffer eliminate inhibition by trace Zn^{2+} .
- Phosphate-based buffer and/or chelator addition maximize KDAC8 activity.
- Proposed buffer conditions are compatible across assays and minimize enzyme use.

Figure 1. Chelators increase deacetylation of ac-{Kac}-AMC by KDAC8 in tris-based buffer
 Deacetylation of ac-{Kac}-AMC by KDAC8 was measured in reaction mixtures (n = 3) supplemented with 5.0 mM citrate, EDTA at the indicated concentration, 1.0 mg ml⁻¹ BSA, and/or 0.5 μM Zn²⁺ where noted. (A) Reactions in Buffer T. Chelators and BSA increase basal activity and reduce Zn²⁺ inhibition, without an additive effect. (B) In Buffer T supplemented with BSA, KDAC8 specific activity is independent of enzyme concentration. (C) Reactions in Buffer P30. KDAC8 activity is independent of BSA and added Zn²⁺.

Figure 2. KDAC8 activity and sensitivity to free Zn²⁺ are dependent on phosphate concentration
 Deacetylation of an unlabeled peptide substrate (ac-FR{Kac}RW-am) by KDAC8 was monitored in phosphate-based assay buffer (n = 3). (A) Reactions in Buffer P30 and 0.5 μ M Zn²⁺ or 0.5 mM EDTA where indicated. (B) Activity of KDAC8 as a function of phosphate concentration. The change in activity of KDAC8 due to phosphate concentration is independent of the zinc inhibition. (C) Zn²⁺ inhibition in Buffers P30 (grey squares) and P10 (black circles). Higher concentrations of phosphate result in lower basal activity, but also reduce the impact of free Zn²⁺ on enzyme activity.

Figure 3. Buffer with low phosphate concentration and chelating agents eliminates the activity artifacts caused by free Zn^{2+}

KDAC8 was reacted with ac-FR{Kac}RW-am in Buffer P10. In the presence of 2.0 μM Zn^{2+} , activity was measured as a function of the concentration of (A) citrate ($n = 3$) and (B) EDTA ($n = 2$). (C) Deacetylation with and without 5.0 μM EDTA was monitored ($n = 2$) in the presence of varied KDAC8 concentrations without added Zn^{2+} . Addition of EDTA did not cause a change in specific activity at either enzyme concentration.

Figure 4. Chelators maximize KDAC8 activity and provide consistency across reaction conditions
 KDAC8 was incubated with ac-{Kac}-AMC in the indicated buffer in the presence of the indicated chelating agent at the following final concentrations: 1.0 mg ml⁻¹ BSA, 2.5 mM citrate, or 5.0 μM EDTA (n = 3). The added chelators increase activity compared to the unchelated reaction (relative activity of 1; Fig 1A) and only the tris with citrate condition is statistically different from any other condition with chelator.