Published in final edited form as:

Methods Mol Biol. 2018 ; 1844: 59–70. doi:10.1007/978-1-4939-8706-1_5.

Competition Assay for Measuring Deubiquitinating Enzyme Substrate Affinity

Michael T. Morgan¹, Cynthia Wolberger²

¹Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD, USA.

²Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD, USA.

Abstract

Assays of the affinity of a deubiquitinating enzyme for substrate, either through binding studies or determination of the Michaelis constant, K_M , can shed light on substrate selectivity and the effects of mutations on substrate interactions. The difficulty in generating sufficient quantities of ubiquitinated substrate frequently presents a barrier to these studies. We describe here an alternative approach that can be used in cases where non-hydrolyzable, chemically ubiquitinated substrate analogs can be more readily generated. The substrate analog can be utilized as a competitive inhibitor in kinetics experiments monitoring cleavage of ubiquitin-AMC (Ub-AMC) by the deubiquitinating enzyme. The resulting inhibitory constant, K_i , provides a reliable approximation of the K_d for ubiquitinated substrate. We show how this approach can be used to assay the affinity of the yeast SAGA DUB module for nucleosomes containing monoubiquitinated H2B.

Keywords

Enzyme kinetics; Deubiquitinating enzymes; Ubiquitin; Enzyme inhibition; Equilibrium binding

1 Introduction

Deubiquitinating enzymes (DUBs) remove ubiquitin from substrate proteins and are involved in virtually every signaling process in eukaryotes. The approximately 90 DUBs encoded by the human genome target a vast variety of substrates and serve distinct roles in biology. There are six distinct classes of DUBs: the USP, UCH, OTU, MJD, and recently discovered MINDY cysteine protease DUBs and the JAMM metalloproteases [1]. All of these enzymes catalyze hydrolysis of the isopeptide linkage that joins the ubiquitin C-terminus to a lysine or, in some cases, to a protein amino terminus [2]. A detailed understanding of the substrate specificity of a particular DUB or the impact of mutations entails in vitro assays of purified components. Methods for measuring specificity include determining the kinetic constants, k_{cat} and K_M , or assaying binding of a catalytic mutant to

substrate using methods such as Förster resonance energy transfer (FRET) or isothermal titration calorimetry (ITC). In many cases, however, obtaining sufficient quantities of ubiquitinated substrate is difficult, if not impossible, thus presenting an obstacle to quantitative measures of substrate affinity and specificity.

We describe an alternative approach to estimating the affinity of a DUB for its substrate that can be implemented in cases where a non-hydrolyzable analog of the ubiquitinated substrate is available. The non-hydrolyzable substrate can be used as a competitive inhibitor in assays monitoring cleavage of the fluorogenic substrate, ubiquitin-AMC (Ub-AMC). The inhibition constant, K_i , which is determined by measuring Ub-AMC cleavage activity as a function of increasing competitor concentration, provides an estimate of the affinity of the DUB for the ubiquitinated substrate.

The protocol described here is for the yeast Spt-Ada-Gcn5 acetyltransferase (SAGA) DUB module, a four-protein subcomplex of the 1.8 MDa SAGA transcriptional coactivator [3]. The DUB module cleaves ubiquitin from Lys123 of yeast histone H2B in the context of the nucleosome core particle (NCP-Ub) [4–6]. Binding assays and kinetic studies of the DUB module are challenging due to the difficulty in generating sufficient quantities of nucleosomes that are specifically monoubiquitinated at H2B-K123. H2B-Ub substrate can be made by a semisynthetic approach that utilizes H2B and ubiquitin fragments generated by native chemical ligation [7, 8], which can be incorporated it into nucleosomes. The yield, however, is limited, and generating significant quantities is highly labor-intensive, making this approach impractical for kinetic or binding studies. While FRET methods require less material, it is first necessary to identify appropriate sites on both the enzyme and nucleosome for the donor and acceptor fluorophores.

We devised an approach for approximating the affinity of the DUB module for NCP-Ub that takes advantage of a method for generating nucleosomes with a non-hydrolyzable linkage between the ubiquitin C-terminus and Lys123 of histone H2B [9, 10]. Cysteine residues substituted for ubiquitin residue G76 and H2B residue K123 (K120 in *Xenopus* H2B) can be cross-linked with dichloroacetone (DCA) [9, 10], thus approximating the native isopeptide linkage. Nucleosomes containing H2B with the cross-linked ubiquitin (H2B-Ub_{DCA}) are then used to inhibit the cleavage of Ub-AMC by the DUB module [10]. The point at which rates of Ub-AMC cleavage are reduced by 50% is described as the inhibition constant, K_i . If NCP-Ub_{DCA} behaves as a competitive inhibitor, then $K_i \approx K_d$.

In this protocol, we describe how to identify the appropriate enzyme concentration for Ub-AMC cleavage assays, determine the Michaelis-Menten constants for Ub-AMC cleavage, and approximate the K_i of the non-hydrolyzable NCP-Ub_{DCA} analog. This method can be generalized to any DUB for which a non-hydrolyzable ubiquitinated substrate can be generated.

2 Materials

All solutions should be prepared with ultra-pure water and analytical-grade materials. Buffers can be prepared in advance, aliquoted in convenient volumes, and frozen at $-20\text{ }^{\circ}\text{C}$

for several months. *See* Note 1 for details of materials used for data acquisition and analysis. The use of a multichannel pipettor is optional.

2.1 Kinetics of Ubiquitin-AMC Cleavage

1. 384-well, low-volume, flat bottom, black polystyrene plates.
2. Purified SAGA deubiquitinating module (DUBm) (as described in [11]) in DUBm storage buffer: 10 mM HEPES pH 8, 150 mM NaCl, 20 μ M ZnCl₂, and 5 mM dithiothreitol (DTT).
3. Ubiquitin-AMC, a fluorogenic substrate in which the ubiquitin C-terminus is covalently linked to an AMC molecule via a peptide bond (Boston Biochem). When this bond is cleaved by the DUB, there is an increase in fluorescence.
4. Dimethyl sulfoxide (DMSO).
5. Assay buffer: 20 mM HEPES buffer (pH 7.5), 150 mM NaCl, 20 μ M ZnCl₂, 1 mM DTT, 0.1 mg/mL bovine serum albumin (BSA).

2.2 Determination of Inhibition Constant by Non-hydrolyzable Substrate

1. Nucleosome storage buffer: 10 mM Tris-HCl (pH 7.5), 50 mM KCl, and 1 mM DTT.
2. Non-hydrolyzable substrate, nucleosome core particle (NCP) with ubiquitin C-terminus conjugated to H2B residue 123 via a DCA linkage (NCP-Ub_{DCA}), prepared as previously described [10], in nucleosome storage buffer.
3. Serial dilutions of Ub-AMC in DMSO (*see* Subheading 3.3.1).

3 Methods

3.1 Determine Range of Enzyme Concentrations Over Which Reaction Velocity Is Linear

It is first necessary to determine the concentration range of enzyme in which the rate of Ub-AMC cleavage increases linearly with increasing enzyme concentration. In practice, enzymes can adhere to surfaces, lose activity during handling, or exhibit prep-to-prep variability in a way that may significantly alter the amount of active enzyme. Measurements of initial rates are most easily obtained when the reaction is slowest, which corresponds to the lowest reasonable concentration of enzyme. It is therefore important to determine the lowest enzyme concentrations at which the activity of the DUB in question is linear. Such an approach ensures that the experimenter is working at DUB concentrations at which the assumptions of kinetic analysis hold and that results are reproducible. In this section, we describe a workflow for determining the linear range of the DUB module.

3.2 Determining the Linear Range of Ub-AMC Cleavage

1. Prepare 60 μ L of 10 μ M Ubiquitin-AMC (Ub-AMC) substrate in assay buffer; this will be a 10 $\times$ stock of Ub-AMC (final concentration of Ub-AMC was chosen to be at least 5 $\times$ the highest concentration of DUBm used in step 2 below).

2. Prepare 90 μL each of 10 nM, 25 nM, 50 nM, 100 nM, 150 nM, and 200 nM DUBm in assay buffer. This volume was chosen to slightly exceed the total amount needed for the measurements below.
3. To perform the first replicate, pipette 27 μL of each of the six DUBm dilutions into six wells of a microplate. Cover with tape.
4. Incubate the plate for 20 min in the plate reader, which has been pre-equilibrated at the assay temperature (30 $^{\circ}\text{C}$ for the yeast DUB module).
5. Initiate the cleavage reaction by adding 3 μL of the 10 $\times$ Ub-AMC stock to each well containing DUB enzyme, giving a final reaction concentration of 1 μM Ub-AMC (*see Note 2*). The pipetting can be done either in rapid succession with a repeater pipette or at once with a multichannel pipette. For cases in which the reaction rate is quite fast, such that a significant amount of Ub-AMC is consumed during the time it takes the plate reader to monitor multiple wells, it will be necessary to assay one concentration at a time (*see Notes 3 and 4 on gain setting*).
6. Record the fluorescence increase in each well as a function of time (*see Note 5*).
7. Repeat steps 2–6 two additional times such that three measurements of each enzyme concentration are made.
8. Identify the linear range of the reaction, which is typically over the period in which approximately 10% of substrate is consumed.
9. Determine the initial velocity (v_i) for each enzyme concentration by measuring the slope of the line in the linear range of the reaction. This is done by plotting the increasing fluorescence values as a function of time and using linear regression in Excel (or similar software) to fit a line to the data points.
10. Using Prism, Excel, or other data analysis software, plot initial velocity (v_i) divided by the corresponding enzyme concentration ($v_i/[E]$) as a function of the enzyme concentration ($[E]$). This is in essence a plot of the first derivative of the rate as a function of enzyme concentration. An example is shown in Fig. 1
11. The linear range of enzyme activity corresponds to the concentration range over which the slope of the plot of $v_i/[E]$ versus $[E]$ is approximately 0. For the data shown in Fig. 1, the activity of the DUB module is linear after an enzyme concentration of 150 nM (*see Note 6*).
12. If the plot does not reveal a concentration regime that reflects the desired stability of $v_i/[E]$, consider altering the buffer conditions, exploring a different concentration range of enzyme, or repurifying the enzyme.

3.3 Determining Michaelis-Menten Kinetic Constants for DUB Module Cleavage of Ub-AMC

Classical enzyme kinetic assays can be used to measure the Michaelis-Menten constants that define the enzyme's activity on Ub-AMC. Since commercially available Ub-AMC is typically dissolved in DMSO, an important consideration is maintaining constant solvent

content while increasing the concentration of the Ub-AMC substrate. It is important to keep the volume of substrate added to the reaction as small as possible because high DMSO concentrations can inhibit enzyme activity. Since the concentration of substrate required to reach maximum enzyme velocity ($V_{\max}$) differs between DUBs, the need to minimize the proportion of DMSO in the reaction can, in practice, limit the maximum amount of substrate that can be used.

In order to accurately determine the relative amount of ubiquitin that has been cleaved as a function of time, it is first necessary to generate a standard curve, which is used to convert arbitrary fluorescence signal into units of concentration.

3.3.1 Generating a Ub-AMC Standard Curve

1. Make 10 μL stocks of 5, 10, and 20 μM Ub-AMC.
2. Make 300 μL of a 1 μM stock of DUB module in assay buffer (*see* Note 7).
3. Pipette 27 μL of the DUB stock into each of 9 microplate wells, forming three sets of three wells.
4. Add 3 μL of 5 μM Ub-AMC to each of the first three wells. Repeat with the 10 and 20 μM Ub-AMC stocks with the next two sets of wells, such that there are three cleavage reactions for each concentration of Ub-AMC (now at 0.5, 1, and 2 μM for each respective Ub-AMC stock).
5. Monitor the fluorescence in each well with the plate reader until there is no further increase in signal in any of the wells (*see* Note 8). At this point, the Ub-AMC in each well is assumed to be fully digested.
6. Record the signal from each well. Each set of three readings should then be averaged.
7. Plot the average fluorescence values and their corresponding errors as a function of Ub-AMC concentration, and use Excel to fit the values to a straight line.
8. The algebraic expression of the line, $y = mx + b$, can then be used to convert fluorescence units into concentration values; given any fluorescence value (y), solving for x gives the concentration of Ub-AMC that has been cleaved. This equation will be used to measure the amount of Ub-AMC consumed in all experiments (Fig. 2).

3.3.2 Measuring Reaction Velocities, K_M , and $V_{\max}$

1. Make a series of 10 μL stocks of Ub-AMC diluted in pure DMSO, at the following concentrations: 10, 20, 30, 40, 50, 70, 100, and 192 μM Ub-AMC (in this case, the Ub-AMC obtained from the vendor was at a concentration of 192 μM).
2. Make a working stock of DUBm by diluting with assay buffer to 1.1 $\times$ the optimal enzyme concentration as determined in Subheading 3.1 (165 nM in this case).

3. Pipette 27 μL of the working enzyme stock into three wells of the microplate. Each of the three wells will constitute a triplicate measurement of each substrate concentration.
4. Incubate the microplate containing the enzyme in the plate reader at the desired temperature (30 $^{\circ}\text{C}$ in this case) for 20 min.
5. Add 3 μL of the 10 μM Ub-AMC stock to each well to initiate each reaction replicate.
6. Immediately begin monitoring the cleavage of Ub-AMC with the plate reader once the substrate is added.
7. For each well, determine the interval during which the fluorescence increases linearly as a function of time (*see* Note 9).
8. Repeat steps 3–7 for 20, 30, 40, 50, 70, 100, and 192 μM Ub-AMC concentrations, until triplicate measurements of each desired substrate concentration are obtained.
9. Determine the slope of the linear region of the cleavage reaction for each well as described in step 9 of Subheading 3.2, and convert the fluorescence units into concentration of product as described in step 8 of Subheading 3.3.1. The result will be the reaction velocity in units of $\mu\text{M/s}$. Average the values for each triplicate point to obtain the average value of v_i for each concentration.
10. Plot the average initial velocities as a function of Ub-AMC concentration as shown in Fig. 3. These values should be plotted during the course of the experiment in order to monitor whether the reaction is approaching saturation, meaning that the reaction velocity does not change appreciably as the Ub-AMC concentration is increased. Since the K_M of the DUB for Ub-AMC can be high, making it impractical to approach saturation, data analysis software such as Prism can be used to fit the data to the Michaelis-Menten equation and monitor the range of 95% confidence intervals for $V_{\max}$ and K_M values to determine when the values are sufficiently constrained.

3.4 Measure the K_i of Nucleosome Containing Non-hydrolyzable H2B-Ub Substrate Analog

The kinetic constants of the deubiquitinating enzyme are used to determine the K_i of a non-cleavable substrate analog, which in this application is the ubiquitinated nucleosome containing a non-hydrolyzable linkage between the ubiquitin C-terminus and H2B (NCP-Ub_{DCA}). The cleavage of Ub-AMC is monitored in the presence of increasing concentrations of NCP-Ub_{DCA}, which binds to the DUBm and competes for substrate cleavage. The effect of increasing the concentration of competitor should be assayed for at least three different Ub-AMC concentrations. Substrate analog concentration should span a range of both above and below the estimated K_d of the substrate analog in order to derive well-determined values. In this case, we used electrophoretic mobility shift assays (EMSA) to estimate the K_d of the DUB module affinity for NCP-Ub_{DCA} and inferred that the K_d likely approximates the K_i (*see* Note 10). If the K_d is not known, it may be necessary to

assay a range in order to identify a set of concentrations over which the apparent K_M of the reaction is reduced by greater than 50% (see Note 11).

1. Prepare a 187.5 nM stock solution of the DUB module in assay buffer (1.25× the final enzyme concentration in each reaction). For the experiment described here, 500 µL of enzyme stock is a sufficient volume to measure Ub-AMC cleavage at five different Ub-AMC concentrations in the presence of three different NCP-Ub_{DCA} concentrations, as well as in the absence of NCP-Ub_{DCA} (storage buffer only added).
2. Make 15 µL Ub-AMC stock solutions at concentrations of 10 µM, 20 µM, 30 µM, 50 µM, and 100 µM in DMSO, each representing a 10× stock solution of the final substrate concentration in each reaction.
3. Prepare 15 µL each of 10× NCP-Ub_{DCA} stock solutions at concentrations of 10, 25, and 50 µM. Add 15 µL of NCP storage buffer to a fourth tube (to serve as the no-inhibitor control).
4. Add 120 µL DUB stock to the 10 µM NCP-Ub_{DCA} stock, giving a total volume of 135 µL. This is the exact volume to be used, whereas previous steps provided a slight excess, so pipette with care or make a slightly larger volume. Incubate for at least 20 min.
5. Aliquot 27 µL of the DUBm-NCP-Ub mixture into each of five wells in the microplate, and incubate for 20 min at 30 °C.
6. To ensure that early time points are captured, initiate one reaction at a time, and monitor fluorescence with the plate reader. To initiate the first reaction, add 3 µL of the 10 µM Ub-AMC stock to the first well (final concentration 1 µM). Monitor the increase in fluorescence as a function of time.
7. Repeat step 6 with the 20 µM, 30 µM, 50 µM, and 100 µM Ub-AMC stocks (giving final substrate concentrations of 2, 3, 5, and 10 µM).
8. Verify that the initial rates are linear by monitoring the fluorescence signal.
9. Repeat steps 4–8 for each concentration of the inhibitor, NCP-Ub_{DCA}, as well as for the NCP storage buffer (no-inhibitor control).
10. Use the standard curve determined in Subheading 3.2.1 to convert initial rates of fluorescence evolution (AU/s) into initial rates of product formation (µM/s).
11. For each inhibitor concentration, plot the initial rates as a function of substrate concentration, and determine the apparent K_M :

$$v_i = V_{\max} \times \frac{[S]}{K_M^{\text{obs}}(I) + [S]}$$

The values of the apparent K_M for each inhibitor concentration are then used to determine the K_i from the following equation:

$$K_M^{\text{obs}}(I) = K_M \times \left(1 + \frac{[I]}{K_i}\right)$$

Data analysis software such as GraphPad Prism have the option to determine the K_i directly from a spreadsheet containing the values for the reaction velocity as a function of substrate and inhibitor concentration, as demonstrated in Fig. 4.

4 Notes

1. The experiments described here were performed on a BMG Labtech POLARstar Omega plate reader. Data are fitted with software for analyzing enzyme kinetics, such as Prism (Graph-Pad). The plate reader or fluorometer used to measure fluorescence should be equipped with a temperature control mechanism, and the instrument should be set to equilibrate at the desired temperature 20 min before use; for yeast enzymes, 30 °C is appropriate. If the fluorescence detection instrument does not have monochromators, filters should be used (345 nm for excitation, 445 nm for emission, both with a 10-nm bandpass to ensure sufficient signal).
2. Throughout this protocol, thorough mixing is necessary to produce high-quality data. We recommend using a second pipette with a fresh tip to mix each reaction immediately after adding the substrate.
3. It is critical to ensure that the rate of the reaction is observable on a time scale matching the instrument's read time. If the reaction is too fast to measure multiple wells at once, one should measure one reaction at a time. We suggest pilot experiments to estimate the speed of the reaction.
4. For all steps in this protocol, we assume that the maximum fluorescence one would want to measure is 10% of the highest overall Ub-AMC concentration used throughout (in this case, 2 μM , or roughly 10% of the highest concentration used in Subheading 3.2.2). Thus, a 2 μM standard of Ub-AMC pre-cleaved with 1 μM DUBm for 30 min should be used to determine the instrument's gain setting, and the same gain value should be used throughout the experiments described.
5. Typically, 10–20 points collected within 30 s of initiation are sufficient for a robust measurement. Longer monitoring windows may be needed for slow reactions. As substrate is consumed, the rate of the first 10% of product formation will be approximately linear with respect to substrate concentration, before curving toward the plateau corresponding to full cleavage. It is the initial, linear phase of the reaction that is used to fit initial velocity values (v_i).
6. Slower reactions are easier to monitor, especially if monitoring multiple wells in a plate reader format. For this reason, it is advantageous to use the lowest enzyme concentration within the linear range. Alternatively, one can lower the temperature of the reaction to slow the kinetics.

7. This enzyme concentration is one that will rapidly digest each sample of Ub-AMC to completion.
8. Progress of the reaction can be periodically checked; however typically the Ub-AMC will be fully digested within 20 min of initiating the reaction.
9. The Michaelis-Menten kinetic approximations hold only when a small proportion (<10%) of substrate has been consumed.
10. Reliability of K_i estimates depends on how well-inhibited the enzyme is. We recommend that substrate analog concentrations cover a range at which the enzyme is (1) <50% inhibited in its initial velocity, (2) approximately 50% inhibited, and (3) >50% inhibited.
11. Note that the protocol below utilizes a single replicate of each condition due to material limitations. Given the high ratio of observations (20) to the single parameter to be determined, K_i , this approach can still yield an accurate measurement. If possible, triplicate measurements of each concentration would be optimal.

Acknowledgments

Supported by the National Institute of General Medical Sciences (GM095822).

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Fig. 1.

Identifying the enzyme concentration to use in assays. To find the minimal concentration of enzyme for use in kinetic assays, the initial rate of Ub-AMC cleavage is plotted as a function of enzyme concentration. The same concentration of Ub-AMC is the same in each assay

Fig. 2.

The standard curve of fluorescence as a function of cleaved Ub-AMC concentration. A plot of fully digested Ub-AMC at different concentrations is used to convert fluorescence units (AU) into concentration units (μM)

Fig. 3. Michaelis-Menten kinetics of DUB module cleavage of Ub-AMC. Plot of initial reaction velocity as a function of Ub-AMC concentration can be fit to the Michaelis-Menten equation to determine values for K_M and k_{cat} .

Fig. 4.

Initial velocity of Ub-AMC cleavage in the presence of increasing concentrations of non-hydrolyzable inhibitor. DUB module cleavage of Ub-AMC is plotted for different concentrations of NCP-Ub_{DCA}, which acts as a substrate analog. The K_i is derived as described in the text