

A study of SIRT1- and SIRT2-Mediated Deacetylation

Using the Agilent RapidFire high-throughput mass spectrometry system

Authors

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Abstract

The Agilent RapidFire high-throughput mass spectrometry (MS) system is well-suited to screening sirtuins because it obviates the need for labeled substrates and coupled reactions. Using this system, kinetic data, including linear conversion ranges, binding constants, and IC_{50} values, were determined for the SIRT1 and SIRT2 enzymes produced by BlueSky Biotech. Also, because high-throughput MS measures analytes directly, multiple deacetylation events on a single peptide could be monitored. These experiments underscore the utility of BlueSky Biotech reagents and high-throughput MS for label-free screening of sirtuins.

Introduction

Histones and other proteins are subject to a variety of post-translational modifications that regulate a wide host of biological processes. One such modification is the deacetylation of specific lysine residues by sirtuins, whose activity has been associated with inflammatory, cardiovascular, proliferative, neurodegenerative, and metabolic disorders.¹ Therefore, sirtuins constitute promising targets for drug discovery, requiring the development of biological assays to identify small molecules that modulate their activity.

Studying sirtuins by RapidFire-MS offers numerous advantages over other existing assay formats. It avoids the costs and special handling procedures required by using radioactive substrates. RapidFire-MS-based assays do not involve secondary or coupled reactions that can complicate data interpretation. Because the peptide species are measured directly by MS, there is no need for fluorescent tags, which have previously caused data artifacts. In situations where multiple products can be formed from a single peptide, which is a common reaction carried out by sirtuins and other epigenetic targets, MS detection enables every modification state to be measured discretely and concurrently.

In this study, the use of RapidFire-MS to study the SIRT1- and SIRT2-mediated deacetylation of native peptides is demonstrated. Using this technology, linear conversion ranges, binding constants, and IC_{50} values were determined. The successive deacetylation of a p53 peptide containing three acetyl (ac) modifications (i.e. 3ac to 2ac to 1ac to 0ac) is also demonstrated, illustrating the utility of BlueSky Biotech (Worcester, MA) reagents and RapidFire-MS in providing superior data for studying sirtuin enzymes.

Experimental

Reagents

Recombinant SIRT1 and SIRT2 were produced in *E. coli*, purified using affinity resin, formulated in stock buffer (40 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.01% Brij-35, 0.01% Triton X-100, 0.005% Tween-20, and 8% glycerol), snap-frozen in liquid nitrogen, and stored at -80°C by BlueSky Biotech.

The acetylated-p53 substrate (TP53 Q9NP68, p53 Mutant Form (372 to 389) Lys382, KKGQSTSRHK-Kac-LMFKTEG) was purchased from Anaspec, Fremont, CA, and the triply-acetylated-p53 substrate (KKGQSTSRH-KacKac-LMF-Kac-TEG) was synthesized by Biopeptide, San Diego, CA.

Reactions

Deacetylase reactions were carried out in reaction buffer (50 mM Tris-HCl pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2 , and 0.05% BSA) plus amounts of NAD^+ , peptide substrate, and enzyme that varied depending on the experiment. All reactions were initiated by the addition of peptide substrate and quenched by the addition of formic acid.

Method

- **RapidFire Buffer A:** H_2O + 0.09% formic acid + 0.01% trifluoroacetic acid, 1.5 mL/minute
- **RapidFire Buffer B:** 80% acetonitrile + 0.09% formic acid + 0.01% trifluoroacetic acid, 1.25 mL/minute
- **RapidFire cartridge:** A
- **MS/MS (+ mode):** acetylated-p53 peptide: 534.4/128.6 (1ac) and 523.9/128.6 (0ac)
- **Triply-acetylated-p53 peptide:** 555.5/638.6 (3ac), 545.0/624.6 (2ac), 534.5/610.6 (1ac), and 524.0/596.6 (0ac)

Results and discussion

SIRT1 assay development with the acetylated-p53 peptide began with an enzyme titration time course to determine the linear range of enzyme kinetics. Figure 1A shows the deacetylation of acetylated-p53 peptide over time at four enzyme concentrations. Formation of product with time was linear at all enzyme concentrations tested, and plotting the initial velocity of the reaction (the slope of the linear regression) against enzyme concentration revealed a linear relationship with $R^2 = 0.9942$ (Figure 1B). Once the optimal enzyme concentration was identified, an experiment to investigate the effect of peptide concentration on initial velocity was conducted.

The resulting velocities were plotted against the peptide concentrations used, allowing the binding constant of the peptide for SIRT1 to be determined as 25 μM (Figure 1C). This determination facilitated the development of a screening protocol for SIRT1, which was tested using nicotinamide, a known SIRT1 inhibitor. Percent conversion was studied at six nicotinamide concentrations, and the resulting data (Figure 1D) revealed an IC_{50} of 62 μM . This value correlates well with 50 μM published elsewhere.²

To further illustrate the utility of using RapidFire-MS to study sirtuin-mediated deacetylation, a reaction was conducted with SIRT1 enzyme and a triply-acetylated-p53 peptide. The time course experiment was analyzed for each of the four possible peptide acetylation states (3ac, 2ac, 1ac, and 0ac). Figure 2 shows the conversion over time of the 3ac substrate (orange diamonds) to 2ac (green circles), 1ac (red triangles) and 0ac (blue squares) products. The ability to differentiate between each of these species is unique to MS and can provide information that is critical to developing robust screening protocols. For example, the ability to distinguish between two modifications to a single substrate molecule versus one modification to two substrate molecules enables the proper calculation of substrate binding constants and provides unparalleled data to assist in choosing the optimal substrate.

The development of the SIRT2 assay proceeded just like the SIRT1 characterization above. To begin, an enzyme titration time course was conducted to determine the linear range of conversion. Figure 3A shows the time-dependent peptide deacetylation resulting from four SIRT2 concentrations. Formation of product was linear with time for each concentration tested, and plotting the initial velocities against the respective enzyme concentration

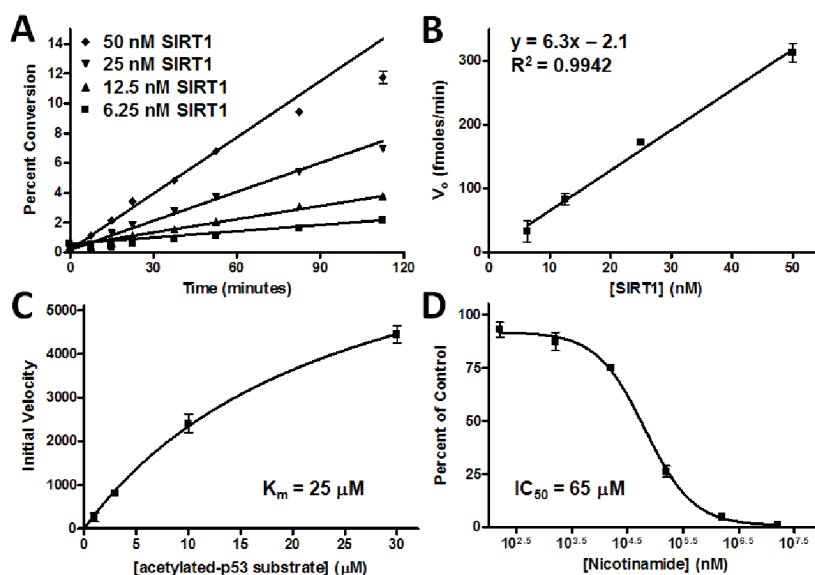


Figure 1. Monitoring the SIRT1-mediated deacetylation of a p53 peptide. SIRT1 titration time course data (A) were used to establish the range of linear enzyme kinetics (B), p53 peptide K_m (C) and nicotinamide IC_{50} (D).

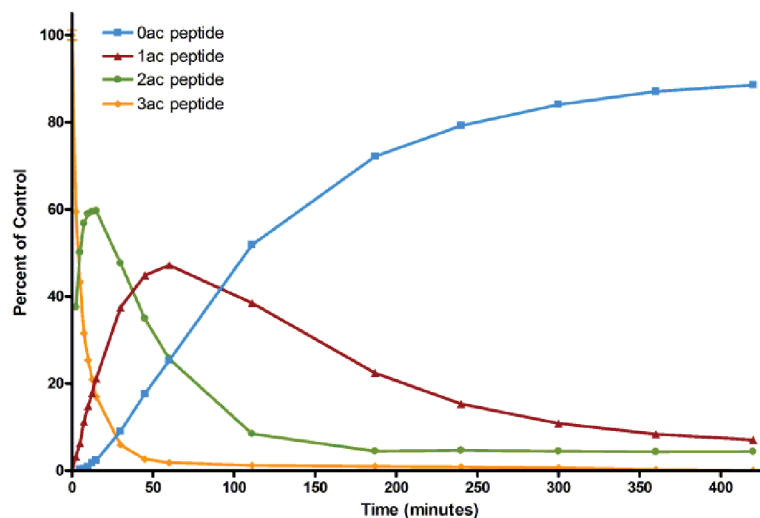


Figure 2. Successive deacetylation of a triply-acetylated p53 peptide by SIRT1. Conversion over time of 3ac peptide (orange diamonds) to 2ac (green circles), 1ac (red triangles) and 0ac (blue squares) species is observed.

used revealed a linear relationship with $R^2 = 0.9971$ (Figure 3B). Next, the effect of peptide concentration on initial velocity was investigated. The resulting reaction velocities were plotted against the peptide concentration used, and a binding constant of the substrate for SIRT2 was extracted as $8 \mu\text{M}$ (Figure 3C). Subsequently, a screening protocol for SIRT2 was developed and tested using a known SIRT2 inhibitor, nicotinamide. Conversion was studied at six nicotinamide concentrations, and the resulting data (Figure 3D) revealed an IC_{50} of $11 \mu\text{M}$. This value was similar to $10 \mu\text{M}$ reported previously.³

Conclusion

Label-free detection by RapidFire-MS provides compromise-free data and facilitates the determination of accurate linear conversion ranges, binding constants, and IC_{50} values for the SIRT1 and SIRT2 enzymes from BlueSky Biotech. The ability to measure reaction substrate and product(s) directly is advantageous for developing robust assays and asserts the utility of these products for drug discovery research.

The specific activity of BlueSky Biotech SIRT1 is 3 U/mg ($= 3 \text{ nmole product/mg/min}$), where one unit (U) is defined as the amount of enzyme required to produce 1 nmole of deacetylated peptide per minute at 25°C in 50 mM Tris-HCl pH 7.5, 137 mM NaCl , 2.7 mM KCl , 1 mM MgCl_2 , $0.05\% \text{ BSA}$, $250 \mu\text{M NAD}^+$, and $10 \mu\text{M}$ acetylated-p53 substrate. The specific activity of BlueSky Biotech SIRT2 is 9 U/mg ($= 9 \text{ nmole product/mg/min}$).

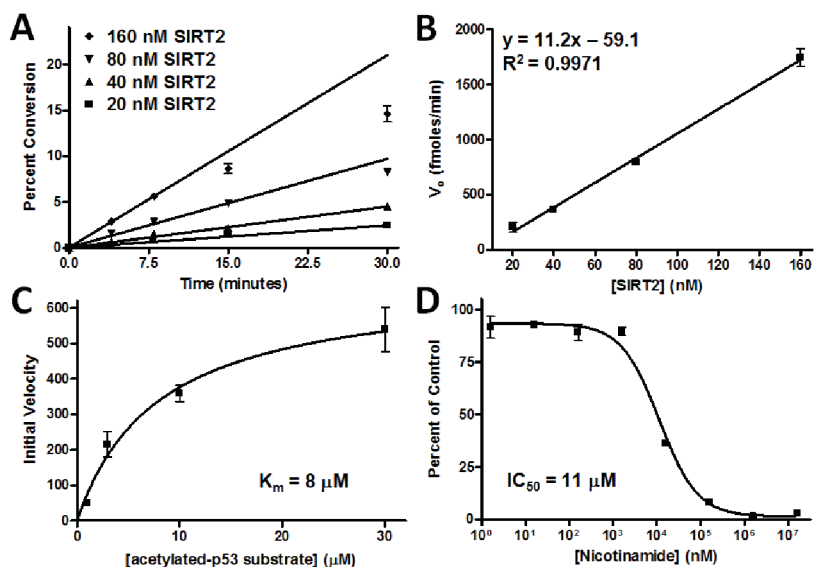


Figure 3. Monitoring the SIRT2-mediated deacetylation of a p53 peptide. SIRT2 titration time course data (A) were used to establish the range of linear enzyme kinetics (B), p53 peptide K_m (C) and nicotinamide IC_{50} (D).

References

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