Using the Agilent RapidFire High-throughput Mass Spectrometry System to study SIRT1- and SIRT2-mediated deacetylation

Application Note

Drug Discovery

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\textsubscript{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.
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.

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 - 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$_2$O + 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)

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 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 \( \mu M \) (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 \( \mu M \). This value was similar to 10 \( \mu M \) 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\textsubscript{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 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\textsubscript{2}, 0.05% BSA, 250 µM NAD\textsuperscript{+}, and 10 µM acetylated-p53 substrate. The specific activity of BlueSky Biotech SIRT2 is 9 U/mg (= 9 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\textsubscript{m} (C) and nicotinamide IC\textsubscript{50} (D).

References