Development of a Fluorogenic Probe with a Transesterification Switch for Detection of Histone Deacetylase Activity

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S *Supporting Information*

ABSTRACT: Histone deacetylases (HDACs) are key enzymatic regulators of many cellular processes such as gene expression, cell cycle, and tumorigenesis. These enzymes are attractive targets for drug development. However, very few simple methods for monitoring HDAC activity have been reported. Here, we have developed a fluorogenic probe, K4(Ac)-CCB, which consists of the histone H3 peptide containing acetyl-Lys and a coumarin fluorophore with a carbonate ester. By the simple addition of the probe to a HDAC solution, enzyme activity was clearly detected through spontaneous intramolecular transesterification, which renders the probe fluorescent. In addition, K4(Ac)-CCB can be applied to the evaluation of HDAC inhibitor activity. This is the first report to demonstrate the monitoring of HDAC activity by using a one-step procedure. Thus, our novel fluorogenic probe will provide a powerful tool for epigenetic research and the discovery of HDAC-targeted drugs.

Histone deacetylases (HDACs) are hydrolytic enzymes that catalyze the removal of acetyl groups from ε-N-acetylated lysine residues of histones and other cellular proteins.[1] The deacetylation of these proteins plays essential roles in the epigenetic regulation of gene expression, signal transduction, and cell homeostasis.[2,3] It has also been demonstrated that HDACs are involved in various diseases such as cancer, metabolic syndromes, and neurodegenerative diseases.[4] Hence, HDACs are the major targets of drug development. Despite the importance of the enzyme assay in the medical and pharmaceutical fields, no method has been reported for monitoring the enzymatic activity of HDACs using a one-step procedure. So far, radio isotopes[5] and antibodies[6] have been utilized to verify the activity of HDACs. However, these methods require laborious handling or multistep procedures, which hinder the immediate measurement of HDAC activity. As an alternative method, a fluorogenic probe based on a substrate peptide was developed and is available from a commercial source.[7,8] Its limitation is that additional protease digestion of the probe is essential for fluorescent detection. In addition, the flexibility of the probe design is restricted in this mechanism, because the conjugation of the fluorophore with the carboxylate of the terminal lysine is essential for fluorogenic digestion by trypsin. Besides the above methods, genetically encoded fluorescent reporters for histone modifications, such as methylation[9] and phosphorylation,[10]

were developed. In these reporters, fluorescence resonance energy transfer (FRET) between two fluorescent proteins (FPs)[11] was utilized. Recently, a biosensor for the evaluation of histone acetylation levels using FRET between FPs was also reported.[12] Although other methods such as HPLC,[13] mass spectrometry,[14] inhibitor competition,[15] etc. have been reported, none of them allows straightforward, facile detection of deacetylase activity. In this study, to overcome these obstacles, we developed a fluorogenic probe for the detection of HDAC activity by using a one-step procedure.

For the fluorescent detection of HDAC activity, it is necessary to design a probe based on a mechanism in which fluorescence output is automatically obtained as a result of the deacetylation of acetyl-Lys. In general, the fluorescence properties of fluorophores are controlled by their electronic state. Therefore, the electronic state of the fluorophore of any potential HDAC probe must be altered by the enzyme reaction. However, this is challenging because the substrate structure consists of an aliphatic amide in acetyl-Lys and does not allow π-conjugation of the amide to the fluorophore to modulate its electronic state feasibly by the enzymatic reaction. Because of this reason, the deacetylation reaction cannot directly affect the fluorescence properties of the fluorophore, connected to the enzyme-cleavable moiety in the probe. To conquer this problem, we focused on the nucleophilicity of the amino group in lysine. While the acetamide of the acetyl-Lys is chemically inert, the primary amine generated by the HDAC reaction is highly nucleophilic and reacts with various electrophilic groups. Considering this, we designed a probe, K4(Ac)-CCB (Figure 1). The probe consists of a substrate peptide derived from the N-terminal region of histone H3, and a coumarin derivative as the fluorophore. The peptide contains an acetyl-Lys (K4(Ac)) at the fourth position in its sequence. In order to convert the deacetylation reaction of the probe into a fluorescence response, an electrophilic carbonate ester was incorporated into the 7-hydroxy group of the coumarin as a fluorogenic switch. Importantly, it is known that the acylation of the 7-hydroxy group of the coumarin derivative results in the loss of its fluorescence.[16] Since the electrophilic carbonate ester is reactive with the intramolecular nucleophile, the ester would be transferred to the amine of the deacetylated probe, DP, through nucleophilic attack by the amine. This transesterification reaction generates a fluorescent molecule, TP. Thus, the amine generated by enzymatic deacetylation triggers
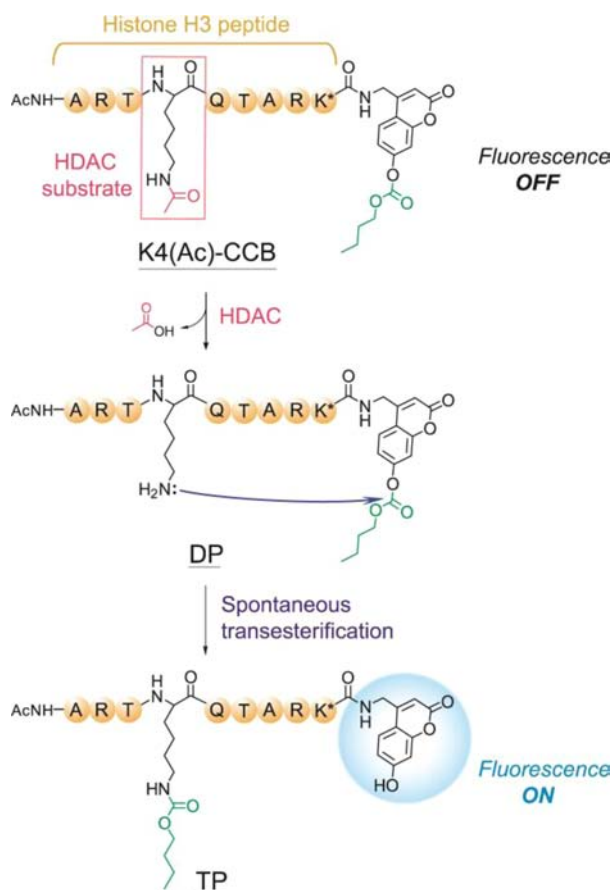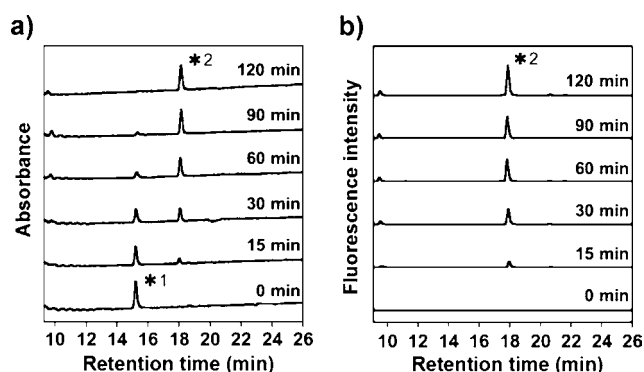
Figure 1. Design of K4(Ac)-CCB and the principle behind the detection of HDAC activity by using a one-step procedure. The trimethylated lysine at the ninth position is marked with an asterisk.

Figure 2. Reversed-phase HPLC analysis of the transesterification reaction of DP. DP (3 μM) incubated for the designated period was analyzed by monitoring absorbance at 305 nm (a) and fluorescence with excitation and emission wavelengths of 338 and 458 nm, respectively (b). The retention times of the peaks marked by asterisks 1 and 2 were 15.5 and 18.2 min, respectively.

spontaneous intramolecular transesterification in the probe, which turns on the masked fluorescence of coumarin. Therefore, it was envisioned that HDAC activity could be detected by the fluorescence enhancement of the probe, induced as a result of enzymatic deacetylation. A lysine at the ninth position of the peptide sequence is trimethylated to prevent undesirable transesterification without altering its positive charge (Figure 1).

First, DP was synthesized to test whether the transesterification reaction occurs. After DP was incubated in 20 mM HEPES buffer (pH 8.0) at 37 °C, aliquots were analyzed periodically by high-pressure liquid chromatography (HPLC) and electrospray ionization mass spectroscopy (ESI-MS) (Figures 2 and S1). As expected, the intensity of the peak corresponding to DP (retention time = 15.5 min) was reduced after the incubation. Instead, a peak appeared at the retention time of 18.2 min (Figure 2a). An eluted compound obtained from this peak showed a mass value that matches those of DP and TP (Figure S1). It is noted that DP and TP contain the same mass value.

Furthermore, the fluorescence chromatogram verified that the newly generated peak of TP exhibits fluorescence, while the DP peak does not (Figure 2b). On the other hand, K4(Ac)-CCB incubated in HEPES buffer did not yield any peak besides the small one derived from the hydrolysis product of the probe (Figures S2 and S3). These results suggest that DP undergoes intramolecular transesterification from the 7-hydroxy group of the coumarin to the ε-amino group of K4 to generate a

fluorescent product, TP, as shown in Figure 1. To confirm this reaction, matrix-assisted laser-desorption/ionization time-of-flight/time-of-flight mass spectrometry using the "LIFT" technique (MALDI-LIFT-TOF/TOF MS) was carried out[17] using the elutant obtained at the retention time of 18.2 min in Figure 2. In the mass spectrum, acylated lysine was clearly detected at the fourth position in the DP sequence. This result confirmed the transesterification to the lysine (Figure S4).

Next, we investigated the reactivity of K4(Ac)-CCB with Sirt1. Sirt1 belongs to the family of NAD^{+}-dependent histone deacetylases and regulates various cellular events such as transcriptional silencing, chromatin remodeling, and lifespan extension.[18] This enzyme is also involved in cancers[19] and type-2 diabetes[20] and is a promising therapeutic target for anticancer drugs.[21] To examine whether K4(Ac)-CCB was deacetylated by Sirt1 and underwent subsequent transesterification, we performed HPLC and ESI-MS analyses of the enzyme reaction mixture. Following a 15-min incubation of the probe with the enzyme, the peak representing the probe (retention time = 19.7 min) completely disappeared (Figure 3). A new peak was transiently detected at a retention time of 15.3 min, which
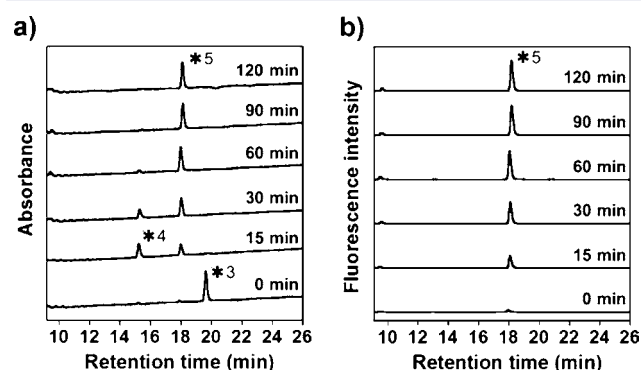
Figure 3. Reversed-phase HPLC analysis of the enzymatic reaction of K4(Ac)-CCB with Sirt1. K4(Ac)-CCB (3 μM) was reacted with Sirt1 (100 nM) in 20 mM HEPES buffer (pH 8.0) containing 300 μM NAD^{+} at 37 °C for the designated period. The aliquots were analyzed by monitoring absorbance at 305 nm (a) and fluorescence with excitation and emission wavelengths of 338 and 458 nm, respectively (b). The retention times of the peaks marked by asterisks 3, 4, and 5, were 19.7, 15.3, and 18.0 min, respectively.

corresponds to DP (Figure 2a). This peak was absent in the chromatogram of the probe incubated without Sirt1 (Figure S2); therefore, these results indicate that the K4(Ac) of the probe was deacetylated by Sirt1. This peak was diminished when the probe was reacted with the enzyme for 90 min. As with the HPLC analysis of DP, the intensity of the peak at the retention time of 18.0 min gradually increased. Strong fluorescence was recorded in this peak (Figure 3b), and its elutant gave the same mass as that of TP (Figure S6). Moreover, the molecular weight and the fragmentation pattern of this elutant were analyzed by MALDI-LIFT-TOF/TOF MS. The acylation of K4 in the probe was clearly detected (Figure S5). Judging from the retention time, the fluorescent property and MS data, this peak was assigned to the transesterification product, TP. These results indicated that Sirt1 catalyzed the deacetylation of the probe, triggering the subsequent intra-molecular transesterification from acylated coumarin to deacetylated lysine, and resulting in the generation of 7-hydroxycoumarin. Although the rate of transesterification was relatively slower than that of the enzyme reaction, we think that this could be overcome by optimizing the distance between the acetyl-Lys and the fluorophore.

Finally, we conducted fluorescence analyses of the enzyme reaction. The fluorescence spectra of the probe were periodically recorded during incubation of the probe with Sirt1. As shown in Figure 4a, the fluorescence intensity of the probe was
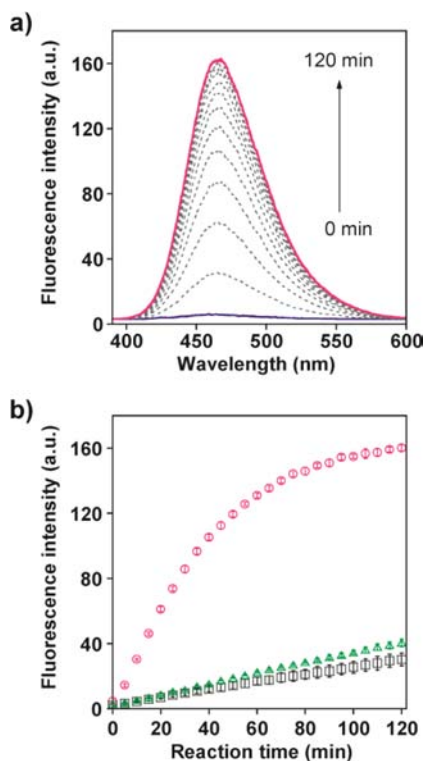
Figure 4. (a) Time course of fluorescence spectra of K4(Ac)-CCB in presence of Sirt1. The measurement was conducted using K4(Ac)-CCB (3 μM) with Sirt1 (100 nM) in 20 mM HEPES buffer (pH 8.0) containing 300 μM NAD$^+$ with an excitation wavelength of 371 nm. Spectra measured every 10 min are displayed. (b) Plot of the fluorescence intensity of K4(Ac)-CCB at 466 nm versus reaction time. The fluorescence intensities of the probe in the presence and absence of Sirt1 are represented by circles and squares, respectively, while that of the probe with Sirt1 and EX-527 is denoted by triangles.

initially weak and increased significantly during the incubation. A small increase in probe fluorescence was also observed in the absence of the enzyme. This is because the carbonate ester of the probe is slightly hydrolyzed under the condition of enzyme reaction. The hydrolysis rate was estimated to be 0.22%/min from time-course experiments of HPLC (Figure S2). Toward cellular application, the stability of the probe in cell lysate was examined. The hydrolysis rate in cell lysate was 0.27%/min, which was comparable to that of the probe in the absence of cell lysate (Figure S7). It is plausible that this side reaction can be suppressed by increasing pK_a of 7-hydroxy group of the coumarin. This pK_a increase would be achieved by introducing electron-donating substituents into the coumarin ring. Alternatively, instead of carbonate ester, more stable carbamate ester may work as the transesterification switch. The optimization of the probe structure is important for the application of this probe to the live-cell imaging of the enzymatic activity with high signal-to-noise ratio. These studies are now under investigation. Importantly, the hydrolysis did not affect the detection of the enzymatic activity under this experimental condition, because the fluorescence enhancement of the probe in the presence of the enzyme was significantly larger than that resulting from probe hydrolysis (Figure 4b). In a control reaction, Sirt1 was inactivated by heat shock at 95 °C for 5 min, and then reacted with the probe. The fluorescence intensity remained low, similar to that of the enzyme-free probe (Figure S8). These results demonstrate that the probe specifically detects the activity of Sirt1. We applied this probe to an inhibition assay using EX-527, a potent and specific Sirt1 inhibitor.[22] When K4(Ac)-CCB was incubated with Sirt1 in the presence of EX-527, the fluorescence enhancement of the probe was significantly restrained to the background level (Figure 4b). HPLC analysis of the reaction mixture demonstrated that no compound corresponding to the deacetylation product, DP, was generated (Figure S9). Thus, it was indicated that the inhibition of the enzymatic activity by EX-527 was detectable by the probe.

In summary, we have developed a novel fluorogenic probe, K4(Ac)-CCB, for HDAC activity. The probe detects enzymatic deacetylation upon merely mixing the probe and enzyme, without additional treatment. Successful detection of enzymatic activity is achieved by utilizing the intramolecular trans-esterification mechanism for the first time. An exciting feature is that this probe can be applied to inhibitor evaluation. This probe should be a useful tool for epigenetic research and the development of HDAC-targeted drugs.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Data on mass spectrometric analyses and HPLC chromatograms, synthetic schemes of compounds, and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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