Lysine deacetylase activity monitored by a fluorogenic assay using the CLARIOstar®

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- **The CLARIOstar® monochromator functions allow convenient adaptation to commercial assay kit constituents and further optimization of assay parameters**
- **Kinetic parameters K_M, k_{cat} and V_{max} and IC_{50} value for a sirtuin enzyme inhibitor calculated**

Introduction

Protein acetylation is a universal regulatory mechanism of protein activities. Recent developments in epigenetic drug discovery have caused increasing interest in methods to study lysine deacetylation, including *in vitro* assays of lysine deacetylases.[1] Here we optimized a commercial peptidase coupled lysine deacetylase assay to establish the enzymatic properties of *Escherichia coli* CobB. CobB is a sirtuin-type enzyme that participates in the regulation of bacterial transcription, translation, metabolism, and chemotaxis.[2-4]

Assay Principle

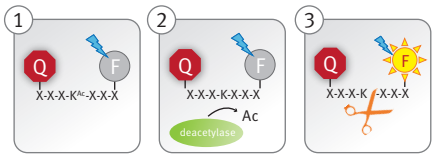

Fig. 1: Schematic illustration of the lysine deacetylase assay principle.

The CycLex SIRT1 assay kit makes use of an acetylated peptide conjugated to both a fluorophore and its quencher (panel 1). Enzymatic deacetylation of a lysine residue within the peptide (panel 2) generates a functional peptidase cleavage site. Fast subsequent cleavage of the deacetylated peptide by the peptidase separates the fluorophore from the quencher and allows fluorescent readout of the reaction (panel 3).

Materials & Methods

- 384-well black flat-bottom microplates (Greiner, #781900)
- SIRT1 fluorimetric assay kit (CycLex, CY-1151V2)
- Recombinant *Escherichia coli* CobB
- CLARIOstar microplate reader from BMG LABTECH

All standard chemicals and disposables were obtained through normal distribution channels. His6-tagged *E.coli* CobB was expressed in *E.coli* BL21(DE3) cells transfected with a pET28 expression vector, and purified using standard protocols involving immobilized metal ion chromatography (HisTrap, GE Healthcare) and size exclusion chromatography (Superdex-75, GE Healthcare). CobB lysine deacetylase activity was measured at ambient temperature (21°C) under the general conditions described in the manufacturer's protocol, with modifications as described below. Enzymatic assays were carried out using 50 nM CobB with the addition of NAD^+ and inhibitor as indicated.

Instrument settings

Detection Mode:	Fluorescence Intensity
Detection Method:	Plate Mode Kinetic
No. of cycles:	120
Cycle time (sec):	60
No. of flashes:	5
Scan mode:	orbital averaging
Scan diameter (mm):	1
Excitation:	355-10
Dichroic:	400 (autoset)
Emission:	445-10
Shaking:	8 sec before each cycle
Shaking mode:	orbital, 400 rpm

Results & Discussion

Optimization of excitation and emission parameters

Spectral scans (Fig. 2) of the fluorogenic peptide provided with the assay kit indicated that the excitation and emission settings could be improved. We used the CLARIOstar monochromators with excitation at 355/10 nm, a dichroic set at 400 nm, and emission at 445/10 nm. This resulted in the highest signal, although it did not change the Z' factor[5] which was 0.78 using either the above settings or the settings recommended by the provider.

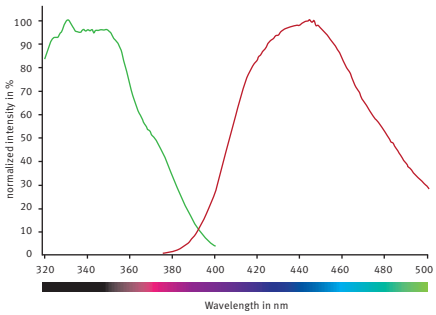

Fig. 2: Excitation and emission spectra of the fluorogenic peptide provided with the SIRT1 assay kit.

Reference curve for kinetic measurements

A dilution series of the deacetylated reference peptide provided with the assay kit was used to calibrate the measurements (not shown).

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Basic kinetic parameters of CobB lysine deacetylase activity

We conducted CobB enzymatic assays in presence of various concentrations of NAD^+ and fluorogenic substrate peptide at the concentration recommended in the SIRT1 assay kit manual. Under our experimental conditions, we determined a K_M^{NAD+} of 71 µM (Fig. 3). We are not aware of published kinetic parameters for NAD^+ cleavage by CobB.

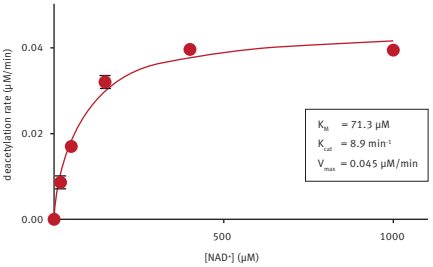

Fig. 3: Determination of the enzymatic properties of *E.coli* CobB.

Inhibition of CobB by a general sirtuin inhibitor

Ex527 is a general inhibitor of sirtuins.[6] We determined the potency of Ex527 for the inhibition of CobB activity. Assays were carried out using 70 µM NAD^+ as co-substrate and varying concentrations of Ex527 with a final DMSO concentration of 1%. The result of this analysis is shown in Fig. 4.

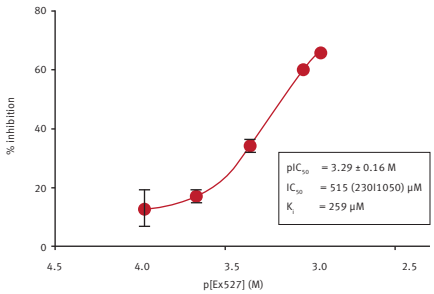

Fig. 4: Determination of the potency of Ex527 inhibition of *E.coli* CobB.

Ex527 is a weak CobB inhibitor (IC_{50} 515 µM; Ki = 259 µM determined using the Cheng-Prusoff equation). The low potency of the inhibitor, in combination with its limited solubility and the DMSO sensitivity of the enzyme, compromised the quality of the determination; the effect of Ex527 concentrations higher than 1 mM could not be measured at 1% DMSO.

Conclusion

We have used the CLARIOstar multimode microplate reader to determine the kinetic properties of the *E.coli* lysine deacetylase CobB and its inhibition by a general sirtuin inhibitor, using a commercial assay kit. Our results illustrate the general method, which should be useful for similar investigations into other lysine deacetylases and, in principle, lysine directed acetyltransferases.

Acknowledgments

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References

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