Kinetic study of the *Escherichia coli* sirtuin enzyme CobB

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Introduction

Proteins are chains of amino acids which can be modified by a post-translational modification process. There are many different post-translational modifications (PTMs) such as phosphorylation, methylation, ubiquitination or acetylation. Protein acetylation is a post-translational modification (PTM) consisting in the transference of an acetyl group from an acetyl donor molecule to the ε-amino group of a lysine residue. The importance of protein acetylation has greatly increased in last years, due to its impact on the function, structure, stability and/or location of thousands of proteins involved in diverse cellular processes.

Protein acetylation can be carried out by an enzymatic way (catalyzed by an acetyltransferase) or by a non-enzymatic or chemist way [1]. Protein acetylation can be reverted by deacetylases or histone deacetylases (HDACs). There are four classes of HDACs, class I, class II, class II and class IV. The class III proteins are known as sirtuins, which are broadly conserved from bacteria to humans. Sirtuins three-dimensional structure consists of a typical Rossmann-fold and a Zn^{2+} binding domain [2]. Sirtuins employ NAD^{+} to catalyze protein deacetylation generating the protein deacetylated, nicotinamide and 2'-0-acetyl-ADP ribose. Sirtuins have been traditionally associated to histone deacetylation in eukaryotes organism, playing an important role in transcription silencing [3]. However, in recent years, protein deacetylation of non-histones proteins by sirtuins has become more relevant, including glucose metabolism and transcription regulation. Moreover, it has been recently reported that human sirt6 is a tumor repressor [4]. Because of the importance and the difficulty to measure the activity of sirtuins, many studies have tried to determine a recognition protein domain unsuccessfully [5].

Bacterial sirtuins are the only known deacetylases in these microorganisms, moreover, the high homology with human sirtuins and microorganisms make them a perfect system to study sirtuins specificity and metabolismregulation. Thus, a recent study has revealed that in *Escherichia coli*, CobB, the only sirtuin to date in this microorganism, is able to deacetylate proteins acetylated by an acetyltransferase or by a non-enzymatic way [6]. The microenvironment of the target acetyllysine and the kinetic parameters of sirtuins have been unsuccessfully studied in bacteria with peptide libraries [6], since sirtuins recognize a large three dimensional protein structure and not an amino acid sequence.

In this work we have overexpressed and purified the *E. coli* sirtuin CobB and its main known substrate, the acetyl-CoA synthetase proteis Acs. Three enzymatic assays have been evaluated to study CobB kinetic successfully.

Methods

Homology alignment

Ten representative sirtuins were selected: Cob bacterial sirtuins from *Escherichia coli* and Salmonella enterica, Hst2 sirtuin from yeast and the seven human sirtuins (Sirt1, Sirt2,

Sirt3, Sirt4, Sirt5, Sirt6 and Sirt7). Uniprot free software was employed to obtain proteins sequences and to align them.

Protein overexpression and purification

CobB and Acs proteins were overexpressed employing the ASKA collection plasmids [7]. *E. coli* BL21 wt and the mutant Δ*cobB* were employing to purify CobB and Acs, respectively. Protein overexpression and purification was previously described [8]. Proteins were dialyzed against Tris-HCl pH 7.5 and stored at -80ºC until the use.

Kinetic assays

1. Discontinuous assays

A chemiluminescent and a fluorescent assay based on the publication of Liu et al., [9] were evaluated.

2. Continuous assay with Nicotinamidase

Acs deacetylation by CobB was measured coupling the sirtuin reaction to Nicotinamidase and Glutamate Dehydrogenase as is described by Smith et al., [10].

Results

The *Escherichia coli* protein CobB showed a great homology with human sirtuins

There are seven known sirtuins in humans: sirt 1-7 with a high homology between them. In this study, a homology alignment between the seven human sirtuins, the yeast Hst2 sirtuin and the *Salmonella* and *Escherichia coli* bacterial sirtuins CobB has been carried out.

All sirtuins aligned showed the conserved domains, the active site consisting in a histidine amino acid and the same binding site (Figure 1A). The mitochondrial Sirt5 showed the highest homology with the bacterial CobB. This fact matches with their evolutive taxonomy (Figure 1B).

Figure 1A. Homology alignment. Conserved domains are in yellow, active site in red, metal binding in pink and binding site in green.

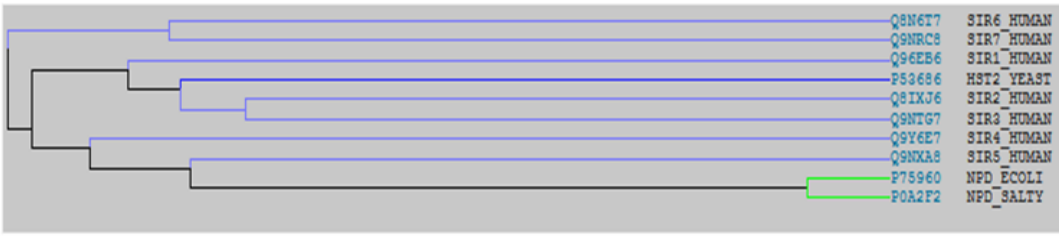

Figure 1B. Sirtuins taxonomy tree.

Discontinuous chemiluminescent and fluorescent assays

A chemiluminescent and a fluorescent discontinuous assay based on the publication of Liu et al., [9] were carried out. The calibration curve for both assays are shown in Figure 2. The chemiluminescent assay (Figure 2A) was a linear behavior from 0 to 60 µM of NAD^+, while the fluorescent assay showed a higher linear region (to 200 µM) because of the higher sensitivity of chemiluminescent signal. However, the measurements showed a low reproducibility. The fluorescent assay was more stable, although the discontinuity of the assay made impossible the measurement of a real deacetylation kinetic.

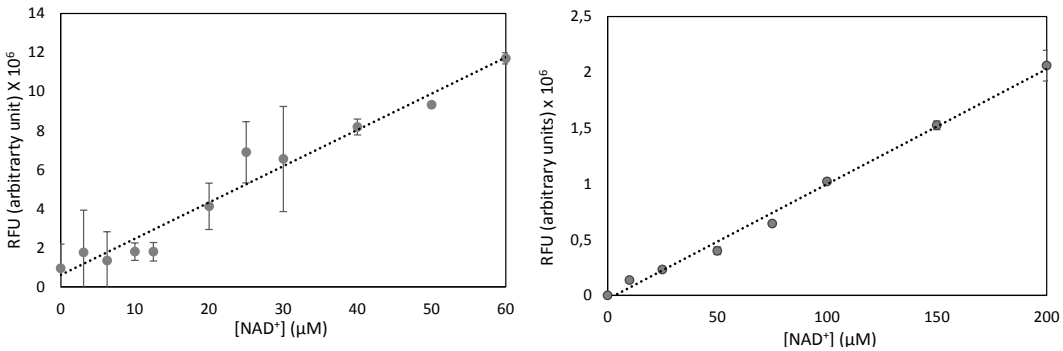

Figure 2. A. Chemiluminiscent calibration curve. **B**. Fluorescent calibration curve.

Continuous assay

The continuous assay based on the work of Smith et al., [10] is shown in Figure 3. This is the only one assay developed to date to measure sirtuins kinetic by a continuous way with a non-fluorescent substrate. Sirtuin deacetylation is coupled to Nicotinamidase and Glutamate Dehydrogenase enzymes, and the absorbance of NADPH at 340 nm is measured.

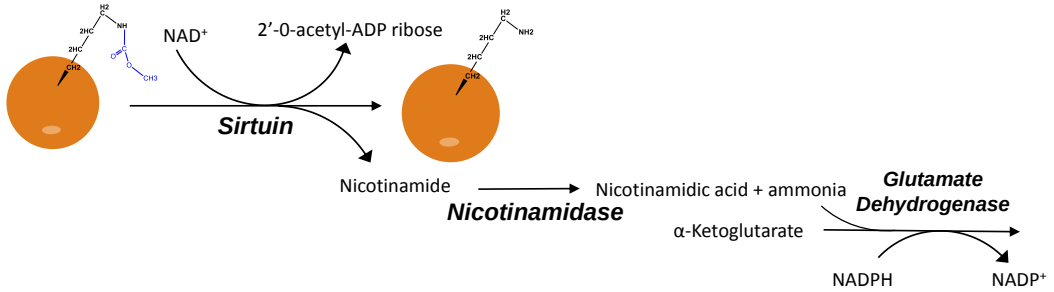

Figure 3. Squeme of sirtuin coupled reaction.

The linearity of the assay was evaluated with different concentrations of nicotinamide (Data not shown). Acs deacetylation by the sirtuin CobB was studied at different CobB concentrations at a fixed Acs (15 µM) for 2 hours (Figure 4).

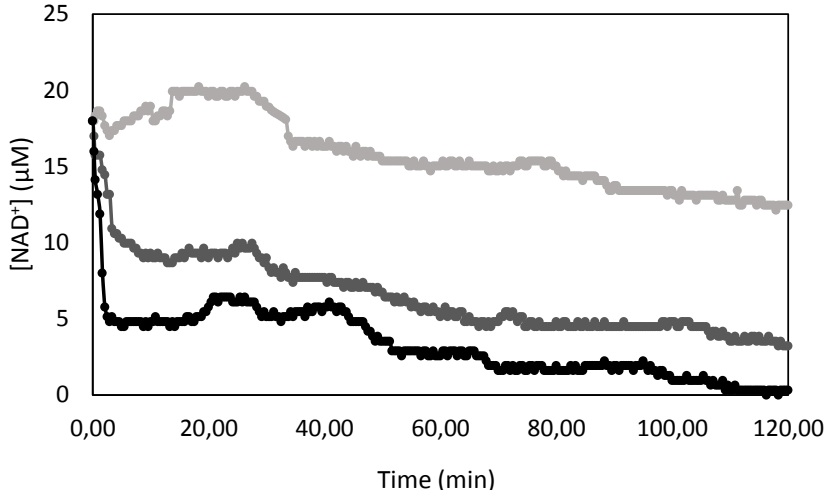

Figure 4. Acs deacetylation curves at different CobB concentrations for 120 minuts.

The deacetylation reactions were initiated with CobB at 1, 2 and 2,5 µM. The consumption of NAD^+ by CobB was calculated since the extintion coefficient of NADPH (ε=6,22 x 10^{-3} M^{-1} cm^{-1}). The total NAD^+ consumed was 18 µM aproximately. As the Acs concentration in the assay was 15 µM, the NAD^+ consumpition was stequiometric with the protein substrate, so probably only a lysine was deacetylated in Acs. The first rate was calculated for the CobB 2 and 2,5 µM. The first rate for the lowest enzyme concentration assaied could not be determined (Figure 5).

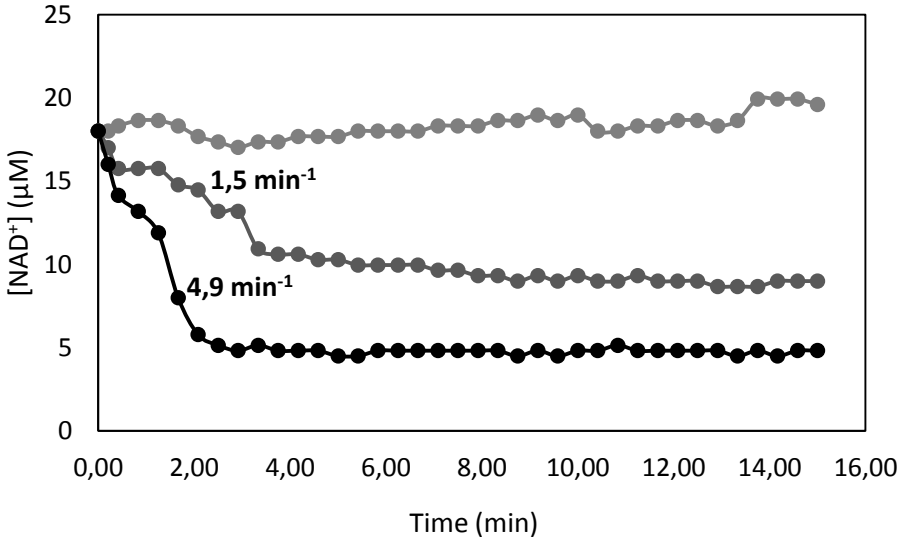

Figure 5. Acs deacetylation curves at different CobB concentrations for 15 minuts. First deacetylation rates are shown in the graph.

The fast rate showed for Acs deacetylation by CobB demonstrate a high specificity not studied to date for a natural substrate acetylated *in vivo*. This study represents a breakthrought in the study of sirtuins specificity.

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