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 Zn²⁺ 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 $\Delta cobB$ 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 -80°C 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).

296EB6 SIR1 HUMAN	285 VDFPDLPDPQAMFDIEYFRKDPRPFFKFAKE-IYPGQFQPSLCHKFIAL3DKEGKL
28IXJ6 SIR2 HUMAN	109 KYHLPYPEAIFEISYFKKHPEPFFALAKE-LYPGOFKPTICHYFMRLLKDKGLL
OSNIG7 SIRS HUMAN	170 OYDLPYPEAIFELPFFFHNPKPFFTLAKE-LYPGNYKPNVTHYFLRLLHDKGLL
9Y6E7 SIR4 HUMAN	87 RRPIQ-HGDFVRSAPIRORYWARNFVGWPQF35HQPNPAHWALSTWEKLGKL
9NXA8 SIR5 HUMAN	82 AQDLATPLAFAHNPSRVWEF HYRREVMGSEEPNAGHRAIAECETRLGKQGRR
SN6T7 SIRE HUMAN	76RGLAPKERVGLL
SANCE SIRT HUMAN	
	131 KGRSVSA
53686 HST2_YEAST	57RLKLPYPEAVFDVDFFQSDPLPFYTLAKE-LYPGNFRPSKFHYLLKLPQDKDVL
75960 NPD_ECOLI	35VEDVATPEGFDRDPELVQAFWNARRQLQQPEIQPNAAHLALAKLQDALGDR
OA2F2 NPD_SALTY	35VEDVATPEGFARNPGLVQTFTNARQQLQQPEIQPNAAHLALARLEEALGDR
SEEBE SIR1 HUMAN	340 LRNYTONIDTLEOVAGIORIIOCHGSFATASELIERYKVDCEAVRG
SIXJE SIR2 HUMAN	162 IRCYTONIDTIERIAGLEOEDIVEA GTFYTSHEVBASCRHEYPLSWMKE
9NTG7 SIRS HUMAN	223 LRLYTONIDGLERVSGIPASKLVEA GTFASATUT
9Y6E7 SIR4 HUMAN	138 YWLVTQNVDALHTKAGSRRLTEL GCMDRVLCL-DCGEQTPRGVLQERFQVLNPTWS
9NXA8 SIR5 HUMAN	135 VVVITQNIDELHRKAGTRNLLEI GSLFKIRGISGGVVAENYRSPICPALS
SNGT7 SIR6 HUMAN	108 RFLVSQNVDGLHVRSGFPRDKLAEL GNMFVEEGAKCKTQYVRDTVVGIMGL
SNRC8 SIR7 HUMAN	162 QHVVSQNCDGLHLRSGLPRTAISEL GNMYIEV@IS@VPNREYVRVFDV
53686 HST2 YEAST	110 KRVYTQNIDTLERQAGVKDDLIIEA GSFAHCHCIGCGKVYPPQVFKS
75960 NPD ECOLI	87 FLLVTQNIDNLHERAGNTNVIHM GELLKVRC3Q3GQVLDWIG
OA2F2 NPD SALTY	87 FLLVTONIDNLHERAGNRNIIHM GELLKVRES-OSGOILEWNG
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96EB6 SIR1 HUMAN	386DIFNQVVPREPREPADEPLAIMKPEIVFFGENLPEOFHRAMKY
SIXJ6 SIR2 HUMAN	212KI-FSEVIPKEEDEQSLVKPDIVFFGESLPARFFSCMQ3
9NTG7 SIR3 HUMAN	271DVMADRVPREPUETGVVKPDIVFFGEPLPQRFLLH-VV
	194 AEAHGLAPDGDVFLSEEQVRSFQVPTGVQGGGHLKPDVVFFGDTVNPDKVDFVHK
9NXA8 SIR5 HUMAN	186 GKGAPEPGTQDASIPVEKLPRELEAGEGGLLRPHVVWFGENLDPAILEEVDR
9NXA8 SIR5 HUMAN	
SNAR SIRS HUMAN SNGT7 SIRG HUMAN	186 GKGAPEPGTQDASIPVEKLPREEAGCGGLLRPHVVWFGENLDPAILEEVDR 160KATGRLG-TVAKARGLRAGRGELRDTILDWEDSIPDRDLAL
9NXA8 SIR5 HUMAN 8N6T7 SIR6 HUMAN 9NRC8 SIR7 HUMAN	186 GKGAFEPGTQDASIEVEKLPREE%GCGGLIREHVVHRGENLDPAILEEVDR 160GLIREKARGIRAGRACHGELRDTILDWEDSLPDRDLAL 211TERTALHRNQTGATGHKGSGLIRDTIVHFGERGTLGQFLNWEA
9NXA8 3IR5 HUMAN 8N6T7 3IR6 HUMAN 9NRC8 3IR7 HUMAN 53686 H3T2 YEAST	186 GKGAEEPGTQDASIFVERLPRÜEEAGÜGGLIRPHVVWFGENLDPAILEEVDR 160RATGRLTVARARGLRARRGELRDTILVMEDSLPDRDLAL 211TERTALX-FWGUCGRTGHKGSGLURDTIVHFGERGTLGGELMMEA 158
9NXA8 SIR5 HUMAN 8NGT7 SIR6 HUMAN 9NRC8 SIR7 HUMAN 53686 HST2 YEAST 75960 NPD ECOLI	186 GKGREPGTQDASIEVEKLPREEXAGGGLIRPHVWHGENLDPAILEVDR 160KATGRLGTVAKAGIRAGRGELRDTILDWEDSLPDRDLAL 211IERTALHRUGTGRTEHKGGTQLRDTIVHFGERGTLGQPLNWEA 158KLAEHPIKDFVKGVVGELVKPAIVFFGED-LPDSFBETWLM 130 -D
9NXA8 SIR5 HUMAN 8NGT7 SIR6 HUMAN 9NRC8 SIR7 HUMAN 53686 HST2 YEAST 75960 NPD ECOLI	186 GKGAEEPGTQDASIFVERLPRÜEEAGÜGGLIRPHVVWFGENLDPAILEEVDR 160RATGRLTVARARGLRARRGELRDTILVMEDSLPDRDLAL 211TERTALX-FWGUCGRTGHKGSGLURDTIVHFGERGTLGGELMMEA 158
SNXA8 SIRS_HUMAN SNGT7 SIRS_HUMAN SNRC8 SIR7_HUMAN S3666 H372_YEAST 75960 NPD ECOLI 2022F2 NPD_SALTY	186 GKGREPGTQDASIEVEKLPREEXAGGGLIRPHVVHFGENLDPAILEVDR 160KATGRLGTVAKAGIRAGRGELRDTILDWEDSLDDRDLAL 211IERTALHRUGTGRTGHKGGTQLRDTIVHFGENGTLGQPLNWEA 158KLAEHPIKDFVKGVVGGELVKPAIVFFGEN-PSGPDETWLN 130 -DVTPEDKEHCCQFPAPIRPHVVHFGENPLGMDEIYM 130 -DVMPEDKEHCCQFPAPIRPHVVHFGENPLGMDEIYM 130 -D
SNXA8 SIRS_HUMAN SNET7 SIRC_HUMAN SNERS SIR7_HUMAN S3686 HST2_YEAST 75860 NPD_ECOLI 0A2F2 NPD_SALTY S6EB6 SIR1_HUMAN	186 GKGAFEPGTQDASIEVEKLPREEAGCGLIRPHVVHFGENLDPAILEEVDR 160 RATGRI
SWXAS SIRS_HUMAN SNGT SIRG_HUMAN SAGES SIRT_HUMAN SAGES HST2_YEAST TSSGO NPD_ECOLI 0A2F2 NPD_SALTY SGEB6 SIR1_HUMAN SIXJ6 SIR2_HUMAN	186 GKGREPGTQDASIEVEKLPREEXAGGGLIRPHVVHFGENLDPAILEVDR 160KATGRLGTVAKAGIRAGRGELRDTILDWEDSLDDRDLAL 211IERTALHRUGTGRTGHKGGTQLRDTIVHFGENGTLGQPLNWEA 158KLAEHPIKDFVKGVVGGELVKPAIVFFGEN-PSGPDETWLN 130 -DVTPEDKEHCCQFPAPIRPHVVHFGENPLGMDEIYM 130 -DVMPEDKEHCCQFPAPIRPHVVHFGENPLGMDEIYM 130 -D
SNXAS SIRS-HUMAN SNET SIRC-HUMAN SNET SIRC-HUMAN S3656 HST2-YEAST 75560 NPD_ECOLI 0A2F2 NPD_SALTY SEEB6 SIR1_HUMAN SIXJ6 SIR2_HUMAN SIXJ6 SIR2_HUMAN	186 GKGAFEPGTQDASIEVEKLPREEAGCGLIRPHVVHFGENLDPAILEEVDR 160 RATGRI
SNIKAS SIRS-HUMAN SNET7 SIRG-HUMAN SNERS SIR7-HUMAN S3686 HST2-YEAST 75960 NPD_ECOLI 0A2F2 NPD_SALTY SEEB6 SIR1_HUMAN SIXJ6 SIR2_HUMAN SNIG7 SIR3_HUMAN	186 GKGAPEPGTQDASIEVEKLPREEAGEGLIRPHYUNFGENLDPAILLEVDR 160
SWARS SIRS-HUMAN SNETS SIRC HUMAN SACES SIRT HUMAN SACES HSIZ_VEAST TSSCO NPD ECOLI DA2F2 NPD_SALTY SEEBS SIR1_HUMAN SIXJS SIR2_HUMAN SIXJS SIR2_HUMAN SIXSF SIR3_HUMAN	186 GKGAFEFGTQDASIEVEKLPREEAGEGLIRPHVURGENLDPAILLEVDR 160
SNXAS SIRS_HUMAN SNET SIRS_HUMAN SNERS SIR7_HUMAN SG686 HST2_YEAST 75960 NPD_ECOLI 0A2F2 NPD_SALTY 96EB6 SIR1_HUMAN SIXJ6 SIR2_HUMAN 99TG7 SIR4_HUMAN 99TC87_SIR4_HUMAN 99TC84	186 GKGAFEPGTQDASIEVEKLPREERGGGLIRPHVVHFGENLDPAILEEVDR 160 KATGRI
SWARA SIRS-HUMAN SNETS SIRG-HUMAN SNECS SIRT-HUMAN SAGSG HST2_VEAST TSSGO NPD ECOLI 0A2F2 NPD_SALTY SEEBS SIR1_HUMAN SIXIS SIR2-HUMAN SNEGT SIR3-HUMAN SNEAT SIR5_HUMAN SNETT SIR5_HUMAN	186 GKGAFEFGTQOASIEVEKLPREEAGEGLIRPHVUNFGENLDPAILLEVUR 160
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SNKAS SIRS-HUMAN SNKCT SIRG-HUMAN SNRCS SIR7-HUMAN SS686 HST2_YEAST 75860 NPD ECOLI 002F2 NPD_SALTY 96EE86 SIR1_HUMAN SIKJ6 SIR2_HUMAN 99KC7 SIR4-HUMAN 99KC7 SIR4-HUMAN 99KC7 SIR4-HUMAN 99KC7 SIR4-HUMAN 99KC7 SIR4-HUMAN 99KC7 SIR4-HUMAN 99KC7 SIR4-HUMAN 99KC7 SIR4-HUMAN 99KC7 SIR4-HUMAN 98KC7 SIR4-HUMAN 98KC7 SIR4-HUMAN	<pre>186 GKGAFEFGTQDASIEVEKLPREEAGGGLIRPHVURGENLDPAILEEVDR 160RATGRL</pre>
29YEE7 SIR4 HUMAN 29NECS SIR5 HUMAN 29NECS SIR7 HUMAN 29SICS SIR7 HUMAN 29SICS SIR7 HUMAN 29SICS SIR7 HUMAN 29SICS SIR1 HUMAN 20NETS SIR2 HUMAN 20NETS SIR2 HUMAN 20NETS SIR4 HUMAN 29NETS SIR5 HUMAN 29NETS SIR5 HUMAN 29NETS SIR5 HUMAN 29NETS SIR7 HUMAN 29NETS SIR7 HUMAN 29NETS SIR7 HUMAN 29NETS SIR7 HUMAN 29NETS SIR7 HUMAN 29NETS SIR7 HUMAN	186 GKGAFEPGTQDASIFVEKLPREERGGGLIRPHVVHFGENLDPAILLEVDR 160 KATGRI

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 μ M of NAD⁺, while the fluorescent assay showed a higher linear region (to 200 μ M) 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.





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 μ M) 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 μ M. 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 μ M aproximately. As the Acs concentration in the assay was 15 μ M, the NAD⁺ consumption 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 μ M. 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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