Structural analysis with DSC and Fluorescence in different mutants in lysine 101 of Catabolite Regulator Protein from *Escherichia coli*.

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Introduction

Nowadays, *Escherichia coli* (*E. coli*) microorganism is a Gram- bacteria widely used in Biotechnology Area. The ease of its transformability and genetic manipulate, the low cost in cultivation and its well-known genome, have established to this bacterium in an organism model and a robust cell factory in this field [1], [2].

The study of Post-translational and Post-transcriptional (PTMs) modifications have gained relevance in last years due to seems to controls the E. coli metabolism [3]. Because PTMs confer properties to the modified proteins, the knowledge of protein modification regulatory mechanisms is crucial for the optimization the biotechnology process where E. coli is used [4], [5]. Recently, the lysine acetylation has been discovered like a very important PTM in all domains in life, even in human where it has been related with the cancer. Actually, the role in protein acetylation and its regulation still remains unknown, although we know that exist two different mechanisms for it. This PTM can be carried out through an enzymatic acetylation, where an acetyltransferase catalyzes the process, and a non-enzymatic acetylation, where the reaction occurs chemically [3].

Until this moment, the study of acetylation like a PTM has been poorly developed due to detection and characterization techniques have begun to expand since few years ago [6]. At present, the increasing power of high throughput proteomic techniques is changing this field, providing a lot of information about the peptides acetylated in several conditions [7]. The recent studies revealed that a vast number of metabolic enzymes are acetylated, which suggests that it is a way of metabolism regulation that is highly conserved [5], [8].

In las years, have been published a lot of proteomics studies in different condition, where we can observe the wide variety in function of acetylated protein [9], [10]. Particularly, the global transcription factor Catabolite Regulator Protein (CRP) from *E. coli* contains acetylated lysine 100 (K100) [11], [12]. The main function that CRP carries out is the control in the carbohydrates metabolism and regulates the Catabolite Repression in this bacterium [13].

Lysine 100 is situated adjacent of Activating Region 2 of CRP. This region is completely necessary for the regulation expression in type II promoters through the possible alteration of the direct contact between RNA polymerase with AR2 [14]. For this reason, knowing the role of the acetylation in K100 in this protein could have an important

impact in metabolism and thus, we could understand how the acetylation affects to the genic expression.

The main objective of this study is purifying the native and CRP mutants and use them to check if the mutations in lysine 100 affect to conformational structure of this protein.

Experimental procedures

Construction of CRP over-expression vector: using Molecular Biology techniques, wild type gene *crp* from *E. coli* BW25113 was cloned into the pRSETC (Invitrogen) vector. The plasmid construct was called pRSETC-CRP N for overexpression CRP N protein.

Site directed mutagenesis: over pRSETC-CRP N vector was performed site-directed mutagenesis in lysine 100 of CRP. Two different mutants were constructed: pRSETC-CRP R and pRSETC-Q. In the first, lysine 100 was substituted by an arginine (R) for miming a permanent deacetylated state, while in the second vector lysine 100 was changed by a glutamine (Q) simulating a permanent acetylated state.

Overproduction and purification of proteins: vectors were transformed in *E. coli* BL21(DE3) plysS strain by heat shock. Cultures were grown at 37°C with orbital shaking (200 rpm) and the expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) during 1 hour, changing the culture temperature at room temperature. The culture medium used was Luria Broth (LB). Protein purification of three different proteins (CRP N, CRP R and CRP Q) was performed using Ni^{2+} chromatography IMAC as described de Diego *et al.* (2015) [15].

Protein dialysis: the purified proteins were dialyzed against a buffer contains: 50mM phosphate buffer, pH 8.5, 200mM NaCl, 10% glycerol, 100µM cAMP and 200µM $MgCl_2$. This was the work buffer for the next experiments.

Fluorescence analysis of CRP N, R and Q denaturalization: Fluorescence intensity of CRP native and its mutans were monitored on a Hitachi Spectrofluorometer (model F4500) at different incubation temperatures using a thermostated cell. Protein samples were previously incubated in the cell for 5 min, then excited at 295 nm and emission registered at 330nm using 5nm bandwidth in both the excitation and emission path. The final concentration in all the media was 0.03 mg/mL of enzyme. The spectrofluorometer automatically provided corrected spectra by comparison with a 1 nM standard solution of rhodamine B in glycerol to avoid changes in lamp output and instrument geometry.

Differential Scanning Calorimetry (DSC): A differential scanning calorimeter DSC 2920 (TA Instruments) was used for calorimetric analysis of CRP native and mutants. Samples were analysed using a programmed heating scan rate of 0.5 °C min^{-1} from 15°C to 105°C. All experiments were carried out in 50mM phosphate buffer, pH 8.5. Phosphate buffer without protein was used as reference. No peaks were visible on the rescan in any case, which means that all transitions were irreversible. For data analysis and conversion Universal Analysis Software (TA Instrument v 0.4) was used and collected DSC data were normalized for protein concentration. Enthalpy (ΔH) was expressed in kcal mol^{-1} (1 cal = 4,184 J).

Results and discussion

Fluorescence analysis: The three purified proteins were used for Fluorescence Analysis, as we have described previously. The results obtained are shown in Figure 1.

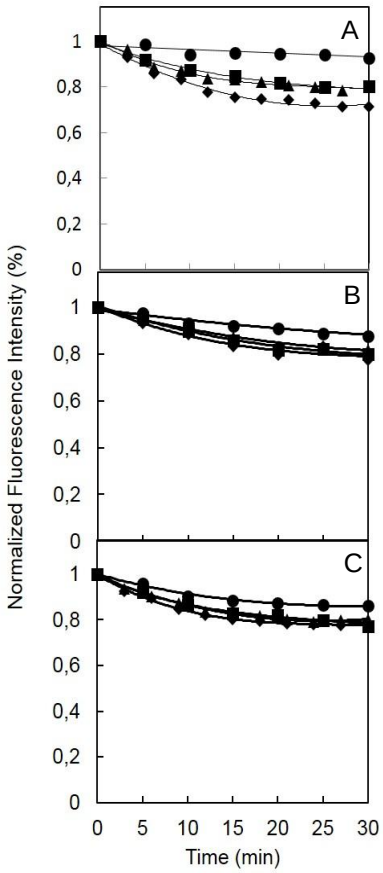

Figure 1. Normalized profiles of fluorescence intensity (%) of CRP native (A), CRP K100Q mutant (B) and CRP K100R mutant (C) with time at different incubation temperatures: (●) 40ºC, (■) 50ºC, (▲) 60ºC and (♦)70ºC.

As we can observe in Figure 1, three mutants have a similar knowledge in denaturalization process. The most changes between them occur at 40ºC (●), although are not significant.

In table 1, we present the denaturalization constant (kd) and half-life ($t_{1/2}$) calculated from data of fluorescence experiment. Both parameters show increased differences between CRP N, R and Q with lower temperatures (40ºC and 50ºC). However, at higher temperatures (60ºC and 70ªC) the changes are minimal.

Nevertheless, the analysis of parameters obtained (kd and $t_{1/2}$) allow us to conclude that the little variation observed between them is minimal. For this reason, we can say that the mutation in lysine 100 doesn't alter the conformational structure of CRP protein.

Differential Scanning Calorimetry (DSC): in Figure 2, we can observe the thermal denaturalization of CRP N, R and Q. The three profiles are very similar between them. For more details, we have to examine Table 2, where we can study the characteristic enthalpy variation (ΔH), melting point (T_m) and half-life ($T_{1/2}$).

Comparing T_m parameter in three mutants, we can conclude that there are few changes, which can be ascribing to experimental error.

Temperature (ºC)	kd (min^{-1})			$t_{1/2}$ (min)		
	N	R	Q	N	R	Q
40	0.0016	0.0046	0.0026	7.13	6.07	6.64
50	0.0048	0.0055	0.0045	6.03	5.90	6.10
60	0.0066	0.0063	0.0062	5.71	5.76	5.78
70	0.0089	0.0071	0.0082	5.41	5.64	5.50

Table 1. Thermodynamic parameters of CRP N and CRP (R and Q) mutants obtained from Fluorescence Analysis.

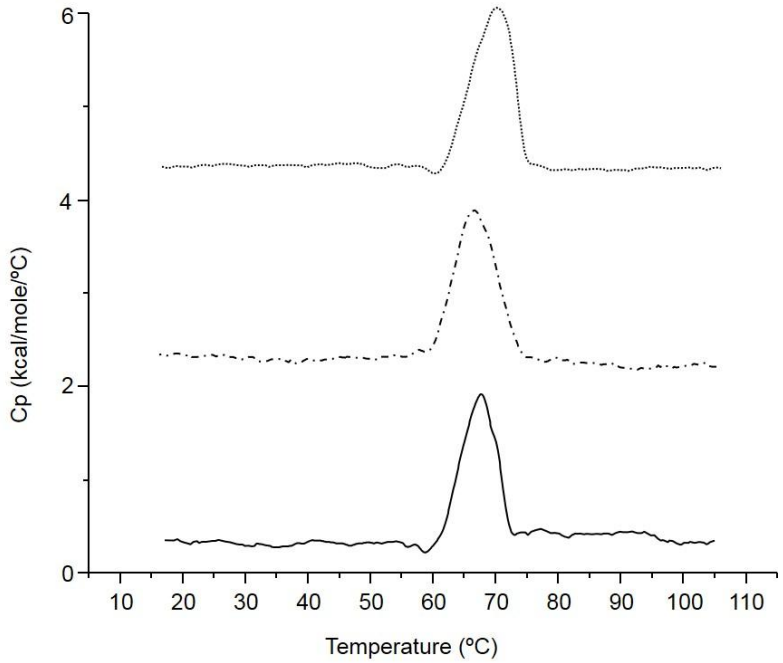

Figure 2. Differential scanning calorimetry. DSC thermograms of:

CRP N (·····)
CRP Q (-·-·-·-)
CRP R (———)

Indeed, this conclusion can be reflected if we observe the ΔH values in CRP N, R and Q, where we have not variation, practically. The same fact occurs when we analyze the half-life ($T_{1/2}$).

	ΔH (kcal/mol)	**T_m (ºC)**	**$T_{1/2}$ (ºC)**
CRP native	19.6	70.5	9.0
CRP Q mutant	18.2	66.0	8.7
CRP R mutant	17.4	67.0	8.3

Table 2. Thermodynamic parameters of CRP and mutants obtained from DSC thermograms

The DSC results allow us to conclude that the mutation in lysine 100 of CRP doesn´t affects significantly to the conformational structure.

Conclussions

The aim of this study was analyzing if acetylation in lysine 100 of CRP would affect in conformational structure of this protein. The results obtained in Fluorescence experiments and DSC allow us to conclude that the three different proteins (CRP N, CRP R and CRP Q) don´t present significant structural changes. One more time, we have to say that the few differences may be assumed as experimental error.

For this reason, we conclude that the acetylation in lysine 100 of CRP doesn´t alters the structural conformation. Thus, the expression of genes controlled by CRP only will be affected by the differential interaction between CRP acetylated/deacetylated and

RNA Polymerase, and not by the possible sructurals changes produced with chemical modification by acetylation in K100.

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