



## Thermodynamic study of the native and phosphorylated regulatory domain of the CFTR

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### ABSTRACT

The regulatory domain (RD) of the cystic fibrosis transmembrane conductance regulator (CFTR), the defective protein in cystic fibrosis, is the region of the channel that regulates the CFTR activity with multiple phosphorylation sites. This domain is an intrinsically disordered protein, characterized by lack of stable or unique tertiary structure. The disordered character of a protein is directly correlated with its function. The flexibility of RD may be important for its regulatory role: the continuous conformational change may be necessary for the progressive phosphorylation, and thus activation, of the channel. However, the lack of a defined and stable structure results in a considerable limitation when trying to build a unique molecular model for the RD. Moreover, several evidences indicate significant structural differences between the native, non-phosphorylated state, and the multiple phosphorylated state of the protein. The aim of our work is to provide data to describe the conformations and the thermodynamic properties in these two functional states of RD. We have done the circular dichroism (CD) spectra in samples with a different degree of phosphorylation, from the non-phosphorylated state to a *bona fide* completely phosphorylated state. Analysis of CD spectra showed that the random coil and  $\beta$ -sheets secondary structure decreased with the polypeptide phosphorylation, at expenses of an increase of  $\alpha$ -helix. This observation lead to interpret phosphorylation as a mechanism favoring a more structured state. We also studied the thermal denaturation curves of the protein in the two conditions, monitoring the changes of the mean residue ellipticity measured at 222 nm as a function of temperature, between 20 and 95 °C. The thermodynamic analysis of the denaturation curves shows that phosphorylation of the protein induces a state of lower stability of R domain, characterized by a lower transition temperature, and by a smaller Gibbs free energy difference between the native and the unfolded states.

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### 1. Introduction

Cystic fibrosis (CF) is a fatal genetic disorder that results from mutations in a gene encoding for the cAMP-activated chloride channel known as the cystic fibrosis transmembrane conductance regulator (CFTR) [1,2]. This protein is the only member of the ATP-binding cassette (ABC) transporter super-family that forms an ion channel. It consists of two membrane-spanning domains (MSD) followed each by a nucleotide-binding domain (NBD) linked by a unique regulatory (R) domain. The MSDs assemble to form a transmembrane pore with deep intracellular and shallow extracellular vestibules that funnel anions towards a selectivity filter [1,2]. Anion flow through the CFTR pore is tightly controlled by the interaction of ATP with two binding sites, located at the interface of the two NBDs [1]. The R domain contains multiple consensus phosphorylation sites on the surface of an unstructured domain. The phosphorylation stimulates CFTR function by enhancing ATP-dependent channel gating at the NBDs.

The complexity of the study of the RD is due to the intrinsically disorder of this domain [4–6]. Intrinsically disordered proteins are characterized by lack of stable tertiary structure when the protein exists as an isolated polypeptide chain under physiological conditions *in vitro*. The existence of a set of possible tertiary structures makes very complicate to create a valid structural model of these peptides.

We have studied the conformational properties of RD in the non-phosphorylated and in the phosphorylated states, by circular dichroism (CD) spectroscopy. Static CD spectra allowed us to obtain information about the average secondary structure of the protein in each state. We characterized also the thermodynamic properties of these two states analyzing the thermal denaturation curves of the RD.

### 2. Materials and methods

#### 2.1. Regulatory domain purification

*Escherichia coli* BL21(DE3) CodonPlus RIL cells (Stratagene) transformed with a plasmid based on the pPROEX HTb vector

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(Invitrogen), encoding a 185-residue fragment, from position 654 to 838, of the human CFTR (UniProtKB P13569) RD, with an N-terminal His6 tag (the construct was generously provided by J.D. Forman-Kay, SickKids Hospital, Toronto, Canada). The RD sequence carried the polymorphism Leu833 to improve solubility [3]. Transformed cells were grown to  $A_{600} \approx 0.6$ – $0.8$  and induced with 1 mM IPTG for 4–6 h at 37 °C. The RD was solubilized from the inclusion bodies fraction using a 6 M guanidinium-HCl, and purified by Ni<sup>2+</sup> affinity chromatography (HisTrap, Ge-Healthcare, Upsalla Sweden) and denaturing size exclusion chromatography on a Superdex 75 / 16–60 column (Ge-Healthcare), followed by ionic exchange chromatography. The purified protein was then dialysed in two steps: first, against 3 M guanidinium-HCl, 20 mM TRIS pH 8, 200 mM NaCl, 2 mM DTT; then, against 30 mM phosphate buffer pH 8, 200 mM NaCl, 2 mM DTT. The product was then concentrated to 2.5 mg/ml by ultra-filtration (Amicon Ultra-10K, Millipore Corporation, Bedford, USA), fast-frozen in liquid nitrogen, and stored at –80 °C for further use. All purification and refolding procedures were done at 6–8 °C. For all successive experiments, protein were freshly thawed, cleared with a 0.45  $\mu$ m filter (Ultrafree-MC, Millipore) and the effective concentration was estimated by the absorbance at 280 nm.

Except when mentioned, all chemical were purchased from Sigma–Aldrich (St. Louis, MO, USA).

## 2.2. Sample preparation: phosphorylation

The catalytic fraction of protein kinase A (PKA, BIAffin GmbH & Co. KG), at several concentrations (100, 200 and 600 PKA units/nM of RD) was added in aliquots of 10–20  $\mu$ l of RD (2–3 mg/ml), supplemented with 50  $\mu$ M ATP and 5 mM MgCl<sub>2</sub>. After an incubation of 30–40 min at 37 °C, the mixture was diluted to a concentration of 0.1–1 mg/ml with 30 mM phosphate buffer (pH 8.0) and 1:3000 of  $\beta$ -mercaptoethanol. The non-phosphorylated RD sample was prepared in the same way, without the addition of PKA.

## 2.3. Circular dichroism experiments

The circular dichroism (CD) spectra were acquired on a Jasco J-815 spectropolarimeter, equipped with a Peltier device for controlling the sample temperature. Scans were collected from 195 to 260 nm at 50 nm/min, with a data pitch of 0.1 nm, in a 0.1 cm rectangular cell. The spectra of the non-phosphorylated and of phosphorylated protein were collected (10 replicate spectra for sample) at 10 °C, and normalized to the concentration, evaluated as from the absorbance at 280 nm, measured immediately before the CD experiment. The analysis of the secondary structure was done by the on-line program Dichroweb [7] using the algorithm SELCON3 [8].

The denaturation curves were obtained from monitoring the mean residue ellipticity at 222 nm,  $\theta_{\text{exp},222}$ , as a function of the temperature, from 20 to 95 °C. The analysis of these curves was done assuming a two state model, defined as the initial native state and the final, denaturated state [9,10]. To identify the initial and final states, the experimental  $\theta_{\text{exp},222}(T)$  curves were first fitted with a sigmoid function:

$$\theta_{\text{exp},222}(T) = \frac{m_1 + m_2}{1 + \exp\left(\frac{T - T_m}{m_3}\right)} + m_1 \quad (1)$$

where  $T$  is the absolute temperature,  $m_1$ ,  $m_2$ ,  $m_3$  and  $T_m$  (melting temperature) are the parameters obtained with the fit. Using these parameters, we normalized the curves to estimate the fraction of denaturated protein,  $X_D$ , as:

$$X_D = \frac{\theta_{\text{exp},222} - m_1}{m_2} \quad (2)$$

Data was fitted according to the van't Hoff plot, as:

$$\ln\left(\frac{1 - X_D}{X_D}\right) = \frac{\Delta H}{RT} - \frac{\Delta S}{R} \quad (3)$$

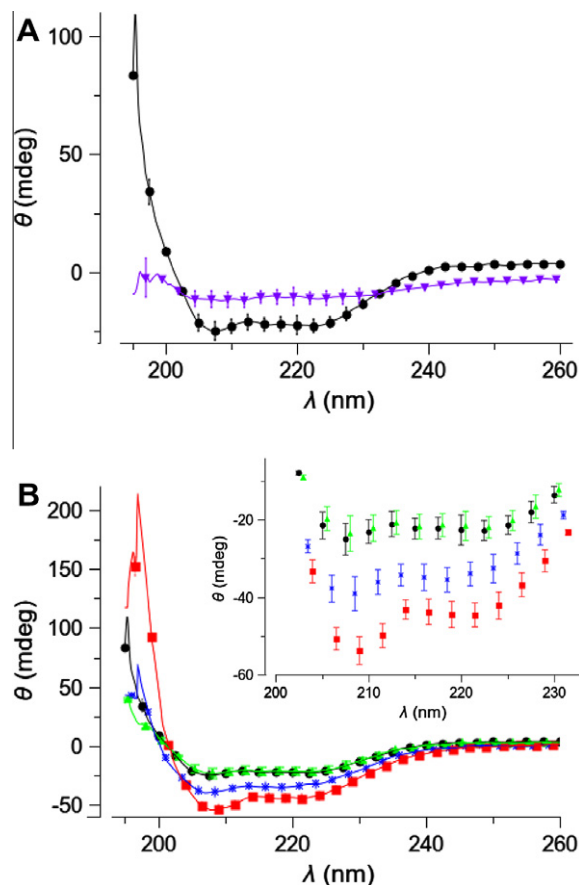
where  $\Delta H$  and  $\Delta S$  are the enthalpy and entropy differences between the initial and denaturated states, and  $R$  is the universal gas constant.

## 3. Results

As mentioned above, the PKA-dependent phosphorylation of the regulatory domain results on the activation of the CFTR channel [1,2]. We aimed to study the structural and conformational properties of the non-phosphorylated and the phosphorylated states of this domain. We used a recombinant protein preparation, similar to that used for the study of the interaction between the RD and other domains by NMR [3,4]. The quality of the RD preparation was verified by the presence of a defined secondary structure, with a significant content of  $\alpha$ -helix and  $\beta$ -sheet motifs, as revealed by the CD spectrum shown in Fig. 1A. Differently, the CD spectrum obtained from a denaturated protein, at the same concentration (Fig. 1A; inverted triangles), is consistent with a random coil polypeptide.

### 3.1. Phosphorylation

Treatment of the RD with PKA significantly modified the CD spectra, indicating that phosphorylation induces a conformational



**Fig. 1.** (A) CD average spectra of the native R domain (circles) and the denaturated protein (down triangles). The errors bars are the standard deviation from the average of 10 different spectra. (B) CD spectra of the RD at four concentration of PKA: 0 units/nM of RD (circles), 100 units/nM of RD (up triangle markers), 200 units/nM of RD (cross markers), 600 units/nM of RD (square markers). In the panel is shown a zoom of the wavelength of the minima typical of  $\alpha$ -helix and  $\beta$ -sheets.

change (Fig. 1B). To assess the cause-effect of the PKA treatment, we performed a series of experiments where the RD was treated with different concentrations of the enzyme for a fixed time (20 min). We observed that the conformational change was more marked as the concentration of PKA is increased. These changes are evident measuring the mean residue ellipticity at 208 and 222 nm. Treatment with low PKA concentration (100 units/nM of RD) does not produce a significant effect on the CD spectra, while an increase on the mean residue ellipticity at 208 and 222 nm is observed when the RD is treated with 200 or 600 units/nM of PKA (see Fig. 2B). Treatment with higher concentrations of PKA (or longer time) does not change the CD spectra further. Treatment of RD with phosphatase results on a CD spectrum that is similar to that obtained in non-phosphorylated conditions (data not shown). We can conclude that RD is not phosphorylated, prior to PKA addition, and the treatment with the enzyme induces a conformational change that is consistent with the degree of phosphorylation of the polypeptide.

The secondary structure content for each spectra was deconvolved with the algorithm SELCON3 [8]. The increase of the phosphorylation level of RD (as PKA concentration is increased) produces a reduction of the random coil content of the protein, favoring a more structured conformation of RD. The random coil decreases from 30% for non-phosphorylated RD, to about 18% for maximum phosphorylated RD. It occurs with a significant increase of  $\alpha$ -helix content from 46% to 71%, as the protein is phosphorylated. Interestingly, phosphorylation of RD reduces the  $\beta$ -sheet secondary structure content from 12% to less than 1%. The *t*-turn content remains about constant (11.6% and 9.8% for non-phosphorylated

and phosphorylated RD, respectively). Results of this analysis are presented in Table 1.

### 3.2. Thermal denaturation curves

To study the stability of protein structure we analyzed the Gibbs free energy difference between the native state and denatured state. Thermal denaturation curves for the non-phosphorylated and the phosphorylated RD are presented in Fig. 2A. The sigmoid shape of these denaturation curves indicates that, in both cases, there is a single transition from the native to the denatured state. From a first glance, it is evident that the phosphorylated RD denatures at lower temperature. Indeed, the melting point,  $T_m$ , is about 20 °K lower for the phosphorylated protein (Table 2). This can be interpreted as a less stable configuration of the RD resulting from the conformational change induced by phosphorylation. We calculated the thermodynamic energy terms of the thermal denaturation process from the linear fit to the van't Hoff plot (Fig. 2B) These plots showed that both  $\Delta S$  and  $\Delta H$  of the native  $\rightarrow$  denatured are higher for the phosphorylated protein than for the non-phosphorylated RD (Table 2).

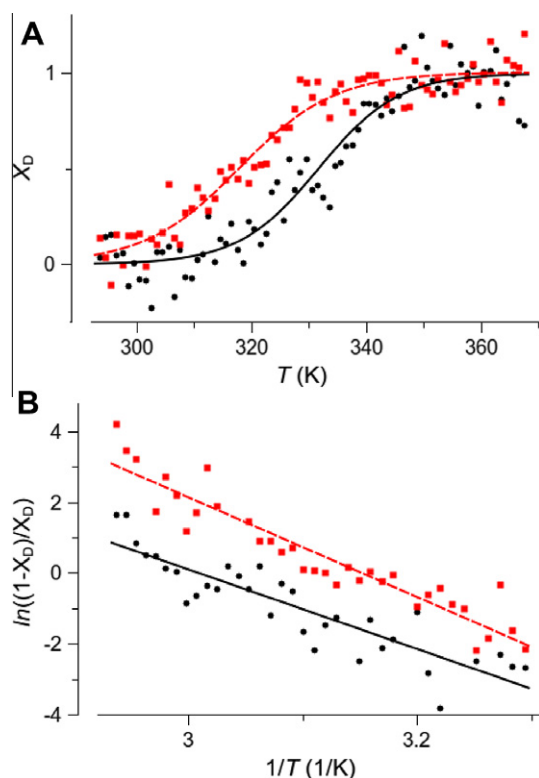
## 4. Discussion

We have demonstrated that RD, being an intrinsically disorder protein, is not an unfolded protein. In fact, the CD spectra (Fig. 1A) of the RD is consistent to the presence of secondary structure. This secondary structure content, defined by the typical minima of the  $\alpha$ -helix and  $\beta$ -sheets, is different depending on the phosphorylation state of RD (Fig. 1B). Differently, the CD spectra for RD in denaturing conditions is consistent to the random coil conformation. The presence of secondary structure organization for RD has coherent with previous CD [6] measures and NMR [3,4] experiments.

It is remarkable the effect of the phosphorylation on the RD, that causes a significant conformation change in the protein. RD becomes more structured upon phosphorylation, as the  $\alpha$ -helix content increase as a function of the concentration of PKA, while there is a reduction of random coil and  $\beta$ -sheet contents. This is opposite to the interpretation of NMR data of phosphorylated RD [3], where a decrease of helicity has been described. Beside the difficulties to interpret the secondary structure content from spectroscopy data with empirical methods [6–8,11], it is however clear that the phosphorylation induces a conformational change on the RD structure.

On the other hand, the phosphorylation of RD induces to a less stable protein, in terms of the native-denatured equilibria. That is evident from the lower melting point of the phosphorylated RD. Indeed, the difference in  $\Delta H$  between the non-phosphorylated and the phosphorylated RD is about 6 Kcal/mol. To have an evaluation of the protein stability, one has to calculate the Gibbs free energy difference from this native  $\rightarrow$  denatured transition. As the Gibbs free energy difference depends on the temperature, we have evaluated it at two different temperatures, 10 and 37 °C.

The non-phosphorylated protein has a more stable conformation than the phosphorylated RD protein. Values in Table 2 indicate



**Fig. 2.** (A) Variation of the mean residue ellipticity at 222 nm during a gradual increase of the temperature from 20 to 95 °C. (B) Analysis of the thermodynamic parameters with the van't Hoff plot (3). The round markers indicate the non-phosphorylated protein while the square markers the R domain phosphorylated with PKA 600 units/nM of protein. The straight lines represent the two fits of non-phosphorylated protein and the dot lines the fits for the phosphorylated one, in (A) we have used the sigmoid curve for fitting the experimental data (1), in (B) a linear fit (3).

**Table 1**

Content of secondary structure conformations present in the CFTR regulatory domain treated with different concentrations of PKA. Data was obtained by deconvolution of the CD spectra with the algorithm SELCON3.

Sample	% $\alpha$ -Helix	% $\beta$ -Sheets	% Turns	% Random coil
RD	45.9	12.1	11.6	30.3
RD + PKA 100 units/nM	48	13.9	12.3	25.8
RD + PKA 200 units/nM	67.6	3.7	9.6	19
RD + PKA 600 units/nM	71.1	0.8	9.8	18.4

**Table 2**  
Thermodynamic analysis of the denaturation curves of the non-phosphorylated and phosphorylated RD.  $T_m$  is the temperature of melting.  $\Delta H$  and  $\Delta S$  are the difference of enthalpy and entropy between the initial and final state, respectively.  $\Delta G_{10}^{\circ}\text{C}$  and  $\Delta G_{37}^{\circ}\text{C}$  is the free Gibbs energy variation calculated at 10 and 37 °C, respectively.

Sample	$T_m$ (K)	$\Delta H$ (Kcal/mol)	$\Delta S$ (Kcal/mol/K)	$\Delta G_{10}^{\circ}\text{C}$ (Kcal/mol)	$\Delta G_{37}^{\circ}\text{C}$ (Kcal/mol)
Non phosphorylated	330.6 ± 0.1	22.3 ± 0.2	0.067 ± 0.006	3.34	1.53
Phosphorylated	308.8 ± 1.2	28.1 ± 0.2	0.088 ± 0.006	3.20	0.82

that the stability of the protein decreases as the temperature increases, and the stability difference is bigger for high than low temperature. It is hypothesized that activation of CFTR is the result of the interaction of the phosphorylated RD and the NBDs [3–5]. We have concluded that the phosphorylation induces a conformational change to RD, that may favor such interactions. Further favoring of the RD-NBD interaction may derive from an increase of RD flexibility, reflected on the diminished thermodynamic stability by phosphorylation.

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