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STUDY OF SOME PHYSICAL PROPERTIES OF GMP KINASE FROM *HAEMOPHILUS INFLUENZAE*

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The proteins guanylate monophosphate kinases (GMPKs) are component parts of cytosolic NMP kinase family with a very important role in the activation of guanosine analog pro-drugs, used both in cancer and virosis therapies. GMPKs have been characterized as monomeric enzymes in prokaryote and oligomeric in eukaryote organisms. The primary structure of GMPK from *Haemophilus influenzae* contains one Trp and 10 Tyr residues, responsible for its fluorescence emission.The fluorescence spectrum of GMPK presents the Trp emission at λ_{em} = 320 nm. The change of protein fluorescence spectrum by the binding of 8-Anilino-1-napthalene sulfonic acid ammonium (ANSA), Guanosine monophosphate (GMP), and Adenosine diphosphate (ATP) ligands to GMPK was monitored. The ligand binding induces a static type quenching of protein fluorescence emission. The affinity constants show that ANSA and GMP are more efficient than ATP in the binding process ($K_{ANSA} = 4.5\times10^4$ M^{-1}, $K_{GMP} = 3.3\times10^4$ M^{-1}, $K_{ATP} = 1.56\times10^2$ M^{-1}). The influence of temperature on the GMPK fluorescence emission was also monitored. The preliminary studies by differential scanning calorimetry (DSC) on GMPK confirm the value of the denaturation temperature, previously deduced from the fluorimetric experiments. Thus, the denaturation of the protein, monitored by DSC, is taking place on a narrow temperature interval, around the value of 50 °C.

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1. Introduction

The nucleoside monophosphate (NMP) kinase family comprises enzymes with a low primary structure homology, but with similar folding, with three separate regions (namely, CORE, LID and NMP - binding regions). One of these enzymes, guanylate monophosphate kinase (GMPK), present both in the prokaryotes and in eukaryotes, is an important enzyme in nucleotide metabolic pathways. Despite of the similar folding, GMPKs from prokaryotes (such as *Escherichia coli*) are monomers, while the GMPKs from eukaryotes are dimers, tetramers or hexamers.

Crystal structures of GMP kinases from *Saccharomyces cerevisiae* [1], *Escherichia coli* [2], *Staphylococcus aureus* [3], *Mycobacterium tuberculosis* [4], yeast [5; 6] and mouse [7] have been solved. The yeast and mouse GMP kinases were crystallized in the complex with their GMP substrate.

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Structural and biochemical characterization of GMP kinase from *Mycobacterium tuberculosis*, $GMPK_{Mt}$, show that this enzyme is a monomer, with an unusual specificity for ATP as a phosphate donor and a lower catalytic efficiency, compared with eukaryotic GMPKs [4]. Guanylate kinase from yeast catalyzes the phosphoryl transfer *via* a sequential mechanism, the reaction rate being largely controlled by the chemical step [8]. Recently, expression, activity, and kinetics of GMPK from *Streptococcus pneumoniae*, using PCR and culture assay, were performed [9]. One of the physiological roles of GMPK is the reversible phosphoryl transfer from ATP to GMP, yielding ADP and GDP. It was revealed the binding site of ATP on GMPK and also the implication of three arginine residues (44, 137 and 148) in the canalization of the phosphoryl transfer reaction [7] from ATP to GMP.

2. Materials and methods

GMP kinase from *Haemophilus influenzae* was been purified at National Institute of Research and Development for Microbiology and Immunology (NIRDMI) Cantacuzino, the molecular weight being assessed at about 23.4 kDa. The ligands, GMP and ATP, were been provided by the same institute. The 8-anilino-1-napthalene sulfonic acid ammonium salt (ANSA) was been purchased from Sigma.

Spectrophotometric, spectrofluorimetric and calorimetric measurements were performed on the following biochemical samples: GMPK, ANSA, GMP, ATP, GMPK - ANSA (2.2 μM, 1 - 100 μM) and GMPK - GMP (2.2 μM, 1.2 - 84 μM), GMPK - ATP (2.2 μM, 1.2 - 84 μM). All samples were dissolved in 50 mM TRIS-HCl, at pH = 7.4. The concentration of each protein sample was determined using a Perkin Elmer Lambda 2S Spectrophotometer, on the basis of Tyr and Trp absorption overlap at 280 nm.

Measurement of fluorescence. Fluorescence emission was been measured with a FluoroMax-3 - Horiba Jobin Yvon spectrophotofluorimeter, equipped with a Peltier device for the temperature control. Solutions of 2.2 μM GMPK and 1 μM ANSA respectively, in 50 mM TRIS-HCl (pH = 7.4) were placed in a 1 cm path-length quartz cuvette. The fluorescence emission spectra were recorded in the spectral range 300 - 500 nm, at the excitation wavelength, λ_{ex} = 290 nm, for GMPK and λ_{ex} = 350 nm, for ANSA. For thermal denaturation of GMPK, the sample was been exposed five minutes at various temperatures, between 20 °C and 70 °C, with steps of 5 °C.

*Calorimetry measurement*s. Differential Scanning Calorimetry (DSC) experiments were been performed with a DSC instrument from Mettler Toledo, measuring the heat flow in the range of 40 - 60 °C, with a scan rate of 1 °C/min.

3. Results and discussions

The purified GMPK from *Haemophilus influenzae* is a small protein, with a number of 208 amino acids in the primary structure, including one tryptophan, ten tyrosine and seven phenylalanine residues. In order to determine the direct GMPK - ligand interaction, the fluorescence emission of the tryptophan and tyrosine was been monitored, while the phenylalanine emission was neglected, being insignificant beyond 270 nm [10; 11]. Fluorescence emission was been monitored for the following samples: GMPK - ANSA, GMPK - GMP and GMPK - ATP.

Binding of ANSA to GMPK

The fluorescence emission spectrum of GMPK at λ_{ex} = 290 nm (Figure 1), where the absorption is less than 0.1 (inner effect is absent) is centered on λ_{max} = 350 nm (characteristic wavelength for Trp emission, exposed to the solvent).

The interaction between a protein and ANSA can principally produce changes in the position or orientation of the tryptophan and/or tyrosine residues, thus changing their exposure to solvent and leading to the alteration of the emission quantum yield. Generally, ANSA emission is

low when it is free, but significantly increases when it is bound to the site of the protein. The emission of ANSA appears at λ_{em} = 460 nm, for λ_{ex} = 350 nm.

Fluorescence spectroscopy was been used to monitor changes on the GMPK tertiary structure by the binding of ANSA. Titration of GMPK with ANSA, in different concentrations, in buffer TRIS – HCl, at pH 7.4, was been performed.

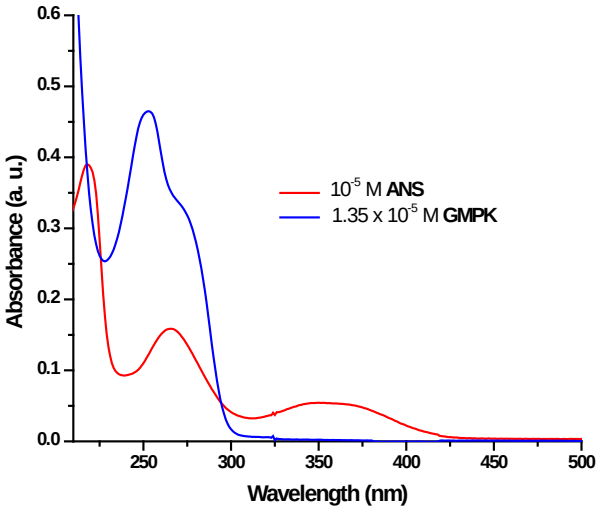

Fig. 1. The absorption spectra of 2.2 μM GMPK and 1 μM ANSA, in 50 mM TRIS - HCl buffer solution, at pH = 7.4.

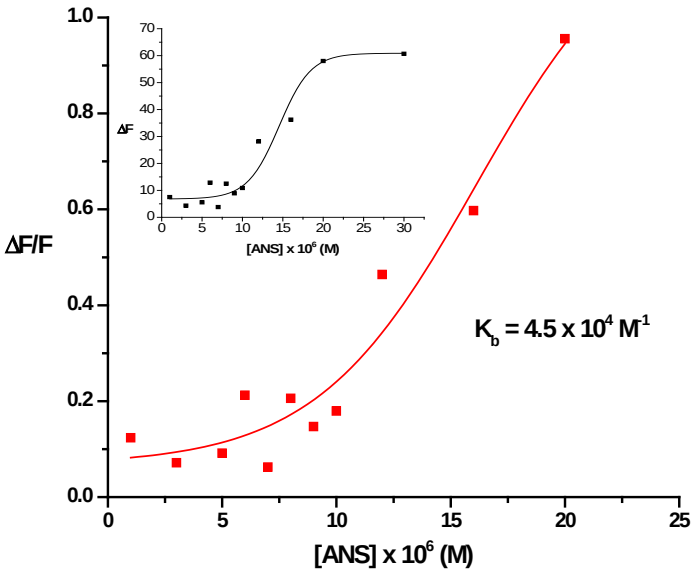

Fig. 2. Scatchard plots for the interaction of ANSA with GMPK (2.2 μM), in 50 mM TRIS - HCl buffer solution, at pH = 7.4. The change of the fluorescence intensity of GMPK as a function of ANSA concentration (inset).

Binding of ANSA to GMPK produces a large increase in fluorescence emission of ANSA, as compared with the intrinsic emission of ANSA. The results are presented in the Figure 2 (inset). It is observed that, at 20 μM ANSA, the saturation of GMPK emission appears. To examine the strength of the binding, the Scatchard method was been used to analyze the experimental data

(Figure 2). The ratio, $\Delta F/F$, linearly increases with the ANSA concentration. The binding constant is 4.5×10^4 M^{-1}. This value of the binding constant indicates a moderate interaction between ANSA and GMPK.

Binding of GMP to GMPK

The changes of the GMPK fluorescence emission intensity by the binding of GMP are shown in the Figure 3 (inset). The binding of the two partners was monitored at protein excitation wavelength, λ_{ex} = 290 nm. It was been observed that the fluorescence emission decreases with the increase of GMP concentration. By Scatchard plot, a linear variation of the $\Delta F/F$ ratio was been obtained (Figure 3). As GMP is one of the GMPK substrates, it is interesting to know how strong is the binding of this ligand to the binding site of this enzyme. One can consider that the binding of the GMP to the protein is moderate, as the binding constant shows (K_b = 3.3 $\times 10^4$ M^{-1}). The affinity constant of the binding of ATP to GMPK was been estimated, too (data not shown). One can say that GMP is more efficient than ATP in the binding process ($K_{GMP} = 1.24 \times 10^4$ M^{-1}, $K_{ATP} = 1.56 \times 10^2$ M^{-1}).

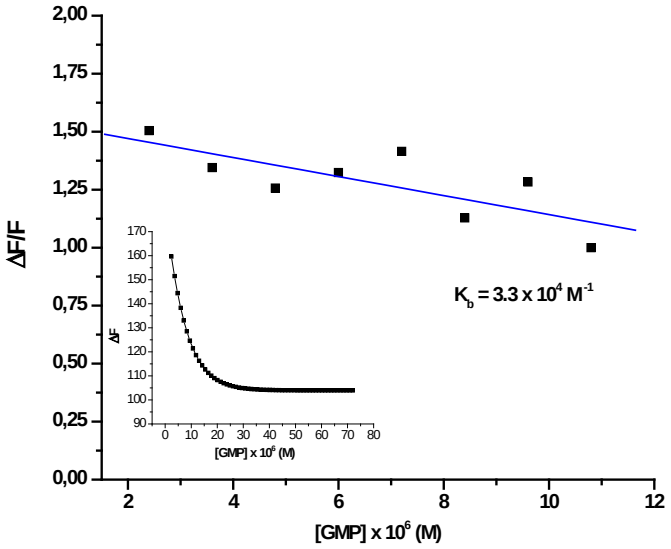

Fig. 3. Scatchard plots for the interaction of GMP with GMPK (2.2 μM), in 50 mM TRIS - HCl buffer solution, at pH = 7.4. The changes of GMPK fluorescence intensity as a function of GMP concentration (inset).

Thermal denaturation of the GMPK structure

Denaturation of all proteins occurs when an external factor is excessively acting on the protein. One of these factors, that can affect dramatically the secondary structure of all proteins, is the temperature.

The temperature dependence of GMPK fluorescence emission was been monitored, in order to determine the denaturation temperature for which 50 % of the GMPK molecules are altered. The temperature fluorescence dependence of triptophan and tyrosine put in evidence a critical denaturation temperature of 50 °C. Consequently, the fluorescence properties of the intrinsic residues significantly changed by denaturation over 50 °C, where other processes, like aggregation of the protein molecules may occur (Figure 4).

Protein secondary and tertiary structures seem to be more stabilised by the ligand binding, especially when the ligand is a peptide. In contrast, the binding of GMP (found in RNA) to the

GMPK manifests a very slow increase in the fluorescence of the protein (Figure 5), meaning that GMP had a small influence on the enzyme 3D conformation.

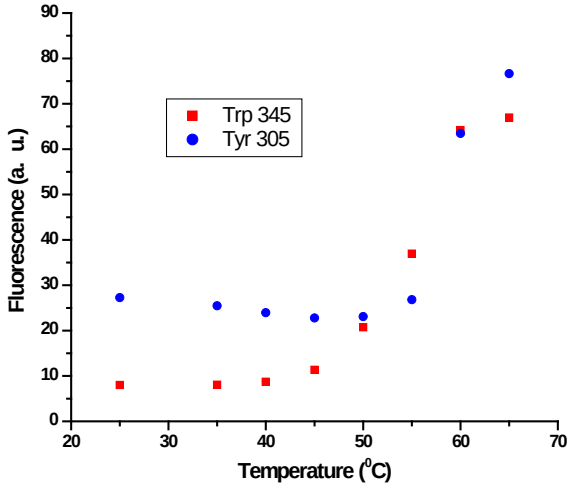

Fig. 4. The emission fluorescence of triptophan and tyrosine during the GMPK denaturation process (20 °C - 70 °C). GMPK (2.2 μM) was dissolved in 50 mM TRIS – HCl buffer solution, at pH = 7.4.

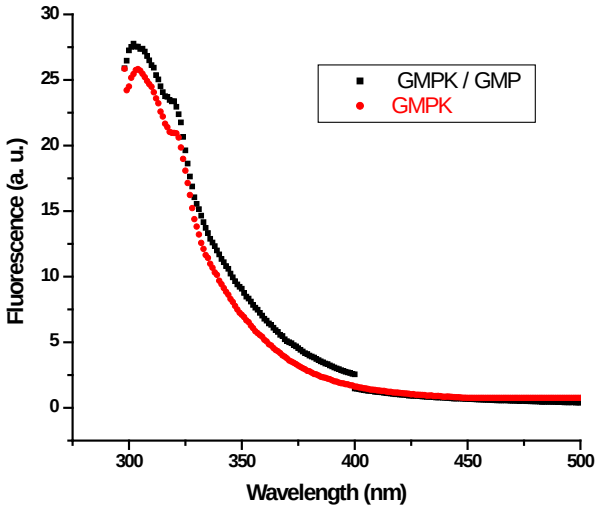

Figure 5. The emission fluorescence of GMPK and GMPK – GMP, at 35 °C. GMPK (2.2 μM) and GMPK – GMP were dissolved in 50 mM TRIS - HCl buffer solution, at pH = 7.4.

Measurement of the caloric capacity of GMPK

The energetics of conformational transitions of biological macromolecules may be studied by the differential scanning calorimetry (DSC). For proteins, the temperature dependence of the heat capacity (a basic thermodynamic parameter) permits the access to the thermodynamic mechanism that governs the equilibrium between the folded and unfolded states of a protein [12]. A typical experiment comprises one temperature scan of the sample.

The behaviour of GMPK from *Haemophilus influenzae* was been investigated by analyzing the protein thermal stability, in a dilute solution. As one can see, the GMPK was been denatured in the 40 - 60 °C temperature interval, observing a transition midpoint at the value of 50 °C (Figure 6 A). The phase transition peak, T_m, is defined as the temperature at which 50 % of the

protein molecules are unfolded or, the temperature at which any molecule spends 50 % of its time folded and 50 %, unfolded. It was been noticed that T_m is the same to that obtained by fluorescence experiments (Figure 4), namely, 50 °C. This value of the transition peak allows to say that GMPK is a somehow thermal resistant enzyme.

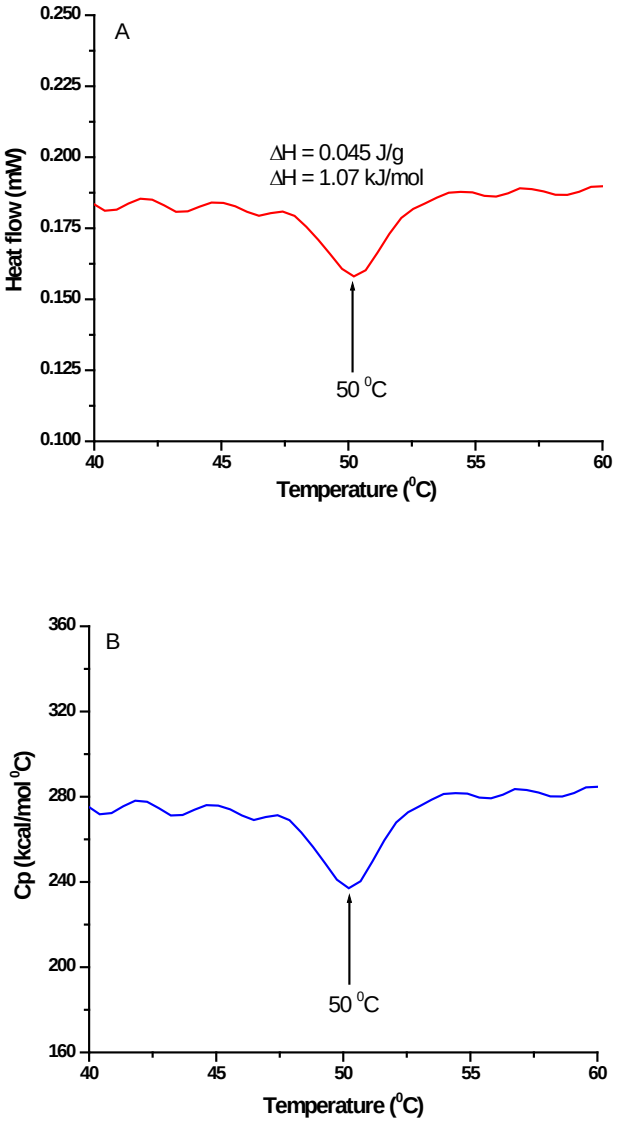

Fig. 6. Heat flow (A) and caloric capacity (B) of the thermal unfolding of GMPK (2.2 μM), in 50 mM TRIS - HCl buffer solution, at pH = 7.4.

After concentration normalization and baseline correction, one can obtain the dependence of caloric capacity, C_p, of temperature. The variation of the caloric enthalpy, ΔH_{cal}, is 1.07 kJ/mol, and of the caloric capacity, at T_m, is 240 kcal/mol K (Figure 6 B). The values obtained for the thermodynamics parameters (C_p and ΔH_{cal}) are much smaller than those obtained for the *Escherichia coli* kinase, also investigated by DSC experiments [2]. This comparison shows that GMPK from *Haemophilus influenzae* may be not as compacted as *Escherichia coli* GMPKs.

4. Conclusions

The binding of GMP kinase from *Haemophilus influenzae* to the ligands takes place with moderated binding constants for both ANSA and GMP.

Fluorescence and calorimetric measurements are consistent each other, putting in evidence the same transition temperature of the protein conformation.

The values of thermodynamics parameters, smaller than those obtained for other kinases, suggest that GMPK from *Haemophilus influenzae* has not a compacted conformation as the other kinases.

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