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Investigating the folding pathway and substrate induced conformational changes in *B. malayi* Guanylate kinase

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Abbreviations:
NMP kinase, nucleoside monophosphate kinase
BmGK, *Brugia malayi* Guanylate kinase
SEC, Size Exclusion chromatography
GdnCl, guanidine hydrochloride
MS, molecular simulation

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ABSTRACT

Guanylate kinase is one of the key enzymes in nucleotide biosynthesis. The study highlights the structural and functional properties of *Brugia malayi* Guanylate kinase (BmGK) in the presence of chemical denaturants. An inactive, partially unfolded, dimeric intermediate was observed at 1–2 M urea while GdnCl unfolding showed monomer molten globule like intermediate at 0.8–1.0 M. The results also illustrate the protective role of substrates in maintaining the integrity of the enzyme. The thermo stability of protein was found to be significantly enhanced in the presence of the substrates. Furthermore, binding of the substrates, GMP and ATP to BmGK changed its GdnCl induced unfolding pattern. Docking and molecular dynamic simulation performed for native BmGK, BmGK bound to GMP and GMP + ATP showed change in the fluctuation in the region between 130 and 150 residues. Arg134 lost its interaction with GMP and Arg145 interaction shifted to ATP after 40 ns simulation upon binding of ATP to BmGK-GMP complex. We, thus, propose the importance of specific rearrangements contributed by binding of substrates which participate in the overall stability of the protein. The work here emphasizes on detailed biophysical characterization of BmGK along with the significant role of substrates in modulating the structural and functional properties of BmGK.

1. Introduction

Understanding the structure-function relationships of an enzyme under different conditions is fundamentally important for both theoretical and applicative aspects. Such studies may provide insight into the molecular basis of the stability of the enzyme. Denaturation is a useful means for studying the characteristics of protein folding and has given a better insight into the mechanism of the folding process [1–3]. Exposure of proteins to denaturing agents such as extremes of pH, temperature, high ionic strength, chemical denaturants and organic solvents has been utilized to study the unfolding/folding pathway [4–6]. Though partially folded forms of numerous proteins have been studied, it has yet not been possible to generalize the concept of 'intermediate states' as universal equilibrium folding intermediates. However, characterization of protein folding intermediates is helpful in identifying and understanding various transition steps and forms [7]. A compact intermediate state, which possesses a high degree of secondary structure and a fluctuating tertiary structure, has been described in several recent studies under various denaturing conditions [8–11].

Ligand binding to proteins is a subject of interest to explain one of the most intriguing and perplexing question in structural and molecular biology i.e. the problem of protein folding into a unique, compact, highly ordered and functionally active form and especially the role of ligands in the structure and in the stabilization of proteins in their native states. The interaction of proteins with small ligands is associated with increase in protein thermostability due to the coupling of binding with unfolding process [12–15]. The binding of proteins to small molecules and protein–protein interactions are key processes in cellular biochemistry. Ligand binding results in change in the conformation of the target protein which in turn produces a given response. Since biological processes are carried out through binding events, therefore the induced structural changes upon ligand binding have been found to be a matter of extensive studies [12].

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Guanylate kinase, GK (ATP:GMP phosphotransferases, EC 2.7.4.8), a nucleoside monophosphate kinase (NMP kinase), catalyzes the reversible transfer of the terminal phosphoryl group from ATP to guanosine 5- monophosphate (GMP) substrate, subsequently releasing guanosine diphosphate (GDP) as product. This is a crucial intermediate step in RNA or DNA synthesis foregoing the formation of the key nucleotide guanosine triphosphate (GTP) or its deoxy form dGTP [16]. The structure of Guanylate kinase comprises of NMP binding region, CORE and the LID region. Crystal structures have been solved for enzymes from many organisms including *Saccharomyces cerevisiae* GK which is available in both nucleotide free form and in complex with GMP and also for mouse GK in an abortive complex with ADP and GMP (mGK-GMP-ADP) [17,18].

This investigation describes the biophysical characterization of BmGK, as an endeavour of understanding the structure-function relationship, the basis and rationale of its distinct physicochemical properties and the folding unfolding mechanism. The study also reports the changes in the conformation and dynamic behaviour of BmGK on binding to substrates. Very little information about the folding aspects of Guanylate kinase is available; hence a detailed analysis of native, intermediate and denatured states of BmGK was carried out by measuring the intrinsic tryptophan fluorescence measurement, near & far-UV CD analysis, 1- aniline-8- naphthalene sulphonic acid (ANS) binding and quenching of intrinsic tryptophan fluorescence.

Large-scale conformational alterations are thought to mediate allosteric regulations, which are related with protein function in signal transduction, immune response, and enzymatic activity [19]. Thus, a fundamental problem is to understand the mechanism for the conformational transitions in BmGK upon binding of substrates which is not known. The relationship between protein stability upon substrate binding and the role of substrate in modulating the intrinsic flexibility of the enzyme using various spectroscopic techniques and bioinformatics tools such as molecular dynamic simulation have been carried out. Such a comprehensive and consistent description for BmGK is largely complementary to previous experimental studies carried out on this enzyme.

2. Materials and methods

2.1. Materials

All the reagents were purchased from Sigma–Aldrich Chemical, USA unless specifically mentioned. Molecular weight markers for SDS–PAGE were purchased from MBI Fermentas, Maryland, USA. Ni–NTA agarose matrix was procured from Qiagen, Germany.

2.2. Protein expression, purification and activity assay

Guanylate Kinase was expressed in *E. coli* BL21 (DE3) and purified to homogeneity by Ni-NTA affinity chromatography as described earlier [20]. The purity of the protein was checked on 12% SDS PAGE. Enzyme activity was measured according to the method of Agrawal *et. al* [21]. For studies involving denaturation with urea or GdnCl, the enzyme was incubated in assay buffer containing desired concentrations of the denaturants for 1 h. It was determined that the auxiliary enzymes (pyruvate kinase and lactate dehydrogenase) during assay remained active under the experimental conditions. The reversibility of the transitions was determined by incubating 20 μM BmGK for 1 h in the presence of the different concentrations of denaturants. The samples were then diluted with 50 mM Tris buffer pH 7.5 to attain the final concentration of 10 mM urea and 2.5 mM GdnCl respectively. The diluted enzyme mixture was then immediately used for the activity. The loss of enzyme activity as a function of temperature was followed, in the presence and absence of substrates in 50 mM Tris-Cl buffer (pH 7.5). The enzyme samples were incubated for 15 min at different temperatures 25 °C to 55 °C and after cooling to 4 °C, the residual activity was measured on spectrophotometer at 25 °C.

2.3. Fluorescence spectroscopy

Fluorescence spectra of BmGK were recorded in Perkin-Elmer LS 5B luminescence spectrometer with a 5 mm path-length quartz cell. Samples containing different concentrations of urea or GdnCl were equilibrated for 5 h in 50 mM sodium phosphate buffer (pH 7.0) at 25 °C. The spectra were normalized for the solvent contribution to fluorescence. Titrations for binding of nucleotides (GMP and ATP) with BmGK were carried out by adding small aliquots (0–2 mM) to 5 μM of BmGK in buffer containing 50 mM phosphate buffer (pH 7.0) after an equilibration period of 2 h. For tryptophan fluorescence measurements, an excitation wavelength of 290 nm was used and the emission was recorded between 300 and 400 nm.

ANS binding was estimated by incubating BmGK previously denatured at different concentrations of urea or GdnCl with optimised concentration (100 μM) of ANS at 25 °C. The excitation wavelength was 380 nm and emission was recorded between 400 nm and 600 nm.

2.4. Fluorescence quenching experiment

Acrylamide quenching experiments were performed by adding acrylamide to BmGK denatured at different concentrations of urea or GdnCl for 5 h and the fluorescence quenching data were analyzed according to the Stern–Volmer equation [22].

2.5. Circular dichroism measurements

CD experiments were performed with a JASCO J810 spectropolarimeter (equipped with peltier temperature controller system) in a 1 cm path length cell at 25 °C. The far UV CD spectra were measured for 5 μM BmGK treated with desired concentration of denaturants for 5 h in 50 mM sodium phosphate buffer (pH 7.0) at 25 °C. Readings obtained were normalized by subtracting the baseline observed for the buffer with similar denaturant concentration under similar conditions. The tertiary structure of the enzyme (20 μM) was monitored in the near UV region in wavelength range 250–350 nm using a cell of path length of 0.5 cm. Each spectrum was recorded as an average of three scans. For binding experiments, the CD spectra of 5 μM BmGK were measured in buffer containing 50 mM phosphate buffer (pH 7.0) with 0.25 mM GMP and (0.25 mM GMP + 0.25 mM ATP). The values obtained were normalized by subtracting the baseline recorded for the buffer having same concentration of substrate under similar conditions. Thermal denaturation was studied by monitoring changes in molar ellipticity at 222 nm as a function of temperature from 30 °C to 85 °C.

2.6. Limited proteolysis

BmGK (0.5 mg/ml) was subjected to limited proteolysis with trypsin for 1 h at 37 °C with protease to protein ratio at 1:500 (w/w). Samples were preincubated with 0.25 mM substrates for 1hr before trypsin digestion. The reaction was stopped by adding PMSF (1 mM) in the reaction mixture and samples were analyzed on 15% SDS-PAGE.

2.7. Size exclusion chromatography

Gel filtration experiments were carried out on Superdex TM 75, 10/300GL column on AKTA FPLC (GE Health care) pre-calibrated