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Glycine-rich loop encompassing active site at interface of hexameric *M. tuberculosis* Eis protein contributes to its structural stability and activity

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ABSTRACT

RvEis is a crucial thermostable hexameric aminoglycoside acetyltransferase of *Mycobacterium tuberculosis*, overexpression of which confers Kanamycin resistance in clinical strains. The thermostability associated with hexameric RvEis is important for the enhanced intracellular survival of mycobacteria. However, the structural determinants responsible for its thermal stability remain unexplored. In this study, we have assessed the role of glycines of conserved glycine-rich motif ($G^{123}GIYG^{127}$) present at the oligomeric interface in the hydrophobic core of RvEis in sustenance of its structural stability, oligomerization and functional activity. Substitution of glycines to alanine (G123A/G127A) result in significant decrease in melting temperature (T_m), reduction in the oligomerization with concomitant increase in the monomeric form and higher susceptibility towards the denaturants like GdmCl and urea relative to wild type. G123A/G127A mutant displayed lower catalytic efficiency (k_{cat}/K_m) and is completely inactive at 60 °C. ANS binding assay and the complete dissociation of hexameric complex into monomers at lower concentration of urea in G123A/G127A relative to wtRvEis suggests that altered hydrophobic environment could be the reason for its instability. In sum, these results demonstrate the role of $G^{123}GIYG^{127}$ motif in structural stability and activity of RvEis.

1. Introduction

Emergence of drug resistant tuberculosis poses a serious threat to the proper treatment of the disease. Enzymes having capacity to modify aminoglycosides, a second line anti-TB drug, have further complicated the drug resistance menace. RvEis (Rv2416c) is one such aminoglycoside acetyltransferase belonging to GNAT family (GCN5-related family of N-acetyltransferases) which acetylates the aminoglycoside antibiotics to make them non-functional. Upregulation of RvEis protein confers Kanamycin resistance to *Mycobacterium tuberculosis* clinical isolates [1]. RvEis enhances the intracellular survival of *Mycobacterium smegmatis* in macrophage like cell line U937 [2]. RvEis was found to be present in culture supernatant of infected macrophages and modulates the secretion of TNF-α, IL-10 [3] and the cross regulation of T-cells [4]. Moreover, RvEis modulates the autophagy, inflammation and ROS dependent cell death [5]. RvEis protein acetylates host's DUSP16, a JNK-specific phosphatase [6] and nucleoid-associated protein (HU) of *M. tuberculosis* [7]. RvEis protein is mainly hydrophilic but has a hydrophobic amino terminus which justifies its localization in cell membrane, cell wall and culture supernatant besides a predominant amount present in the cytoplasm [8]. RvEis is a hexameric protein which consists of two threefold symmetrical trimers and has an unprecedented ability to multiacetylate variety of aminoglycosides [9]. The amino acids Y126, W197 and Y310 present in hydrophobic core are important for catalytic activity [9]. RvEis has remarkable structural and thermal stability. Thermostability of RvEis has been reported to be important for enhancing intracellular survival of Mycobacteria [10].

Structural stability of proteins is determined by a number of factors including oligomerization [11,12] hydrophobic interactions [13,14] hydrogen bonding and polar surface area [15,16] and secondary structure elements such as helical propensities [17] and loop regions [18,19] etc. The loops are important unstructured regions which connect the secondary structural elements and provide the required flexibility to sustain the oligomerization [20] and stability [21–23] of the proteins. Flexible glycine rich regions are

Abbreviations: wtRvEis, wild-type RvEis; Eis, enhanced intracellular survival; GdmCl, guanidinium chloride; T_m, melting temperature; C_m, melting concentration; KAN, kanamycin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ANS, 8-anilino-1-naphthalenesulfonic acid.

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often found in the loops [24] and may influence the conformation and function of proteins [25]. The substitution of glycines present in the loop region with any other residues usually restrict the required flexibility by affecting the local dynamics of the protein [26] that may reduce the stability and catalytic activity of enzymes [27–30].

M. tuberculosis Eis protein also has some conserved glycine-rich loops and motifs. The correlation of glycine rich loops with the stability and catalytic activity of enzymes prompted us to investigate the role of these conserved glycine motifs in the homo-oligomerization, maintenance of structural and thermal stability and regulation of acetyltransferase activity of RvEis protein.

2. Materials and methods

2.1. Materials

All enzymes used in site directed mutagenesis were obtained from Thermoscientific and New England Biolabs. All other chemicals and reagents used in the study were of biochemical research grade and purchased from Sigma. Superdex 200HR 10/300 GL column and molecular weight markers for size exclusion chromatography were purchased from GE Healthcare Life Sciences, USA.

2.2. Construction of RvEis protein mutants

The gene encoding RvEis protein (Rv2416c) cloned previously was used as a template for site directed mutagenesis [4]. G51A/G55A, G123A/G127A, G155A/G159A and G156A/G160A mutations of glycine-rich motifs were introduced using Phusion site directed mutagenesis kit and confirmed by nucleotide sequencing.

2.3. Expression and purification of wild type RvEis and its mutant proteins

Wild-type RvEis (wtRvEis) protein was purified as described previously [4]. Similarly, mutant proteins of RvEis were purified using Ni-NTA agarose resin. The concentration of wtRvEis and mutant proteins (μg/ml) was estimated using Bradford assay and calculated with respect to the standard curve prepared using different concentrations of BSA.

2.4. Circular dichroism measurements

Far-UV CD spectra of either 3.0 μM purified wtRvEis or its mutant proteins, equilibrated with 20 mM phosphate buffer (pH 8.0) was recorded at 25 °C using 1 mm cell on a Jasco J810 spectropolarimeter calibrated with ammonium (+)-10-Camphorsulfonate. The obtained data is reported as MRE (Mean Residue Ellipticity) in deg cm^2 $dmol^{-1}$, calculated using equation:

$$[\mathrm{MRE}] = \theta \times 100 \times M_r/c \times d \times n$$

where θ is the observed ellipticity in degrees, c = protein concentration in mg/ml, and d = path length in cm, M_r = Protein molecular weight and n = number of amino acids.

For thermal stability experiments, wtRvEis or its mutant proteins were incubated from 25 °C to 90 °C as measured by sample internal probe attached with thermal Peltier controller and CD ellipticity at 222 nm was recorded at 3 °C/min heating rate using 1 mm quartz cuvette. For the effect of SDS on temperature induced denaturation of wtRvEis and G123A/G127A mutant protein, samples were incubated with 2 mM SDS at 25 °C for 12 h. For guanidium hydrochloride (GdmCl) and urea denaturation studies, 3.0 μM protein samples were incubated with increasing concentrations of GdmCl or urea, for 12.0 h at 4 °C. Fraction of unfolded protein corresponding to observed ellipticity at 222 nm versus either temperature or concentration of denaturant was calculated using following equation:

$$f_{D=(y-y_N)/(y_U-Y_U)}$$

where y is the experimental observed signal at either a specific temperature or denaturant concentration. The signal observed for the protein in native and fully denatured state is represented by y_N and y_U, respectively. The following equation was used to calculate the transition midpoint (T_m) for all the transition curves [31].

$$f_D = y_N + \frac{y_U + Y_N}{1 + e^{-[(T-T_m)/b]}}$$

where b is the slope factor.

2.5. Homology modelling of G123/127A mutant protein

Glycines at 123th and 127th position were replaced with alanine in primary amino acid sequence of RvEis and submitted to SWISS-MODEL software (https://swissmodel.expasy.org/). Overall 657 templates were found and two templates with highest sequence similarity (PDB ID: 3R1K and 3SXO) were selected for building the mutant protein model having coverage value of 1.00. Comparative analysis of structure of both mutant and wild type RvEis was performed using PyMOL software.

MolProbity software (http://molprobity.biochem.duke.edu/) [32] was used to prepare Ramachandran plot to find out the torsion angles for G123 and G127 of wtRvEis protein (PDB code: 3R1K).

2.6. Fluorescence spectroscopy

For intrinsic tryptophan fluorescence studies, protein samples were excited at 280 nm wavelength and the emission spectrum was recorded from 300 nm to 450 nm with 5 nm slit width at 25 °C. The baseline recorded for the buffer was subtracted from all the sample values. For the effect of temperature, protein samples were heated at different temperatures from 25 °C to 100 °C for 10 min. Similarly, the effect of SDS was measured after incubating the respective protein with increasing concentration of SDS at 25 °C for 4 h. For the combined effect of SDS and temperature, protein samples were first treated with 2 mM SDS for 12 h at 25 °C, then heated to the respective temperature before recording the emission spectra. The values corresponding to native RvEis without any treatment were taken as 100%. For ANS (8-anilino-1-naphthalenesulfonic acid) binding assay, protein samples were incubated in 1:50 molar ratio with ANS in 20 mM phosphate buffer (pH 8.0) for 5 min at 25 °C. The ANS fluorescence emission spectra were measured from 400 nm to 600 nm following excitation at 360 nm. The slit widths were set at 5 nm for excitation and 10 nm for emission spectra acquisition.

2.7. SDS-PAGE assay

25 μg each of boiled or unboiled samples of either wtRvEis or G123A/G127A mutant containing 1% SDS in 0.125 M Tris (pH 6.8) were electrophoresed on 8% SDS-PAGE as recommended [33]. Coomassie Brilliant Blue R250 stain was used to visualize the bands. SDS-PAGE assay was modified to examine the effect of urea on oligomerization of RvEis. 10 μg of wtRvEis or G123A/G127A mutant protein was treated with increasing concentration of urea for 12 h at 4 °C and oligomeric stability was analyzed by using SDS-PAGE assay.