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Estimation of Förster's distance between two ends of Dps protein from mycobacteria: Distance heterogeneity as a function of oligomerization and DNA binding

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Abstract

Dps protein (DNA binding Protein from Starved Cells) from *Mycobacterium smegmatis* (Ms-Dps) is known to undergo an *in vitro* irreversible oligomeric transition from trimer to dodecamer. This transition helps the protein to provide for bimodal protection to the bacterial DNA from the free radical and Fenton mediated damages in the stationary state. The protein exists as a stable trimer, when purified from *E. coli* cells transformed with an over-expression plasmid. Both trimer as well as dodecamer are known to exhibit ferroxidation activity, thus removing toxic hydroxyl radicals *in vivo*, whereas iron accumulation and non-sequence specific DNA binding activity are found in dodecamer only. This seems to be aided by the positively charged long C-terminal tail of the protein. We used frequency domain phase-modulation fluorescence spectroscopy and Förster Resonance Energy Transfer (FRET) to monitor this oligomeric switch from a trimer to a dodecamer and to elucidate the structure of DNA–Dps dodecamer complex. As Ms-Dps is devoid of any Cysteine residues, a Serine is mutated to Cysteine (S169C) at a position adjacent to the putative DNA binding domain. This Cysteine is subsequently labeled with fluorescent probe and another probe is placed at the N-terminus, as crystal structure of the protein reveals several side-chain interactions between these two termini, and both are exposed towards the surface of the protein. Here, we report the Förster's distance distribution in the trimer and the dodecamer in the presence and absence of DNA. Through discrete lifetime analysis of the probes tagged at the respective regions in the macromolecule, coupled with Maximum Entropy Method (MEM) analysis, we show that the dodecamer, upon DNA binding shows conformational heterogeneity in overall structure, perhaps mediated by a non-specific DNA–protein interaction. On the other hand, the nature of DNA–Dps interaction is not known and several models exist in literature. We show here with the help of fluorescence anisotropy measurements of labeled DNA having different length and unlabeled native dodecameric protein that tandem occupation of DNA binding sites by a series of Dps molecules perhaps guide the tight packing of Dps over DNA backbone.

Keywords: *M.smegmatis* Dps; DNA binding; Fluorescence lifetime; Anisotropy; Rotational correlational time; FRET

1. Introduction

One of the major outcome during evolution of life was the utilization of molecular oxygen from atmosphere instead of other oxidants generated from respiratory or fermentation processes. On the flip side, this advantage turned out to be deleterious at times due to the unavoidable production of reactive oxygen species (ROS) which can damage the biological macromolecules irreversibly, particularly the DNA molecule. In order to prevent such free radical mediated damages, different organisms adopt different pathways, and bacteria offer a plethora of adaptive responses which are very interesting to

Abbreviations: PAGE, Poly Acrylamide Gel Electrophoresis; MLP, Mono Labeled Protein; DLP, Doubly Labeled Protein; HEPES, 4-(2-Hydroxyethyl) Piperazine-1-ethanesulfonic acid; TCEP, Tris(2-carboxyethyl) phosphine hydrochloride; DTT, Dithiothreitol; IAEDANS, 5-[2-(iodoacetamido) ethylamino] naphthalene-1-sulfonic acid; FITC, Fluorescein Isothiocyanate; ds, double strand; nt, nucleotide; FRET, Förster Resonance Energy Transfer; MEM, Maximum Entropy Method; FPLC, Fast Protein Liquid Chromatography; Da, Dalton; kDa, KiloDalton; kb, kilobasepair.

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study. DNA binding protein from stationary phase cells (Dps) is a group of such proteins which appears progressively as a function of growth of the bacteria and generally are believed to protect the genetic material upon binding them non-specifically. In addition, however, Dps from different organisms are known to exhibit ferroxidation activity *in vivo*, thus protecting the DNA from oxidative radicals generated by Fenton's reaction. Existence of such a novel protein was first reported in the year 1992 by Kolter's group in starved stationary phase cultures of *E. coli* [1]. In our laboratory the Dps homolog from *Mycobacterium smegmatis* was first discovered and a bimodal protection of DNA was reported [2,3]. Dps proteins are known from many other organisms, Gram-positive or Gram-negative alike, and they show DNA binding as a predominant mode of masking DNA [1–5], although protection of the cells are known, in some cases, without binding to DNA [6–8].

In spite of our knowledge of Ms-Dps from functional [3] as well as structural [4] point of view, some interesting questions still remain elusive with respect to Ms-Dps, in particular, how the DNA binding ability of the protein is manifested in its sequence or the molecular mechanism behind trimer to dodecamer conversion. This work was motivated with the thought that a suitably labeled Ms-Dps with fluorescent tags may help in deciphering its DNA recognition as well as dodecamerization mechanisms through Förster's energy transfer (FRET) and anisotropy measurements. The proximate aim, obviously, is to decipher the mechanism of action of Ms-Dps and its peculiar bimodal action in mycobacteria.

Ms-Dps does not have Cysteine residue in its sequence. Thus, we labeled the protein after mutating the 169th Serine residue to Cysteine and subsequently tagging it with a thiol specific probe 1, 5-IAEDANS. The position 169 was important as it is located at the beginning of the putative DNA binding C-terminal domain of Ms-Dps [3]. The other fluorescent moiety, FITC, was placed at the N-terminal of Ms-Dps and both 1, 5-IAEDANS and FITC form an excellent Förster's pair [10–12]. Finally, phase-modulation technique was used to calculate the lifetimes of the donor [13] and they were also subjected to Maximum entropy Method (MEM) analysis [15,16,20,23] to get distribution of lifetimes. Steady state anisotropy and rotational correlation time measurements, on the other hand, revealed some interesting features of DNA recognition.

2. Materials and methods

2.1. Construction of Cysteine mutation by site directed mutagenesis

Serine169 residue at C-terminal tail of the full-length native unlabeled Ms-Dps protein was mutated to Cysteine by site directed mutagenesis according to the *QuickChange* protocol (Stratagene). pET21b specific reverse primer (5′-CCAACTCAGGTACCTTTCGGGC-3′) and forward primer (5′-GAGGGGCAG*TGT*ACCGAGAAG-3′) containing mutation were used in PCR. The mutation was confirmed by DNA sequencing (University of Delhi, South Campus).

2.2. Purification of the mutant protein

The mutant C-terminal His tagged S169C Ms-Dps, was purified following similar protocol as for the native unlabeled protein [3]. *E. coli* strain BL21 DE3 (pLys) was transformed with the vector Cys-pET-*ms-dps*. These cells were grown at 37 °C in Luria Bertini (LB) medium to an A_{600} of 0.6 and then induced with 1 mM isopropyl-1-thio-β-galactopyranoside. Single step purification was performed using the Qiagen Ni–NTA affinity matrix according to the manufacturer's instructions. The purity of the protein was checked on a 12% SDS-polyacrylamide gel, and then the protein was dialyzed against 50 mM Tris–HCl (pH 7.9), 150 mM NaCl overnight and used for further analysis. Protein concentration was determined by the method of Lowry (1951) [9]. For the formation of the higher oligomer, protein at a concentration of 1 mg/ml was incubated at 37 °C for 12 h in 50 mM Tris–HCl (pH 7.9), 150 mM NaCl [3].

2.3. Modification of the single Cysteine with 5-[2-(2-iodoacetamidoethylamino]-1 naphthalenesulphonic acid, {1,5-IAEDANS}

Covalent modification of single Cysteine residue was carried out at pH 7.9 in 40 mM HEPES-KOH, 50 mM KCl, 0.1 mM EDTA and 5% glycerol. Protein sample was incubated with 5-times molar excess of TCEP [Tris2-(carboxyethyl)phosphine hydrochloride] for 20 min on ice. Then 20-fold molar excess of 1,5-IAEDANS was added to protein solution and incubated for another 1 h at room temperature in dark followed by a further incubation of 12 h at 4 °C under rotation. To stop the reaction a 10 fold molar excess of DTT over IAEDANS was added. The unbound probe was removed by passing the reaction mixture through a PD10 gel filtration column (Biorad). The labeling was checked by measurements of O.D. of the sample at 280 nm for protein ($\varepsilon_{monomer}$=22,190 M^{-1} cm^{-1}) and 336 nm for IAEDANS (ε=6300 M^{-1} cm^{-1}). The labeled protein was checked for its oligomerization, ferroxidation and DNA binding activities.

2.4. Modification of the N-terminus of the IAEDANS labeled S169C Ms-Dps with Fluorescein IsothioCyanate (FITC)

Specific covalent modification of only the free NH_2 group at the N-terminus of S169C Ms-Dps trimer and its IAEDANS labeled derivative were carried out at neutral pH in 40 mM HEPES-KOH (pH 7.4), 50 mM KCl, 0.1 mM EDTA and 5% glycerol. To the dialysed protein solution, 50-fold molar excess of FITC was added. The mixture was then stirred for an hour at room temperature in dark. The unbound probe was then removed by passing the reaction mixture through a PD10 gel filtration column (Biorad). The purified labeled proteins were then subjected to O.D. measurements at 280 nm for the protein and 494 nm for the dye to calculate the expected labeling ratio of dye: protein. The ε values are 22,190 M^{-1} cm^{-1} and 64,000 M^{-1} cm^{-1} for the protein monomer and FITC respectively. Both the proteins were checked for their oligomerization, ferroxidation and DNA binding abilities.