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Research paper

Characterization of the structure of the phosphoprotein of Chandipura virus, a negative stranded RNA virus probing intratryptophan energy transfer using single and double tryptophan mutants

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ABSTRACT

The phosphoprotein (P protein) of Chandipura virus (CHPV), a negative stranded RNA virus, is involved in both transcription and replication phases of the viral life cycle. The two Tryptophan (Trp) residues of CHPV, located at 105 and 135 respectively and two single Trp mutants W135F and W105F and a double Trp mutant W135F/W105F have been characterized by steady state and time-resolved fluorescence and phosphorescence at 298 K and 77 K. Results indicate that Trp135 is more buried with less polar and more hydrophobic environment whereas the Trp105 is solvent exposed. Quantum yields (Φ) suggest that the singlet–singlet (S ↔ S) non-radiative energy transfer (ET) from the Trp135 to the Trp105 occurs with 66% efficiency. The simulation of the fluorescence spectra of the WT and the time resolved studies support the results. Lifetime and Φ of the single Trp mutants suggest an intrinsic static quenching of the Trp105. The results at 77 K indicate that the ET takes place from the lowest triplet state (T_1) of the Trp105 to the T_1 of the Trp135 apart from the backward S ↔ S ET from the Trp105 to the Trp135. The triplet–triplet (T ↔ T) ET implies a distance of $<$10 Å between the Trp105 and the Trp135. Using the crystal structure of Vesicular Stomatitis Virus (VSV) phosphoprotein exhibiting about 34% similarity with the CHPV P protein, a homology modelling of CHPV supports the observed distance between the Trp residues, the S ↔ S ET efficiency and the environments of the Trp residues in CHPV.

1. Introduction

Chandipura virus (CHPV), a negative stranded RNA virus belonging to the Rhabdoviridae family [1], has been implicated in the deaths of many young children in the western parts of India [2,3]. Recurrence of infection among the young children is becoming a significant health concern due to the unavailability of any preventive measure or vaccine against the virus and calls for detail molecular understanding of the viral life cycle and the events associated with it. The life cycle of the Chandipura virus involves the delicate balance between the transcription and the replication phases, a common feature of the negative sense RNA viruses [4]. The viral RNA dependent RNA polymerase (RdRp) acts both as the transcriptase and replicase [5,6], whereas Phosphoprotein (P protein) acts as a modulator of the RdRp [7]. Studies have shown that the P protein acts as a transcriptional activator. Ser 62 residue was identified as the site of the phosphorylation by the host kinase, casein kinase II (CK II) that was necessary and sufficient for *in vitro* transcriptional activity [7].

Phosphorylation of the P protein renders it as a dimer [7,8] and there is a measurable increase in the helical content also associated with the phosphorylation [9]. Our previous work has demonstrated that the phosphorylation changes the conformation of the P protein from an 'open' to more 'closed' form with a significant change in the available hydrophobic area and protease sites [9]. While in the phosphorylated form, the P protein forms dimer, recent studies

Abbreviations: CHPV, Chandipura virus; P protein, phosphoprotein; Trp, tryptophan; Tyr, tyrosine; Phe, phenylalanine; ET, energy transfer; VSV, Vesicular Stomatitis Virus; LTP, low-temperature phosphorescence; EG, ethylene glycol; ASA, accessible surface area; WW, wild-type protein; CK II, casein kinase II; RNA, ribonucleic acid.

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have established that the unphosphorylated form of the protein also undergoes concentration dependent oligomerization [10].

It has been demonstrated that the unphosphorylated form of the CHPV P protein specifically binds the leader RNA and this binding could be abolished by CKII-mediated phosphorylation [10,11]. This differential binding of the phosphorylated and the unphosphorylated form of the P protein has been proposed to be the modulator of the viral life cycle switch between transcription and replication phase respectively. We have shown earlier that the interaction of the unphosphorylated form of the P protein with the leader RNA of CHPV results in the formation of two complexes [10]. While at lower concentration the protein forms a complex with the RNA that consists of a monomeric P protein and leader RNA, at higher concentration the complex incorporates two P protein molecules. Formation of the second complex has been proposed to be the determining factor for the transcription to replication switch over in the viral life cycle.

Although it is clear that the Phosphoprotein plays a major role in the life cycle of the Chandipura virus, no structural information of this crucial molecule is yet available. A recent crystal structure of the dimerization domain of the unphosphorylated form of the related, Vesicular Stomatitis Virus Indiana serotype (VSVind) Phosphoprotein has been solved [12] and it implicated the amino acid residues 107–177 in the dimerization surface in the truncated protein. According to this structure, this central domain consists of an alpha helix surrounded by two beta-hairpins that form four stranded beta-sheet structures by domain swapping and provides stabilizing force for the assembly. However, Chen et al. has mapped the self-association domain of the P protein of VSVind to amino acids 161–210 within the hinge region [13] using both *in vitro* and *in vivo* experiments. VSV and CHPV Phosphoproteins share only 34% similarity at the amino acid level but substantial similarities have been demonstrated by various structure–function studies. Thus structural information available from the VSV domain acts as the closest reference for the structure of the CHPV Phosphoprotein.

When the sequences of the different Phosphoproteins from CHPV and VSV (both Indiana and New Jersey serotype) are aligned together, a number of conserved residues become apparent. Among these residues, the Trp residues are very much intriguing as they can be used as a tool for the investigation of different structural properties of the molecule. The conserved nature of the Trp residues indicates the importance of these residues in different related viral Phosphoproteins. In case of CHPV, the two Trp residues are at amino acid positions 105 and 135 that may belong to the putative dimerization domain or might be in close proximity.

The Trp residues in proteins are often characterized by steady-state and time-resolved fluorescence of the Trp residue that acts as an intrinsic probe, the fluorescence features being sensitive to the environment of the Trp residues. The low-temperature phosphorescence (LTP) (77 K) spectra of the Trp residues in proteins in a suitable cryosolvent always give structured spectra with definite (0,0) band characteristics of the Trp environment [14–19]. Several cases have also been reported where multi-tryptophan proteins give rise to more than one (0,0) band corresponding to different Trp residues indicating inefficient photoinduced energy transfer between the Trp residues. The narrowness of the LTP bands, in contrast with the poorly resolved fluorescence of the tryptophan, is attributed to the smaller excited-state dipole moment in the case of the triplet [20–26].

Low-temperature triplet state spectroscopy studies of biopolymers or biopolymer–substrate complexes in a proper cryosolvent often provide spectra that correspond to the structures obtained by X-ray diffraction and NMR spectroscopy [27–33]. It may be mentioned that a protein or protein–substrate complex in a proper cryosolvent upon cooling will seek lower-energy conformations on the potential energy landscape. Some of these will represent conformations of the systems at ambient temperatures, but the higher-energy representatives will not contribute at low temperature. This picture is also true in X-ray crystallographic structures where crystal packing forces restrict the number of conformations available in the natural environment [34].

In this work, we characterized the two Trp residues at 105 and 135 of the wild-type (WT) CHPV P protein by steady state, time-resolved fluorescence and low-temperature phosphorescence studies. The analysis was aided by similar studies on two single Trp mutants W105F (Trp105 replaced by Phe), W135F (Trp135 replaced by Phe) and a double Trp mutant W135F/W105F (where both the Trps are replaced by Phe residue).

The main objectives of this study were to find out the,

(i) photophysical features of the Trp residues in the wild-type P protein and in the mutants leading to detail knowledge of the environments of the Trp residues.
(ii) whether the site directed mutagenesis at one point (substitution of Trp by Phe) affects the environment of the other Trp residue.
(iii) contribution of each Trp residue to the total fluorescence of the wild-type protein.
(iv) possibility of the non-radiative photoinduced intramolecular energy transfer among the Trp residues either through the singlet state or the triplet state in the wild-type P protein leading to an estimate of Trp105-Trp135 distance in the protein.

Homology modelling of the structure of CHPV P protein (which has 34% homology with VSV P protein) has been carried out. Calculation of ASA and the knowledge of the nature of the immediate residues (within 5 Å) of the two Trp residues in VSV P protein (from the crystal structure data) and similar calculation for the Trp105 and the Trp135 in CHPV P protein (from the structure generated by homology modelling) have been utilized to interpret our results. Using the distance between the two Trp residues and the orientation with respect to each other in the CHPV P protein the ET efficiency [19,35] has been calculated and compared with that obtained from the experiments.

Since the crystal structure of the P protein of CHPV is not known, a detailed study of the environment and the relative orientation of the Trp residues will be valuable to understand the overall structure and its interaction with other viral component such as nucleocapsid protein and the leader RNA.

2. Materials and methods

2.1. Construction of the tryptophan mutants

The two Tryptophan residues in the wild-type Phosphoprotein of the Chandipura virus were mutated into Phenylalanine and site directed mutagenesis using overlapping primers were carried out for this purpose. Wild-type Phosphoprotein gene cloned in the pET3a vector (pET3a-Pc) [7] was used as the template for the mutagenesis. For the introduction of the Tryptophan to Phenylalanine mutation at the amino acid position 105, overlapping primer pair P1: 5′ GCCAAACAGA CTTTCAAACCGGTGATAG 3′ and P2: 5′ CTATCACCGGTTTGAAA GTCTGTTTGGC 3′ were used. Polymerase chain reactions (PCR) were carried out following the method as described by Ho et al. [36]. Primer CHP1: 5′ TTTATACATATGGAAGACTCG CATCTGTATCAAGCTCTCA 3′ and CHP2: 5′ TTTATATCTAGAG GATCCTCAATTGAA CTGGGGGCTCAAG 3′ containing NdeI and BamHI sites respectively were used as the forward and the reverse primers of the Phosphoprotein gene. After the final PCR reaction, product was gel excised, digested with NdeI and BamHI and ligated