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Wolbachia translation initiation factor-1 is copiously expressed by the adult, microfilariae and infective larvae of *Brugia malayi* and competitively inhibited by tetracycline

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ABSTRACT

The intracellular alphaproteobacteria, *Wolbachia*, is considered to be a future antimacrofilarial drug target as it is obligatory for filarial endurance. Characterizing wolbachial proteins is necessary to understand wolbachial mechanisms and also for discovering new drug entities. Translation initiation factor-1 (Tl IF-1) is an indispensable prokaryotic factor concerned with bacterial viability. This factor is prioritized as one of the most potent antibacterial drug target. To investigate its role in filarial biology, recombinant Wol Tl IF-1 was purified on metal ion column. The factor was found folded in its monomeric native conformation, and contained a buried fluorophore. Molecular modeling revealed that the factor belonged to the Oligomer Binding family, and consisted of the highly conserved S1 domain with 81.6% of the amino acids occupying the allowed regions in Ramachandran plot. In addition, Wol Tl IF-1 exhibited selective binding to the 30S ribosomal subunit, which declined progressively with tetracycline addition. Tetracycline perturbs interaction of Thr18 and Asn32 of the factor with ribosomal protein S4. The factor was immune-localized in adult, microfilariae (Mf) and infective larvae (L3) of *Brugia malayi* by immunoblotting. High expression was also observed in *Wolbachia* within *B. malayi* Mf, L3 and female adult parasite along the gravid uteri by the confocal microscopy. Therefore, Wol Tl IF-1 appears to be an essential *Wolbachia* factor whose inhibition leads to extensive cell apoptosis and premature killing of adult worms, validating the antifilarial potential of the factor.

1. Introduction

Lymphatic filariasis (LF) is a vector borne, neglected tropical disease of public health significance and can have severe socio-economic consequences in developing nations. The species responsible for LF include *Wuchereria bancrofti*, which are prevalent in the tropics, *Brugia malayi* that are confined to South-East Asia and *Brugia timori* that are confined to Timor and the Flores areas of Indonesia. Currently, more than 1.3 billion people in 81 countries are at risk, over 120 million people are infected, and about 40 million disfigured and incapacitated (WHO, 2010). India alone accounts for 40% of the global disease burden (Michael et al., 1996). The current antifilarial treatments comprise of diethylcarbamazine (DEC) and ivermectin in conjunction with albendazole. This treatment interrupts the transmission cycle of filarial parasite by killing predominantly the microfilariae (Mf). However, the limited macrofilaricidal efficacy allows adult worms to survive in human hosts for decades. Current treatments need to be administered annually for several years that may instigate drug resistance, thus immense impediments lie to cure this fatal disease (Schwab et al., 2007; Osei-Atweneboana et al., 2007). A better understanding of molecular processes underlying parasite development and survival can facilitate identification of novel targets, particularly pertaining to adult parasites. Human filarial parasites harbor intracellularly the obligate alphaproteobacterial endosymbiont, *Wolbachia*. Experiments that show the vital role of these microbes in filarial survival, development and embryogenesis make *Wolbachia* an attractive antifilarial drug target (Taylor and Hoerauf, 2001; Hoerauf et al.,

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2003). Furthermore, *Wolbachia* is a major contributor to the inflammatory host pathology, as its depletion causes reduction in the level of vascular endothelial growth factors (VEGFs) essential for lymphangiogenesis (Hoerauf, 2008; Supali et al., 2008; Turner et al., 2006).

Thus, characterization of new wolbachial proteins is a prerequisite to unravel the host-parasite associations and identify novel targets required for prevention of the disease. Translation initiation factor-1 (Tl IF-1), encoded by *infA* gene, is an indispensable protein for translation in prokaryotes. It is involved in bacterial viability and growth (Cummings and Hershey, 1994). It prevents the dissociation of Tl IF-2 from 30S ribosomal subunit and increases Tl IF-2 dependent binding of fmet-tRNA to 30S ribosomal subunit by several folds. This factor exists solely in prokaryotes and not in eukaryotes, which makes it a lucrative drug target. Due to its role in protein biosynthesis, it been exploited well in several other organisms, including *Mycobacterium tuberculosis*, *Escherichia coli*, *Sulfolobus solfataricus* and *Methanococcus jannaschii* (Hatzopoulos and Dieckmann, 2007; Sette et al., 1997; Hasenöhrl et al., 2006; Li and Hoffman, 2001; Croitoru et al., 2006; Phadtare and Severinov, 2009; Sommerville, 1999). In the current manuscript, the *infA* gene of *Wolbachia* encoding for Wol Tl IF-1 is over expressed and purified by affinity chromatography to investigate its native folded conformation. The binding ability of Tl IF-1 with the A-site of 30S ribosomal subunit was also investigated with reference to its inhibition by antirickettsial antibiotic tetracycline. To ascertain *in vivo* distribution in adult female parasites, infective larvae (L3) and Mf of *B. malayi*, the Tl IF-1 was immune-localized using antibody raised against the recombinant factor by confocal microscopy. The current manuscript explores the complete competitive inhibition mechanism of tetracycline from an obligate mutualist alphaproteobacterium, *Wolbachia* of filarial parasite, *B. malayi.*

2. Materials and methods

2.1. Ethics statement

The animals used in the study were maintained at the Laboratory Animal Division of CSIR-Central Drug Research Institute (CDRI), Lucknow, under pathogen-free conditions. All the animals, experimental procedures of animal use were approved by the Animal Ethics Committee of CDRI duly constituted under the provisions of CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals), Government of India. The study bears the approval number IAEC/2011/145 dated 30.11.2011.

2.2. Parasite

The subperiodic strain of *B. malayi* was maintained cyclically in the experimental rodent host, *Mastomys coucha* (GRA 'Giessen' strain) through laboratory bred mosquito vector, *Aedes aegypti*. L3 of *B. malayi* were recovered from the mosquitoes fed on donor Mastomys 9 ± 1 day earlier. L3 were isolated from mosquitoes crushed gently by Baermann technique, washed and counted in Ringer's solution. Adult parasites and Mf were obtained by lavaging the peritoneal cavity of jirds, which were inoculated 3–6 months earlier with *B. malayi* L3 as described previously (McCall et al., 1973).

2.3. Over-expression, purification of recombinant Wol Tl IF-1

Over-expression and purification of recombinant Wol Tl IF-1 was done as described earlier (Nag et al., 2013). In brief, the Wol infA-pET28a(+) construct was transformed into the *E. coli* strain, Rosetta (Novagen, USA) that illustrated optimal protein expression. Protein synthesis was induced with 0.5 mM IPTG (isopropyl β-D-thiogalactoside) (Sigma, USA) for 6 h at 25 °C with constant shaking at 220 rpm in culture in logarithmic phase (OD600 = 0.5–0.6). The cells were harvested by centrifugation at $1844 \times g$ for 10 min and the pellet was suspended in 50 mM NaH_2PO_4 buffer (pH 8.0) containing 250 mM NaCl, 1 μM PMSF and 10 mM imidazole. The cell suspension was sonicated (Soniprep 150, Labexchange, Germany) with 10 s pulses and 30 s pauses at 8 μm amplitude for 25 min period of time, centrifuged at $11{,}529 \times g$ for 30 min at 4 °C and the supernatant was loaded on nickel nitrilotriacetic acid (Ni-NTA) agarose affinity column pre-equilibrated with 50 mM NaH_2PO_4 buffer (pH 8.0) along with 250 mM NaCl and 10 mM imidazole. The column was washed with the same buffer containing 25 mM imidazole followed by 50 mM imidazole and the recombinant protein was eluted with 250 mM imidazole. The purity of eluted recombinant protein was analyzed using 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein content was determined by the Bradford method.

The purified recombinant Wol Tl IF-1 was localized with monoclonal anti-His antibody in Western blot. The eluted fraction of the recombinant was electrophoretically transferred to nitrocellulose (NC) membrane in a dry blot apparatus as per manufacturer's instructions (Invitrogen, USA). The membrane was blocked in 3% nonfat milk for 2 h and incubated at 37 °C with 1/5000 dilution of mouse anti-His antibody (Novagen, USA), re-incubated with goat anti-mouse IgG-HRP (horseradish peroxidase) (1/10,000; Sigma, USA) for 2 h at 37 °C. The blot was developed with the substrate, 3,3′-diaminobenzidine tetra hydrochloride (DAB) in the presence of 20 μl of H_2O_2 (Sigma, USA).

2.4. Fluorescence spectroscopy

The fluorescence spectra of Wol Tl IF-1 were recorded on a fluorescence spectrometer (Perkin Elmer LS55, USA) at an excitation wavelength of 275 nm and an emission wavelength in the range of 280–380 nm. The slit width for both excitation and emission were set at 5 nm. All measurements were made in a quartz cell of 5 mm path length at 25 °C and the fluorescence intensity obtained was normalized by subtracting the baseline recorded for buffer devoid of protein under similar conditions.

2.5. Size exclusion chromatography (SEC)

Approximately 200 μl (10 mg/ml) of purified recombinant protein (Wol Tl IF-1) was loaded on a Sephadex-75 GL column pre-equilibrated with buffer (50 mM NaH_2PO_4, 50 mM NaCl, 0.1% NaN_3 at pH 6.5) using a manual injector. Chromatography was performed using an AKTA purifier system (GE Healthcare, USA) at a flow rate of 0.5 ml/min at 25 °C and the absorbance was monitored at 280 nm. The column was calibrated with standard molecular weight markers.

2.6. Wol Tl IF-1 structural model evaluation

The 3D model of Wol Tl IF-1 was predicted by homology modeling using Bioinformatics Tool MODELLER9v11 on windows-based operating system (Sali and Blundell, 1993). The crystal structure of *E. coli* Tl IF-1 (PDB code:1HR0), retrieved from the Protein Data Bank (http://www.rcsb.org/pdb) (Carter et al., 2001), was used as template. *In silico* model of Wol Tl IF-1 was validated further by ERRAT, ProQ, ProSA and PROCHECK. To determine amino acid sequences of Wol Tl IF-1 in allowed and disallowed regions, Ramachandran plot was generated using PROCHECK program (Laskowski, 1993). The ProSA web server was employed to evaluate energy profiles and verify the structure in term of *Z* scores, which represent the overall quality and measures the deviation of total energy (Wiederstein and Sippl, 2007). The domain present in Wol Tl IF-1 was predicted by Bioinformatics Tool SMART