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Production and biophysical characterization of a mini-membrane protein, Ost4V23D: A functionally important mutant of yeast oligosaccharyltransferase subunit Ost4p

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ABSTRACT

N-linked glycosylation of proteins is an essential and highly conserved co- and post-translational protein modification reaction that occurs in all eukaryotes. Oligosaccharyltransferase (OST), a multi-subunit membrane-associated enzyme complex, carries out this reaction. In the central reaction, a carbohydrate group is transferred to the side chain of a consensus asparagine residue in the newly synthesized protein. Genetic defects in humans cause a series of disorders known as congenital disorders of glycosylation (CDG) that include mental retardation, developmental delay, hypoglycemia etc. Complete loss of N-glycosylation is lethal in all organisms. In *Saccharomyces cerevisiae*, OST consists of nine non-identical protein subunits. Ost4p is the smallest subunit containing 36 residues. It bridges catalytic subunit Stt3p to Ost3p/Ost6p subunit. Mutation of Valine (V) at position 23 in Ost4p to Aspartate (D) causes defects in the N-glycosylation process. To understand the structure, function and role of Ost4p in N-glycosylation, characterization of Ost4p and its functionally important mutant/s are critical. We report the mutagenesis, heterologous overexpression, purification, reconstitution in DPC micelles and biophysical characterization of Ost4V23D and compare its secondary structure and conformation to that of Ost4p. CD and NMR data suggest that mutation of Val^{23} to Asp impacts the secondary structure and conformation of Ost4p.

1. Introduction

Asparagine-linked glycosylation, also known as N-linked glycosylation, is a co- and post-translational protein modification reaction, catalyzed by a membrane-associated multi-subunit enzyme complex, oligosaccharyltransferase (OST). The central step in this reaction involves the transfer of a high mannose oligosaccharide moiety from a donor substrate to the side chain of an asparagine residue present in the -Asn-X-Thr/Ser- (X≠ Pro) recognition motif in a newly synthesized polypeptide chain [1]. N-Glycosylation of nascent proteins is one of the most abundant protein co- and post-translational modification reactions, which occurs in all eukaryotic cells [2] and some of the prokaryotic cells [3]. Apart from enhancing protein folding and strengthening the thermodynamic stability of proteins [4], N-glycan mediate the interaction of polypeptides with the components of endoplasmic reticulum (ER) associated quality control and degradation system [5]. Thus, N- glycosylation is an essential process for protein folding, stability, intercellular recognition and immune response [5,6]. Genetic defects in the N-linked glycosylation pathway in humans cause a series of diseases, called congenital disorders of glycosylation (CDG), which include but are not limited to mental retardation, developmental delay, hypoglycemia, liver dysfunction, etc. [7–9].

Oligosachharyltransferase (OST), localized next to the ribosome in the endoplasmic reticulum (ER), catalyzes the N-glycosylation reaction [10]. In yeast, OST is composed of nine non-identical integral membrane protein (IMPs) subunits: Ost1p, Ost2p, Ost3p, Ost4p, Ost5p, Ost6p, Swp1p, Wbp1p and Stt3p [10]. Biochemical and genetic studies have shown these IMPs to be well organized into three sub-complexes. Ost3p and Ost6p subunits are homologues with 46% sequence similarity and 21% sequence identity. Thus, only one of the two subunits are present in the enzyme at any given time, resulting in two functionally distinct OST complexes [11]. Ost4p is reported to play a critical role in the incorporation of Ost3p or Ost6p in the OST complex. Ost4p is localized between Stt3p and Ost3p in the Stt3p-Ost4p-Ost3p sub-complex, acting as a bridge stabilizing this complex [12]. Ost4p is a mini-membrane protein having only one transmembrane domain containing 36

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amino acid residues [12]. Sequence alignment have shown that most yeast OST subunits have high sequence similarity with OST subunits identified in higher eukaryotes, while Ost4p has the highest sequence similarity (Fig. 1) in *C. elegans*, *M. musculus*, and *H. sapiens* [13,14]. Mutagenesis studies have shown that substitution of any residue in positions 18–24 in Ost4p with a charged residue results in temperature sensitivity, impaired cell growth and disrupts *in vitro* OST activity [12,15].

Despite the advancements in the structural biology field, structural and functional characterizations of integral membrane proteins remain challenging. The key limiting factors for structure determination at atomic resolution are the production of pure, homogeneous membrane proteins and their reconstitution in suitable membrane mimetic. This remains the major obstacle and is the primary reason why only 0.5% of the PDB structures belong to unique membrane proteins despite the fact that 30% of the genome is composed of membrane proteins and over 60% of current drug targets are membrane proteins [16,17].

The three-dimensional structure of chemically synthesized Ost4p was determined in our laboratory in mixed aqueous-organic solvents [14]. Ost4p folded into a well-formed kinked helix in this system. This structure explained the results of mutational studies. Mutation of any residue present in positions 18–24 in helix-2 to a charged residue in Ost4p resulted in severe growth defects in yeast affecting OST activity [15]. These mutations were reported to cause destabilization of the Stt3p-Ost4p-Ost3p sub-complex [12, 15]. In contrast, mutation of any residue from 2 to 17 had no effect on OST activity and stability of the complex [15]. In the Stt3p-Ost4p-Ost3p sub-complex, Ost4p was reported to interact with Ost3p through residues M19, T20, I22 and V23, and with Stt3p through residues M18, L21 and I24 [14,15]. Thus, mutation of any of these hydrophobic residues to charged residue disrupted the interactions of Ost4p to either Ost3p or Stt3p in the complex [12,15]. Mutation of Met^{18} to Lys or Val^{23} to Asp resulted in a severe cell growth defect but mutation of Met^{18} to Leu or Val^{23} to Gly did not affect cell growth suggesting the importance of hydrophobic residues in these positions for maintaining the stability of the OST complex [15]. Based on the analysis of the NMR structure of yeast Ost4p, it was suggested that the α2 helix of Ost4p interacts with Stt3p on one side and Ost3p on the other side via i+4 "ridges-into grooves" helix packing mechanism [15]. The NMR structure of human Ost4 determined in mixed aqueous-organic solvent shows a similar kinked helix as in yeast Ost4p [14,18]. Thus, point mutation of any residue in α2 helix might disrupt the ridges-into-grooves fit resulting in the disruption of the Stt3p-Ost4p-Ost3p sub-complex.

Although the high-resolution NMR structures of chemically synthesized Ost4p from yeast and human are available, the effect of functionally important mutations on the 3-D structure of this protein remains unanswered. For example, what would happen to the "ridges into groove" interaction between Ost4p and Stt3p/Ost3p upon mutation of any residue in α2 helix? What is the impact of point mutation on the overall 3-D structure of Ost4p? The structure-function studies of Ost4p and its functionally important mutants would provide insight into the protein-protein interactions involved in stabilization of the Stt3p-Ost4p-Ost3p subcomplex and consequently the OST complex.

In eukaryotes, the NMR structures of yeast Ost4p [14], human Ost4p [18], crystal structure for soluble N- terminal domain of Ost3p/Ost6p [19], and solution NMR structure of membrane-anchored C-terminal catalytic domain of yeast Stt3p [20] have been determined. Low-resolution structures of the mammalian and yeast oligosaccharyltransferase complex have been determined by cryo-electron microscopy [21,22]. The X-ray structure for soluble N-terminal domain (also known as the thioredoxin domain) of N33/Tusc3 subunit of human OST has been reported [23]. However, except for the chemically synthesized Ost4p, atomic resolution structures of the full-length proteins for rest of the eight yeast subunits (Ost1p, Ost2p, Ost3p, Ost5p, Ost6p, Stt3p, Wbp and Swp) or their counterparts in other higher eukaryotes have not been determined. Despite the fact that subunits of yeast OST were identified, cloned and sequenced over 3 decades ago, there is a gap in our knowledge and understanding of the individual role and most importantly function of each subunit of OST in N-glycosylation protein modification reaction. The major obstacle in membrane protein research is the heterologous expression and production of pure, homogenous IMPs and their reconstitution in suitable membrane mimetic. As a result, biochemical, biophysical and high-resolution structural characterizations of IMPs remain a challenging task.

The heterologous expression and purification of recombinant Ost4p has already been reported from our laboratory [24]. Here, we report overexpression, purification, reconstitution and biophysical characterization of a functionally important mutant protein, Ost4V23D. Comparison of the secondary structure and conformation of Ost4V23D with Ost4p suggest that the mutation affects both secondary and tertiary structure of the wild type protein.

2. Results

2.1. Mutagenesis, overexpression and purification of GB1-Ost4V23D

The pGEV2-Ost4V23D mutant plasmid was constructed from the pGEV2-Ost4 vector by site-directed mutagenesis. The incorporation of mutation was confirmed by DNA sequencing (Fig. 2). Native as well as mutant plasmids were transformed into *E. coli* BL21 (DE3) pLysS cells. Protein expression was optimized using temperatures and IPTG concentrations. The GB1-Ost4V23D mutant

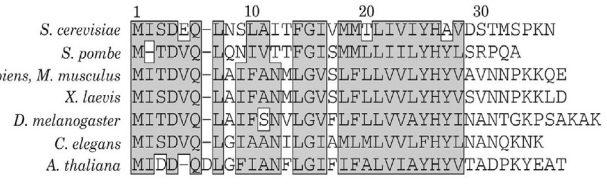

Fig. 1. Sequence alignment of Ost4p from the *S. cerevisiae* (Yeast) and analogs of Ost4p from other species: *Schizosaccharomyces pombe* (fission yeast), *Homo sapiens* (human), *Mus musculus* (house mouse), *Xenopus laevis* (clawed frog), *Drosophila melanogaster* (fruit fly), *C. elegans* (nematode), and *Arabidopsis thaliana* (thale cress) [14]. The residues highlighted in grey are similar across different species.

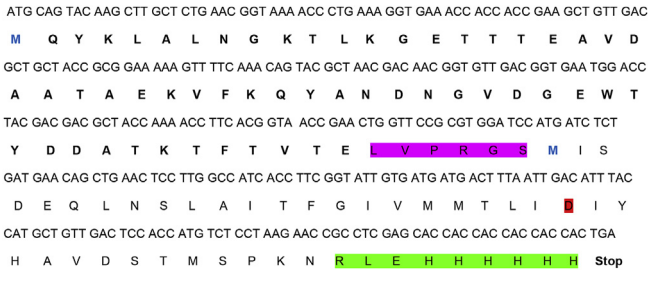

Fig. 2. Nucleotide (top) and corresponding amino acid (bottom) sequences of GB1-Ost4V23D. Letters in **bold** represent GB1 amino acid sequence. Amino acids in **blue** are the first amino acids in the GB1 or Ost4p sequence. Amino acids in purple background represent thrombin cleavage site. Amino acids in green background are those which are not present originally in Ost4 sequence including the hexa-histidine tag. The Asp23 residue shown in red background represents mutation site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)