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Unusual Stability of Anabaena Sensory Rhodopsin Transducer from *Anabaena PCC7120*

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----------ABSTRACT----------
Advances in biotechnology generated wide range of microbial genome and their related protein database. Freshwater cyanobacterium Anabaena PCC7120 sensory rhodopsin, ASR in contrast to classical haloarchaeal sensory rhodopsins interacts with putative soluble transducer, ASRT. The 125 amino acid transducer exists as a soluble protein and is involved in photoreceptor binding. Recombinant DNA tools in biotechnology conventionally support the use of affinity tags for ease of protein purification and subsequent studies. The ASRT exists as a stable tetramer. Both X-ray crystal structure and solution NMR results with ASRT utilizing hexa-histidine affinity tag reveal it as a primarily β-stranded protein We have observed that the affinity tagged ASRT exhibits altered oligomeric stability. In this communication we outlined the effect of commonly used denaturant, Sodium Dodecyl Sulfate (SDS) on the tetrameric packing of ASRT. Our results support that N-terminus hexa-histidine tagged ASRT displayed unusual SDS-resistant structure. The unusual stability of ASRT and its homologues present in other microbial population could provide further insight towards their role in receptor, other ligand binding and signaling.

Keywords*: Cyanobacteria, Domain of Unknown Function [DUF], Hexa-histidine tag, Oligomeric assembly, SDS-resistant transducer*

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I. INTRODUCTION

Genome mining has broadened our understanding of the abundance and diversity of microbial rhodopsin family members and their mode of signaling [1], [2]. Anabaena sensory rhodopsin [ASR] is the first photosensory rhodopsin discovered in eubacterial domain, in the genome of freshwater cyanobacterium, *Anabaena sp. PCC 7120*. Unlike in its microbial homologs, the cytoplasmic half of ASR is unusually hydrophilic and contains several water molecules [3]. Interestingly, a 125 amino acid soluble protein, Anabaena Sensory Rhodopsin Transducer [ASRT] co-expresses with ASR in a single operon [2] and acts as chaperone for expression of membrane receptor, ASR. Our biochemical and biophysical studies showed that ASRT tetramer binds to ASR and affect its photochemistry [2], [4]-[6]. Besides interaction and dissociation from photoreceptor, ASR-ASRT complex [7], ASRT also display novel carbohydrate and DNA binding features [8], [9]. Besides its novel ligand binding, the stability of its quaternary structure as tetramer is not well explored.

Besides the atomic resolution structure of ASRT [5], [9], the homologues of ASRT in other microbial population are termed as "DUF" (Domain of Unknown Function) with least available functional information. ASRT-like proteins are not unique to cyanobacteria. Another member with known crystal structure is in DUF1362 protein belonging to *Thermotoga maritima* (TM 1070 PDB code 1NC7). The structural insight of TM11070 is very much similar to ASRT, except minor differences. The amino acid sequence of these two proteins is depicted in Figure 1.

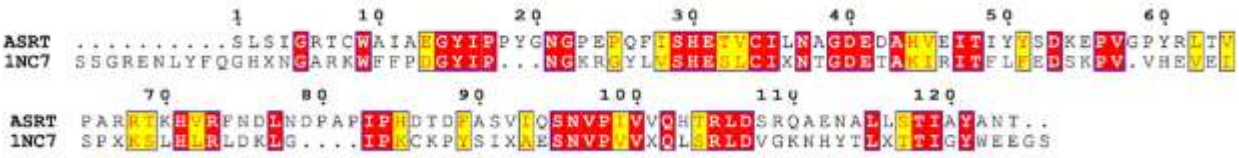

Figure1: Primary sequence comparison of ASRT and TM1070. The red boxed amino acids are conserved among these two organisms. Yellow boxed amino acids share similar features, if not identical.

In this report, we attempted to compare the structural difference to correlate with unusual high stability of ASRT in solution. The oligomeric stability was compared using conventionally hexa-histidine tagged ASRT either at N, amino or at C, carboxyl terminal. We propose that presence of affinity tag at amino terminus allows ASRT to fold into stable oligomer and the putative helical face [a strongly helical motif between amino acid 108-120 at the end of sequence 20% coding region, 100-125], located at the end of coding region of ASRT may permit a larger assembly, may be critical in signaling.

II. MATERIALS AND METHODS

The structural comparison was performed by using available PDB codes of TM1070 of *Thermotoga maritima* (PDB code: 1NC7; chain A, unpublished report) as a template. ASRT tetramer models were made by replacing two monomers in the crystal structure of ASRT (PDB code: 2II7 [5]) with the modeled monomers in either opposing or adjacent positions.

As indicated by Wang et al. [9], the structural model of monomer state of ASRT was calculated using 3D-JIGSAW [10] of TM1070 of *Thermotoga maritima* (PDB code: 1NC7; chain A).

Bacterial strains and plasmids:

The *Escherichia coli* transformants were grown in LB (Luria–Bertani) medium in the presence of ampicillin (50 mg/ml) at 35^{o}C. The ASRT construct were expressed under the plac1 promoter of pMS107 as indicated earlier [2] *E. coli* strains BL21 (Stratagene) or UT5600. All bacterial strain and ASRT plasmid with N-terminus and C-terminus hexa-histidine affinity tag were generously provided by Spudich lab [Center for Membrane Biology, University of Texas Medical School Houston]. All reagents were of high purity obtained from Sigma and/or Fischer Scientific.

ASRT expression and purification:

ASRT was overexpressed in *Escherichia coli strain BL21* harboring plasmid containing the ASRT coding sequence with N-terminus and/or C-terminus hexa-histidine affinity tag under IPTG-inducible promoter. The overnight inoculated cells were diluted to 1:100 in 600 ml of LB in a 1L flask with ampicillin (50 mg/ml) at 35^{o}C C on a gyratory shaker at 180 rpm. Cells were induced at mid log phase (an absorbance A_{600} = 0.4) by adding 1 mM IPTG. After a period of 4-5 h, the cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl, pH 7.8, containing 250 mM NaCl, 10% glycerol and 0.5mM protease inhibitor phenylmethylsulphonyl fluoride (PMSF) (binding buffer).

The *Escherichia coli* cells were lysed by sonication at 4^{o}C, followed by centrifugation [4000 rpm for 20 minutes then 18000 rpm for 30 minutes] to remove cell debris and other fractions. The ASRT containing hexa-histidine affinity tags were purified separately using Ni^{2+}-NTA agarose beads (Qiagen). The soluble fraction in binding buffer with additional 10 mM imidazole to avoid non-specific binding was used at 4^{o}C. The ASRT in binding buffer was mixed with Ni^{2+}-NTA agarose beads for 4 hours in cold. Finally the ASRT was purified by using Bio-Rad column. The unbound fraction was flown out in flow-through. The column was washed with binding buffer with subsequent wash using increasing 20 and 40 mM imidazole to remove other non-specific bound fraction prior to the elution step. The elution buffer containing 250 mM imidazole in binding buffer was used for remove affinity bound ASRT.

ASRT estimation and characterization:

The purified ASRT samples were dialyzed extensively against 50 mM Tris, pH 7.8, with 100 mM NaCl buffer. ASRT concentrations were determined by using Peirce protein estimation kit or by measuring absorbance at 280 nm using an extinction coefficient of 13,610 M^{-1} cm^{-1} [5]. Purified ASRT were characterized by SDS-PAGE electrophoresis and size exclusion chromatography as indicated earlier [2], [5]

SDS induced unfolding by fluorescence spectroscopy:

The SDS- resistant form of N-terminal hexa-histidine tagged ASRT was studied by using intrinsic fluorescence of lone tryptophan [Trp^{9}] by using excitation wavelength of 279 nm in 50 mM Tris-HCl, pH 7.8, with 100 mM NaCl. The equilibrium unfolding measurements in absence and presence of increasing SDS concentration were performed on Shimadzu RF-6000 spectrofluorometer at ambient temperature using 2.5µM protein concentration.

III. RESULT AND DISCUSSION

Three crystal lattices were observed for ASRT packing [5]. The tetramer in the *P4* cell was formed by crystallographic symmetry. The *C2* ASRT crystal and TM1070 crystal contain one tetramer in the asymmetric unit. Interestingly, the large $P2_12_12_1$ asymmetric unit contains two such tetramer facing each other. ASRT and its only known DUF1362 member available structure are shown in Figure 2.

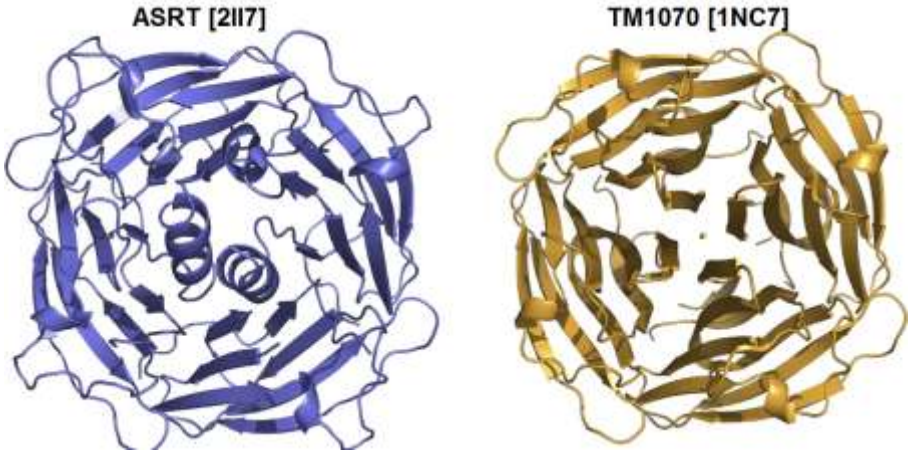

Figure 2: The ribbon representations of the $P2_12_12_1$ [LEFT] and TM 1070 [RIGHT]. The central alpha helical face of ASRT is not evident in *P4* and *C2* packing [5].

The key striking difference is the presence of divalent Mg^{2+} ion in the central space of TM1070 packing. The structure is predominantly β strand. The electron density for all three C-terminal α-helical face is not well resolved. The helix pointing away could be due to presence of hexa-histidine affinity tag impairing the packing of quaternary structure.

Based on the structural analysis, it is evident that ASRT tetramer may assemble further via helical face of C-terminus domain. It is supported by SDS-PAGE profile of purified ASRT with C-terminus tag and N-terminus hexa-histidine affinity tag [Figure 3].

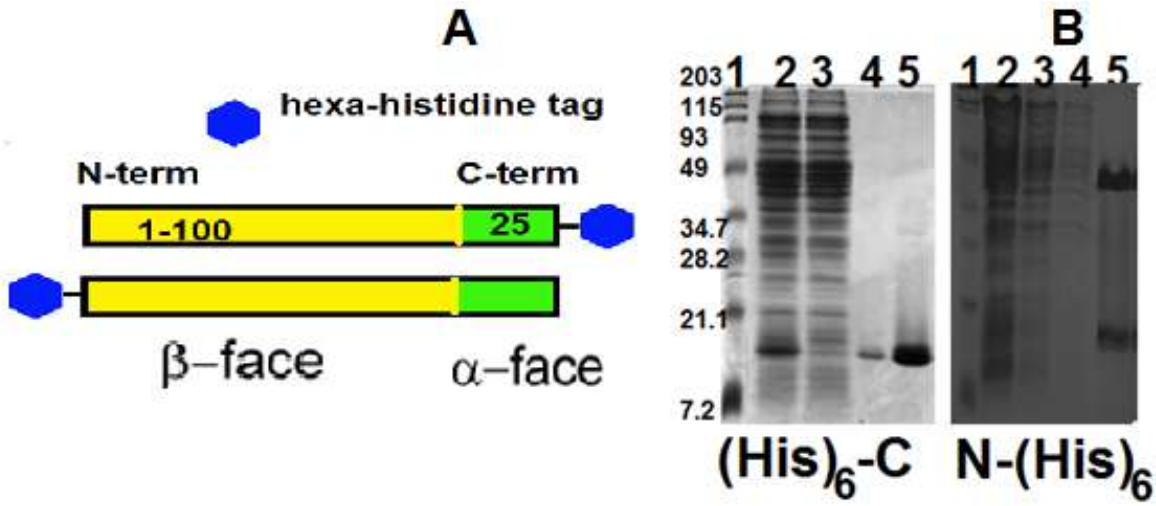

Figure 3: [**A**] The ASRT constructs with hexa-histidine, $(His)_6$ affinity tag on either terminus for ease of purification. The α-helical face of ASRT as shaded fluorescent green is critical for unusual stability. **[B]** Purification of ASRT [$(His)_6$-C and N-$(His)_6$] in soluble fraction used by Ni^{2+}-NTA Agarose chromatography. The standard molecular weight markers are shown in lane 1. Lane 2 represents cell lysate mixed with Ni^{2+}-NTA beads, while lane 3 indicates the flow through after binding. The wash fraction and elution with 40 mM and 250 mm imidazole are depicted in lane 4 and 5 respectively. All samples [without heating were incubated with SDS loading buffer, final concentration 0.5%] were loaded on 15% SDS-PAGE gel and stained by Bio-Rad protein stain.

The 125 amino acid ASRT with hexa-histidine sequence for affinity purification (monomeric molecular weight of ~14kDa) appeared as SDS-resistant oligomer, when the affinity tag is located at N-terminus of the protein. As

can be seen from figure 3 that presence of hexa-histidine,$(His)_6$ tag at C-terminus results in dissociation of stable oligomer in the presence of SDS during electrophoresis. The switch of hexa-histidine affinity tag at N-terminus increased the stability of oligomeric state even in the presence of SDS. It supports the hypothesis that helical face in presence of affinity tag could not establish key contact and impairs the stability of quaternary structure.
The size exclusion chromatographic profile of both ASRT form reflects similar hydrodynamic radii corresponding to tetrameric state, however the packing of N-terminal tagged ASRT show slightly smaller stokes radii compared to C-terminal tagged molecule [unpublished data, not included]. It may be related to compact packing of N-tagged ASRT.
The comparative profile of SDS mediated unfolding of N and C-tagged ASRT is depicted in Figure 4. Both ASRT preparations indicated the emission maxima of ~330 nm compared to ~340 nm for globular proteins, suggesting that lone Trp residue is masked within oligomeric assembly. The presence of hexa-histidine tag at N-terminus resulted in comparable emission maxima, an indicative of identical folding and packing of both tagged proteins in solution. Upon incubation with SDS, the fluorescence intensity maximum for both ASRT preparations shifts from 330 nm to 345 nm, indicative of increased exposure accompanying unfolding [11].

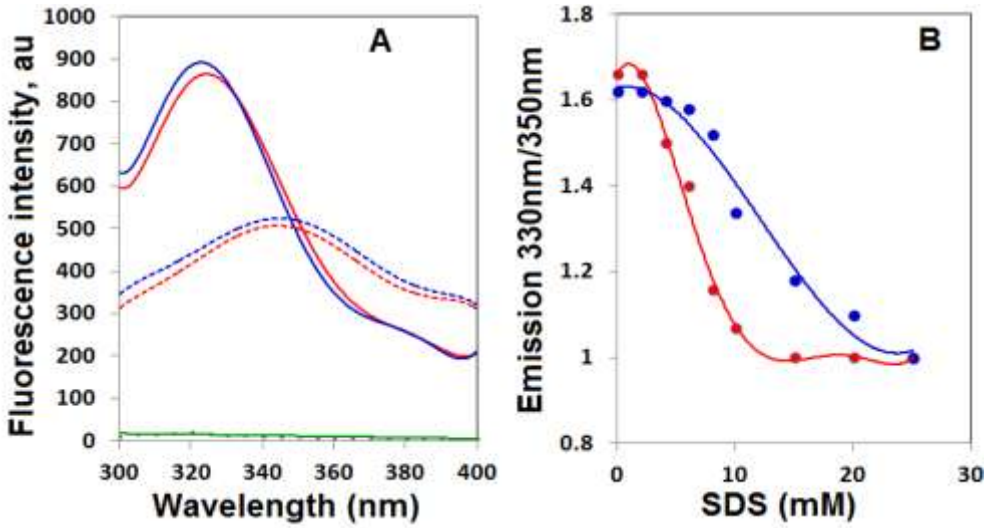

Figure 4: [A] Fluorescence emission spectra of N terminus hexa-histidine tag [blue] and C-terminus tagged ASRT [red] in absence [solid line] and presence of 25 mM SDS [dotted line] in 50 mM Tris-HCl buffer pH 7.8, with 100 mM NaCl [green baseline]. The excitation wavelength was 279 nm. **[B]** Equilibrium unfolding of N terminus hexa-histidine tag [blue circle] and C-terminus tagged ASRT [red circle] followed by fluorescence emission as a function of SDS concentration.

The equilibrium unfolding for C-terminus tagged ASRT supports SDS-PAGE trends, as it unfolds the stabilized oligomer closer to critical micelle concentration, cmc of SDS. Interestingly only a partial unfolding was observed for N-terminus tagged ASRT, highlighting its unusual higher stability even in range of cmc of detergent. The midpoint of equilibrium unfolding by SDS for shifted from ~7mM for C-tagged ASRT to over 12mM for N-terminus hexa-histidine tagged ASRT. Typically binding of monomeric detergents only leads to local changes in protein conformation, while global and cooperative unfolding occurs around the critical micelle concentration, cmc [12], [13]. It is certain that the presence of affinity tag towards α-face/helical face is responsible for proper native like folding and destabilization of oligomeric assembly of ASRT. It is likely that helical face of ASRT may further stabilize the quaternary structure of ASRT in native cynobacteria, PCC 7120. The putative model of helical face interaction is illustrated in figure 5, may be crucial for signaling pathway in native environment.

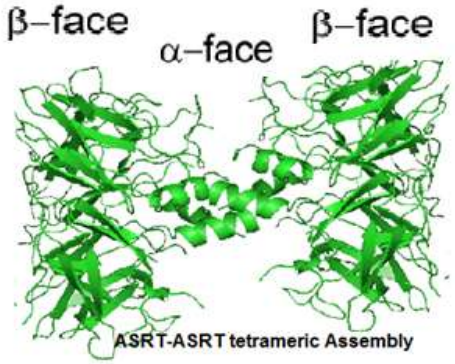

Figure 5: Putative native like oligomeric assembly of ASRT via interaction with helical face. The unresolved helical face in most of the crystal packing is likely due to affinity tag impaired native like folding of ASRT molecule.

It seems unlikely that C and N-terminal affinity tags would have exactly the same effect on protein folding without disturbing the rest of the tetramer. Considering the TM1070 folding pattern to the ASRT sequence the C-terminal affinity tag could impair the packing of putative helical face of ASRT. The secondary structure predictions with the bioinformatics programs [SSpro] suggest an α-helical C-terminal segment for ASRT, although it should be noted that SSpro predicts a C-terminal α-helix for TM1070 as well.

The evidence does not completely rule out the possibility that the N and C-terminal affinity tags are the cause of the partially unstable fold observed in ASRT crystal forms; but, considered together with the fact that the better ordered homolog TM1070 was expressed with an N-terminal hexa-histidine tag as well, it seems very unlikely. Interestingly the characterization of ASRT homologue and other DUF family members is not available and obscure. The present study outlined the significant role of conventionally used affinity tag as hindering force during folding and assembly of native like state of signaling protein and raises concern towards fusion and affinity tags [14].

IV. CONCLUSION

Since the dawn of time, or at least dawn of recombinant DNA technology (biotechnology), investigators have been cloning, expressing heterologous proteins in variety of cells for diverse purposes including structural studies. This report using ASRT suggests that subsequent folding of secondary structure into tertiary and quaternary is critical for functional state of a signaling molecule. Thus selection of affinity tag for ease of purification requires a much closer consideration and evaluation [15] for structure-function relationship of other DUF members of ASRT protein family.

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