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Human SLC26A4/Pendrin STAS domain is a nucleotide-binding protein: Refolding and characterization for structural studies

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ABSTRACT

Mutations in the human SLC26A4/Pendrin polypeptide (hPDS) cause Pendred Syndrome /DFNB4, syndromic deafness with enlargement of the vestibular aqueduct and low-penetrance goiter. Here we present data on cloning, protein overexpression and purification, refolding, and biophysical characterization of the recombinant hPDS STAS domain lacking its intrinsic variable sequence (STAS- Δ IVS). We report a reproducible protein refolding protocol enabling milligram scale expression and purification of uniformly ¹⁵N- and ¹³C/¹⁵N-enriched hPDS STAS- Δ IVS domain suitable for structural characterization by solution NMR. Circular dichroism, one-dimensional ¹H, two-dimensional ¹H-¹⁵N HSQC, and ¹H-¹³C HSQC NMR spectra confirmed the well-folded state of purified hPDS STAS- Δ IVS in solution. Heteronuclear NMR chemical shift perturbation of select STAS- Δ IVS residues by GDP was observed at fast-to-intermediate NMR time scales. Intrinsic tryptophan fluorescence quench experiments demonstrated GDP binding to hPDS STAS- Δ IVS with K_d of 178 μ M. These results are useful for structure/function characterization of hPDS STAS, the cytoplasmic subdomain of the congenital deafness protein, pendrin, as well as for studies of other mammalian STAS domains.

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1. Introduction

SLC26 family anion transport proteins are conserved throughout phylogeny from bacteria to humans [1,2]. In prokaryotes and plants this family of proteins is known as SulP (Sulfate Permease). SLC26 and SulP proteins consist of a short cytoplasmic N-terminus, a complex polytopic transmembrane domain of 14 trans-bilayer spans, and a cytoplasmic C-terminal region of 125–250 aa largely comprising a Sulfate Transporter and anti-Sigma factor antagonist (STAS) domain [3]. The 10 known protein-coding human SLC26 genes encode anion transporters, exchangers and channels expressed in epithelial cells and other cell types throughout the body. Mutations in at least four human SLC26 genes cause early-onset Mendelian diseases, including chondrodysplasia (SLC26A2/DTD), chloride-losing diarrhea (SLC26A3/DRA), deafness with enlargement

Abbreviations: hPDS, Human pendrin polypeptide; IVS, Intrinsic variable sequence; HSQC, Heteronuclear single quantum correlation spectroscopy; CD, Circular dichroism; MS, Mass spectroscopy

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of the vestibular aqueduct and low-penetrance goiter (SLC26A4/pendrin), and a rare form of impaired male fertility (SLC26A8). Many of these mutations involve the respective STAS domains. The human disease phenotypes are modeled in knockout mice with varying degrees of clinical fidelity, and genetic inactivation in mice of other *Slc26* genes unassociated as yet with human disease also causes pathogenic murine phenotypes. In addition, human SLC26A9 variants have been identified as strong risk modifiers of cystic fibrosis lung disease, whereas human pendrin (hPDS/SLC26A4) has been investigated as a risk modifier of systemic fluid balance, blood pressure, and asthma [2].

The STAS domains of SLC26/SulP transporters are homologous to bacterial anti-sigma factor antagonists such as SpoIIAA of *B. subtilis* [4,5]. Anti-sigma factor antagonists counter the activity of repressors (anti-sigma factors) of sigma (transcription) factors in the bacterial sporulation stress response pathway [6]. STAS domains are essential for plasmalemmal targeting, contribute to anion transport function and, like bacterial anti-sigma factors, serve as protein-protein interaction modules. Mutations in STAS domains of mammalian SLC26 transporters and bacterial SulP transporters impair function and/or surface expression of these transporters [3,7,8–10].

SpoIIAA-like STAS domain structures solved by X-ray crystallography include those from dicarboxylate and bicarbonate transporter [11,12], DauA(YchM) of *E. coli* [13] and from dicarboxylate transporter SLC26Dg [3], as well as a STAS- Δ IVS structure from the cochlear outer hair cell Cl⁻-dependent amplifier SLC26A5/prestin of rat [14]. NMR solution structures have been solved for rat prestin STAS- Δ IVS [14] and for the intact putative sulfate transporter Rv1739c of *M. tuberculosis* [15]. The presence of the unstructured "intervening sequence" (IVS) located between STAS helices α 1 and β 3 (nomenclature from [14]) distinguishes mammalian and metazoan STAS domains from those of bacterial anti-sigma factor antagonists and SulP transporters. No function has yet been reported for the IVS, and its deletion was required for protein overexpression and production of the first STAS domain crystals diffracting to high resolution [14]. All mammalian STAS domains reported subsequently have lacked the IVS, and along with bacterial STAS domains have yielded monomeric solution structures. Two-dimensional ¹H–¹⁵N HSQC spectra of wildtype and mutant STAS from human SLC26A3/DRA have been presented without NMR assignment [8].

In addition to their enabling contributions to SLC26-mediated anion transport, SulP STAS domains are also nucleotide binding proteins [12,15], as previously demonstrated for SpoIIAA and ASAs [4,5]. Mammalian STAS domains have been shown to interact with multiple proteins in pulldown assays, including CFTR [8], OASTL (for *A. thaliana* Sultr1) [16] and IQGAP-1 [11]. Towards an understanding of the structural basis and functional utility of STAS domain nucleotide binding, we have initiated a structural characterization of the hPDS/SLC26A4 STAS domain by heteronuclear solution NMR, both in the absence and presence of the IVS. Here we present data on the molecular cloning, purification, and preliminary structural characterization of recombinant hPDS STAS domain lacking the IVS region (STAS- Δ IVS). The reported purification strategy yields well-folded hPDS STAS- Δ IVS in quantities and of stability sufficient for structural characterization by solution NMR. We also report biophysical experiments that demonstrate nucleotide binding by hPDS STAS- Δ IVS.

2. Materials and methods

2.1. Molecular cloning of recombinant hPDS STAS domain (Δ IVS)

To delimit operational boundaries of the STAS domain of SLC26A4/hPDS (Uniprot # O43511), the aa sequence of hPDS STAS was aligned with STAS domains of other human SLC26 polypeptides and with other structurally characterized STAS domains [5,17]. The secondary structure of hPDS STAS was predicted using Pspred (<http://bioinf.cs.ucl.ac.uk/psipred/>). These analyses suggested a STAS domain encompassing aa residues 517–734, with aa 575–652 as the IVS region. Initial hPDS STAS- Δ IVS constructs encompassing aa 512–780 (hPDS_L STAS- Δ IVS) and aa 512–743 (hPDS_S STAS- Δ IVS) were cloned in-frame into pET52b(+) (EMD Chemicals) with a C-terminal His₁₀-tag. A third STAS- Δ IVS construct encompassing aa 517–738 (hPDS_{ST} STAS- Δ IVS) was cloned into pET28-a(+) in-frame with an N-terminal His₆-tag. Digested ligation products were transformed into *E. coli* BL21(DE3) cells (EMD chemicals). Plasmid sequence integrity was validated by DNA sequencing. Recombinant DNA was quantitated by Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA), and DNA size and purity analyzed by 2% agarose gel.

2.2. Expression and isolation of purified hPDS STAS

E. coli BL21(DE3) cells harboring STAS- Δ IVS plasmids in pET52-b(+) and pET28-a(+) were grown overnight in LB medium

containing kanamycin (30 μ g/L). A 20 mL overnight inoculum was added to 2 L LB medium. Cultures were maintained at 37 °C until reaching OD 0.6–0.8. Protein expression was then induced by IPTG (empirically optimized at 750 μ M) for 16 h at 13 °C. Induced cells were harvested by centrifugation (GSA rotor, Sorvall RC5B) at 6000 rpm for 15 min at 4 °C, and stored at –80 °C until further use. Harvested cells were freeze-thawed and suspended in buffer A (25 mM Tris-HCl, pH 8.5, 150 mM NaCl, 10 mM imidazole) supplemented with complete protease inhibitor cocktail tablet (Roche), 0.01% PMSF, and lysozyme (Novagen) (10 μ l/l cell culture), then agitated 30 min at 4 °C, and sonicated (Branson digital sonifier, S-450D) at 4 °C, 7 \times 7 s cycles at 35% amplitude with 1 min on ice between cycles.

2.3. Purification of insoluble STAS- Δ IVS polypeptide

Lysate was centrifuged 30 min at 17,000 rpm at 4 °C to separate soluble and insoluble (inclusion body-containing) fractions. SDS-PAGE of preparations from all three STAS- Δ IVS constructs revealed that each accumulated predominantly in inclusion bodies which were washed 3x in buffer A and solubilized in buffer A containing 6 M Guanidine HCl. The cleared, denatured lysate was loaded onto pre-equilibrated Ni-NTA columns (Qiagen). STAS- Δ IVS protein was purified as previously described for Rv1739c STAS [18], except for inclusion of 2 M urea in wash and elution buffers, and additional modifications described in Supplementary Information. Eluate fractions were evaluated by SDS-PAGE. STAS- Δ IVS-containing fractions were pooled and dialyzed in refolding buffer B (25 mM Tris-HCl, 50 mM NaCl, pH 7.9) at 4 °C with three buffer replacements at 12 h intervals. The dialyzed STAS- Δ IVS protein was subsequently purified by anion-exchange chromatography (Q-sepharose fast flow matrix, GE Healthcare, Marlborough, MA). Purified protein was eluted in buffer B with a salt gradient increasing to 1 M NaCl. Q-sepharose eluates were dialyzed in buffer C (25 mM Tris-HCl, 150 mM NaCl, pH 7.9).

Immunoblot analysis was performed with mouse monoclonal anti-(His)₆ antibody (Sigma-Aldrich, EMD Millipore, Billerica, MA) diluted 1:3000. HRP-conjugated secondary antibodies (Sigma-Aldrich) were diluted 1:1000. Proteins were separated on 16% SDS-PAGE. ECL signal was detected with reagents from Amersham Biosciences by Kodak X-omat 2000 A.

Protein purification was assessed by size exclusion chromatography using a Biologic Duo flow FPLC system (Bio-Rad) at 4 °C on a Superdex 75 column (Amersham Biosciences) of 125 mL bed volume at 1 mL/min flow-rate, pre-calibrated with molecular weight standards (Bio-Rad) and pre-equilibrated in buffer C.

For expression of ¹⁵N-enriched protein constructs, *E. coli* BL21 (DE3) cells harboring hPDS_{ST} STAS- Δ IVS plasmid in PET28-a(+) were grown in M9 minimal media containing ¹⁵NH₄Cl (1 g/l), ¹²C-glucose (2 g/l) (Cambridge Isotope Laboratories; CIL) supplemented with 10 mL/L Bioexpress cell growth media (U-¹⁵N, 98%; 10x concentrate, CIL) as sole carbon and nitrogen sources. ¹³C/¹⁵N-enriched STAS- Δ IVS proteins were produced using ¹⁵NH₄Cl (1 g/l), ¹³C-glucose (2 g/l), and U-¹⁵N, ¹³C (98%, 10x concentrate, CIL). Purification of ¹⁵N or ¹³C/¹⁵N-enriched hPDS_{ST} STAS- Δ IVS was conducted by methods identical to those used for unlabeled protein. The His₆-tag was removed by thrombin cleavage (GE Healthcare; 2U/mg, 16 h at 4 °C), then quenched by 1 mM PMSF. Tag-free hPDS_{ST} STAS- Δ IVS protein was recovered from repassing the cleaved reaction mixture through freshly prepared, buffer B-equilibrated Ni-NTA beads. For solution NMR experiments, samples additionally purified by anion exchange chromatography were dialyzed in Buffer D (40 mM potassium phosphate, 150 mM NaCl, pH 6.25). Purified hPDS_{ST} STAS- Δ IVS samples were stable at pH 6.2, allowing collection of high quality heteronuclear NMR data of good signal-to-noise ratio. Purified STAS- Δ IVS protein was

concentrated with Millipore stirred cell membranes or with Amicon 10 kDa NMWL membrane centrifugal devices (Millipore), then quantitated by Bradford assay [19].

2.4. MALDI-TOF MS

hPDS_{ST} STAS-ΔIVS protein identity and homogeneity were tested by MALDI-TOF MS by determination of the *m/z* ratio on an AB/MDS Sciex 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems, Carlsbad, CA). His₆-tagged protein was diluted 1:20 into a solution of standard MALDI matrix sinapinic acid (prepared from 5.0 mg/mL stock), spotted onto the 4800 OptiTOF metal target plate and dried. Data were collected as total ion current (TIC) from 500 laser shots.

2.5. CD spectroscopy

Protein folding and intrinsic secondary structural characteristics of recombinant hPDS_{ST} STAS-ΔIVS domain were assessed by Far-UV (260–190 nm) CD measurements at 25 °C on a Jasco-810 spectropolarimeter (Tufts University School of Medicine, Boston, MA). Anion exchange chromatography-purified and Buffer C-dialyzed sample was used for CD analysis. Prior to data collection, protein samples were clarified by centrifugation at 13,000 rpm, 1 min, 4 °C (Eppendorf 5424 R). Data were collected using a 1 mm path length quartz cuvette (Starna Cells, Atascadero, CA) with 1 nm bandwidth, 2 s response time, 20 nm/min scan speed, and 4 scans in continuous mode. Buffer-corrected spectra were recorded for 23 μM STAS-ΔIVS. Data were converted into Molar Residue Ellipticity (MRE) in units of θ (deg cm² dmol⁻¹) and analyzed using the program K2D2 [20]. Data were collected in triplicate and averaged for analysis.

2.6. Solution NMR spectroscopy

One-dimensional ¹H, 2D ¹H–¹⁵N HSQC [21] and ¹H–¹³C HSQC (constant time t1 evolution) [22] NMR experiments were performed at 27 °C on a Bruker Avance spectrometer operating at ¹H frequency 600.133 MHz equipped with a 5 mm triple resonance (z-axis) pulsed-field gradient probe. NMR samples of ¹⁵N or ¹³C/¹⁵N-enriched STAS domains (0.25 mM) contained 10% (v/v) D₂O, 1 mM DTT-d₁₀, 0.25 mM DSS as internal standard, 0.05% (w/v) NaN₃, and 1X protease inhibitor. One dimensional ¹H NMR spectrum was acquired using a *zgcppr* pulse sequence with a *presaturation* scheme for water suppression employing a composite pulse. 512 scans were collected for 1D ¹H NMR data interpretation. 2D ¹H–¹⁵N HSQC spectra were acquired using a relaxation delay of 1.00 s, with 8 scans (39 min acquisition time), 32 scans (2 h 45 min acquisition time) or 128 scans (10.3 h acquisition time). The 2D ¹H–¹³C HSQC spectrum was acquired using the Echo-AntiEcho mode of quadrature detection (¹³C dimension), with respective spectral widths of 13 ppm and 80 ppm in ¹H and ¹³C dimensions. Data were processed on an Intel PC workstation running OpenSuse11.1 using NMRPipe/NMRDraw processing software [23]. A Gaussian filter with a line-broadening parameter of 12 Hz was applied in direct and indirect acquired dimensions. All data sets were zero-filled once in each dimension to yield a final matrix of 2048*256 real data points. All ¹H chemical shift values were referenced with respect to internal standard DSS [24]. NMR data were analyzed with NMRDraw [23] and CCPNMR [25]. The hPDS_{ST} STAS-ΔIVS interaction with GDP nucleotide was measured by collecting a series of 2D ¹H–¹⁵N HSQC experiments of 32 scans. hPDS_{ST} STAS-ΔIVS (0.25 mM) was titrated with nucleotide to a saturating concentration of 20 mM.

2.7. Fluorescence spectroscopy

One native Trp and six native Tyr residues of hPDS_{ST} STAS-ΔIVS served as intrinsic fluorescence intensity probes. STAS protein purified by Ni-affinity- and anion exchange chromatography was used in Buffer C for fluorescence experiments. Samples were centrifuged before use. GDP free acid (Sigma) of highest available purity was prepared as stock solutions in buffer A. hPDS_{ST} STAS-ΔIVS (20–23 μM; initial volume 200 mL) was preincubated in 48-well plates at 24 °C with nucleotide. Steady-state intrinsic fluorescence intensity of hPDS_{ST} STAS-ΔIVS was then recorded at λ_{em} 290–400 nm at 2-nm intervals with fixed λ_{ex} 280 nm (SpectraMax M5, Molecular Devices, Sunnyvale, CA) in the presence of sequentially increasing nucleotide concentration, or titrated with buffer C alone. Protein and ligand were mixed and agitated 15 min prior to data collection. hPDS_{ST} STAS-ΔIVS fluorescence at each nucleotide concentration was corrected for dilution and for inner filter effect (IFE) contributions of added nucleotide, on the intrinsic fluorescence of a mixture of a free tryptophan plus 6 M eq of free tyrosine (the 1:6 Trp: Tyr molar ratio reflected in the native hPDS_{ST} STAS-ΔIVS aa sequence), with identical data acquisition parameters and instrument settings, as described [26]. IFE corrected and normalized fluorescence intensities were plotted as a function of nucleotide concentration and fit (SigmaPlot, Systat) to a single site ligand-binding model to determine dissociation constant (K_d) as described for nucleotide binding by Rv1739c STAS [15].

3. Results and discussion

3.1. Molecular cloning of hPDS STAS domain

All the primers used in this study are shown in Table 1. We initially planned NMR structural characterization of hPDS STAS-ΔIVS using two STAS constructs hPDS_S (aa 512–743) and hPDS_L (aa 512–780) as shown in Fig. 1. Empirical testing of these STAS-ΔIVS constructs revealed low protein yields with high tendency to precipitate during dialysis for imidazole removal (*see below*). In view of the potentially destabilizing role of N-terminal Arg 512 of the above STAS constructs, we generated hPDS_{ST} STAS-ΔIVS constructs in pET-52b(+) and in pET28a(+) (Supplementary Fig. S1) that preserved the predicted secondary structure of the longer constructs (*see Supplementary Fig. S2*).

3.2. Expression and isolation of hPDS STAS domain

Growth and induction of *E. coli* expressing STAS-ΔIVS polypeptides hPDS_L, hPDS_S, and hPDS_{ST} respectively yielded 3.6, 3.3,

Table 1

Primer sequences used in PCR amplifications of IVS excluded regions of hPDS STAS. Mutagenesis primers #1–5 were to amplify STAS-ΔIVS sequences for cloning hPDS_L and hPDS_S constructs into pET52b. Primers #6 and 7 were used to shuttle hPDS_{ST} STAS sequence (excluding disordered IVS region) from pET52b(+) into pET-28a(+) for structural studies.

SLC26A4 STAS	Primer sequence
1. <i>mut_For</i>	5'-GACTGTGACCTTGCCATGGCCGAGAGTTTCAGTTTCTTCTTGG-3'
2. <i>mut_Rev</i>	5'-GCGTGGCACCAGAGCGAGCTCGGATGCAAGTGTACCCATAGC-3'
3. <i>mut_Rev-short</i>	5'-GCGTGGCACCAGAGCGAGCTCTAAAATGGAACCTTGACCCTC-3'
4. <i>mut_fuse_Rev</i>	5'-GCACAAGGCTATGGATTGGCACGGCATCAAATCCAACCTGTGGAC-3'
5. <i>mut_fuse_For</i>	5'-GTCCACAGTTGGATTGATGCCGTGCCAATCCATAGCCTTGTGC-3'
6. <i>28a_For</i>	5'-TCACATATGGCTAGCTCTTGAATGGCCTTGAAGC-3'
7. <i>28a_Rev</i>	5'-CAGGAGCTCGAATTCCTAACCTCTTGAGATTTCACTTG-3'

and 3.3 g wet cell paste per liter culture. SDS-PAGE revealed bands of expected size (in kDa) for hPDS_L (24.3), hPDS_S (20.1) (data not shown), and for hPDS_{ST} (18.8; Fig. 2A). Protein induction was more efficient at 13 °C than at 25 °C or 37 °C. Polypeptide products of each construct partitioned 95% into the insoluble fraction.

Inclusion body lysates were bound to Ni-NTA beads, and eluted with imidazole step gradients. Yields of eluted STAS-ΔIVS polypeptide were 28% for hPDS_L, 21% for hPDS_S, and 53% hPDS_{ST}. The larger imidazole-resistant retained fractions of STAS-ΔIVS

constructs hPDS_L and hPDS_S were not released even upon addition of ~0.1% (w/v) octyl glucoside (OG) or dodecyl maltoside (DM). Moreover, as much as 88% of initially soluble Ni-NTA-purified STAS-ΔIVS constructs hPDS_L and hPDS_S precipitated during dialysis. The remaining soluble protein was too unstable to allow completion of CD experiments. Purification in buffers containing OG or DM marginally improved protein yield, but CD spectra of these preparations revealed absence of well-folded protein (not shown). We therefore focused subsequent efforts on purification

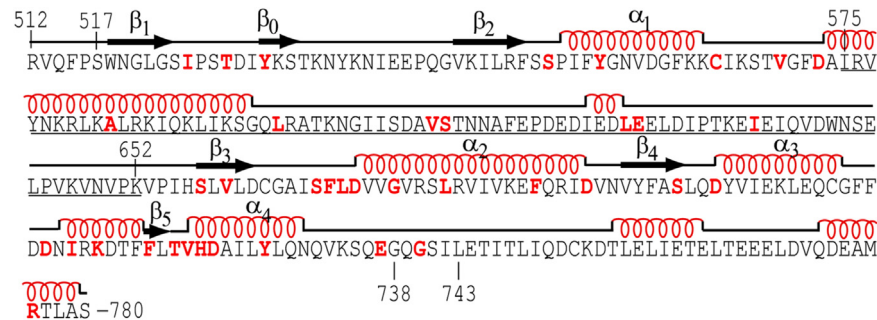


Fig. 1. Amino acid (aa) sequence encoded by SLC26A4/hPDS STAS domain cDNA used for molecular cloning and protein expression. Highlighted in red are sites of disease-associated missense mutations. Underlined aa sequence corresponds to the structurally uncharacterized IVS region (aa 575–652), believed to be disordered.

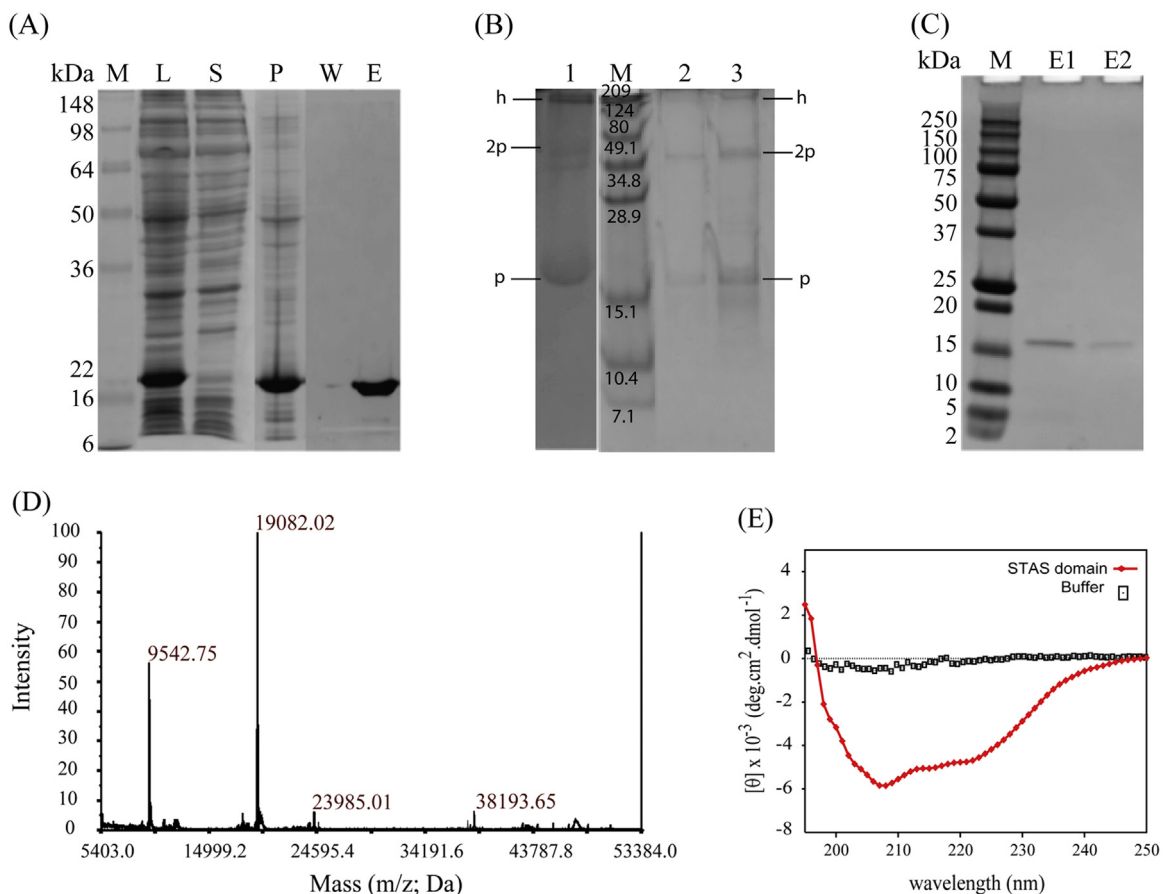


Fig. 2. Protein expression and purification, mass and CD spectra of hPDS_{ST} STAS-ΔIVS. Construct includes N-terminal (His)₆-tag. (A) Coomassie blue-stained SDS-PAGE (12% w/v) in the presence of 50 mM DTT; Lane M, protein standards; lane L, Lysate post-IPTG induction at 13 °C; lane S, supernatant fraction after centrifugation at 17,000 rpm, 4 °C, 1hr; lane P, insoluble (pellet) fraction after centrifugation; lane W, wash fraction from STAS-bound Ni-NTA beads; lane E, STAS-ΔIVS eluted from Ni-NTA by 500 mM imidazole in 500 mM NaCl. (B) Immunoblot showing protein monomer (p), dimer (2p), and higher order oligomer bands (h) in lane 1. Size exclusion chromatography fractions in the absence of reducing agent (lanes 2 and 3) show a similar pattern (band labeling similar to lane 1 on 16% w/v SDS-PAGE). (C) hPDS_{ST} STAS-ΔIVS in 1 M NaCl fractionated by anion exchange chromatography and separated in the presence of 50 mM DTT on SDS-PAGE (4–20% w/v), then Coomassie blue-stained. Lane M, protein standards; Lanes E1&E2, eluate fractions. (D) Mass spectroscopic chromatogram shows 19.1 kDa monomer peak of His₆-hPDS_{ST} STAS-ΔIVS and 38.2 kDa dimer peak as K⁺-adducts. A doubly charged species near 9.5 kDa is also seen. Additional peaks of higher mass may represent aggregates. (E) Far-UV CD spectrum of hPDS_{ST} STAS-ΔIVS at 25 °C.

and characterization of hPDS_{ST} STAS- Δ IVS (see Supplementary Table. S1). All the data presented below are from this construct.

Immunoblot of samples in near physiologic salt concentration demonstrated both monomeric and dimeric forms of hPDS_{ST} STAS- Δ IVS (Fig. 2B), with some higher order oligomers. hPDS_{ST} STAS- Δ IVS exhibited less precipitation during dialysis (<23%) than did larger STAS- Δ IVS polypeptides. Size exclusion chromatography revealed oligomeric forms of protein in solution, corroborating the immunoblot data.

Protein fractionated by size exclusion chromatography was subjected to dialysis for subsequent anion exchange chromatographic purification, with <7% precipitation during dialysis. Reducing SDS-PAGE of 1 M NaCl eluates from anion exchange chromatography revealed only monomers, suggesting that high salt can shift the conformational equilibrium towards the monomeric state (Fig. 2C). Reducing [NaCl] to \leq 150 mM restored oligomeric heterogeneity. Maintaining [NaCl] \geq 500 mM reliably reduced oligomerization of hPDS_{ST} STAS- Δ IVS.

3.3. Yields of His6-tag-free STAS hPDS_{ST} polypeptide

His₆ tag removal from the STAS- Δ IVS polypeptides led to major protein loss (<25% recovery). Tag-free samples exhibited moderate precipitation at later stages of purification, and resulting sample quantities were insufficient for 1D ¹H NMR studies. Therefore, further attempts to optimize purification of His-tag-free STAS- Δ IVS polypeptides were discontinued.

3.4. MALDI-TOF MS

Mass spectrometry of hPDS_{ST} STAS in buffers lacking reducing agent was not successful. Data collected in sample buffers containing \geq 9 mM tris-carboxyethylphosphine resulted in mass detection of potassiated monomeric and dimeric species of protein (Fig. 2D), accompanied by 2 forms of uncertain oligomeric stoichiometry.

3.5. CD spectroscopy

Fig. 2E shows the CD spectrum of hPDS_{ST} STAS- Δ IVS. The data show secondary structural elements suggesting a folded conformation in solution. The characteristic double ellipticity minima at 208 and 220 nm are diagnostic for helical structure. The spectral lineshape pattern, including the noted ellipticity differences at the two minima, shows the presence of β -strand structure. Spectral deconvolution analysis predicts that secondary structural elements constitute \sim 41% of the polypeptide. Correcting for the His6-fusion tag at the N-terminus, the measured % secondary structure is close to that predicted (\sim 46%) from the hPDS_{ST} STAS- Δ IVS model built on the rat prestin STAS- Δ IVS template. These results show that purified, recombinant hPDS_{ST} STAS- Δ IVS polypeptide refolded from inclusion body lysate adopts a well folded, structured conformation in solution.

3.6. NMR spectroscopy of hPDS_{ST} STAS domain

The folded state of hPDS STAS was further examined by applying 1D ¹H and 2D heteronuclear solution NMR methods (Fig. 3). First we attempted 2D ¹H-¹⁵N HSQC spectra on the hPDS_{ST} STAS- Δ IVS sample in buffer C, pH 7.9 (as used for CD measurements). Although the data showed good dispersion of ¹H-¹⁵N correlation crosspeaks of weak intensity on a directly detected dimension, the observed number of ¹H-¹⁵N backbone correlation crosspeaks in the 2D ¹H-¹⁵N HSQC spectrum was less than half the expected 140 non-proline residues (see Fig. S1 for aa sequence of STAS- Δ IVS), likely due to amide proton exchange with solvent and/or to the

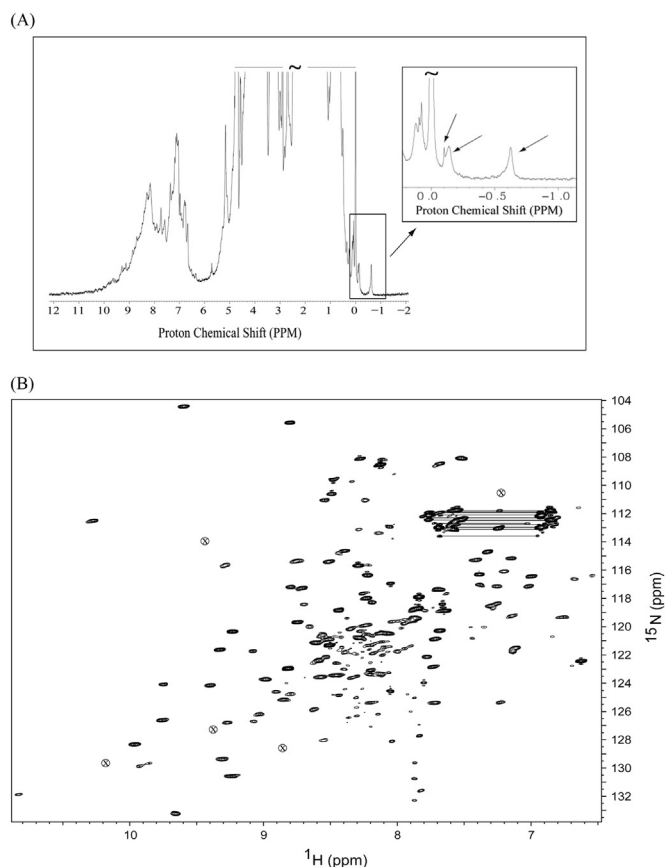


Fig. 3. (A) One-dimensional NMR spectrum of a uniformly ¹⁵N-enriched sample of hPDS_{ST} STAS- Δ IVS. The proton signals above 9.0 ppm and below 0.5 ppm indicate protein in a compact folded state. Methyl proton signals below 0.0 ppm likely reflect ring current effect (arising from interaction with aromatic ring protons), further supporting the folded state in these solution conditions. Inset shows magnified region of methyl peaks below 0.0 ppm. (B) Heteronuclear two-dimensional ¹H-¹⁵N HSQC spectrum of hPDS_{ST} STAS- Δ IVS. The 5 additional crosspeaks seen at higher contour level are highlighted as circled X's. Horizontal lines connect side chain ¹H-¹⁵N correlation crosspeaks arising from Asn and Gln residues.

presence of higher order STAS- Δ IVS oligomers in solution. The results nonetheless suggested a folded state for purified protein in this condition. Empiric assessment of buffer pH-dependence of STAS- Δ IVS stability in solution led to subsequent NMR data collection at pH 6.2.

One-dimensional ¹H NMR spectrum dispersion of \sim 10.80–6.50 ppm clearly demonstrates a folded conformation of hPDS_{ST} STAS- Δ IVS in solution (Fig. 3A). The moderately sharper line-widths of most peaks within the backbone amide region suggests the likely predominance of monomeric protein. The few peaks at 4.5–6.0 ppm likely correspond to ¹H $^{\alpha}$ resonances of residues constituting β -strand structures in the protein structure fold, corroborating the CD data. Select ¹H peaks below 0.0 ppm probably represent methyl proton peaks of residues experiencing a ring current shift due to interaction with aromatic residue ring protons within the tertiary structural fold (region magnified in Inset). These parameters show that hPDS_{ST} STAS- Δ IVS is well-folded, with regions of tertiary structure.

The 2D ¹H-¹⁵N HSQC spectrum of STAS- Δ IVS (Fig. 3B) shows at least one ¹H-¹⁵N backbone correlation crosspeak for most of the polypeptide's non-proline amino acid residues. The ¹H-¹⁵N side-chain correlations from Asn, Gln, and Trp residues are also evident in the spectrum. The chemical shift dispersion of these correlation crosspeaks for a well-folded STAS domain may exhibit a dispersed ¹H ppm spectral window (Fig. 3B), which for an intrinsically disordered or unfolded/partially folded protein would shrink to a

narrow ppm window (~ 8.6 – 7.0 ppm). The highly resolved ^1H – ^{15}N HSQC spectrum (10.3 h acquisition at pH 6.2) shown in Fig. 3B exhibits 151 cross-peaks (excluding the side-chain correlations) and excellent chemical shift dispersion of 10.83–6.57 ^1H ppm. Five additional backbone $^1\text{H}^{\text{N}}$ correlations crosspeaks of higher contour are also evident (circled X's in Fig. 3B). The ^1H – ^{15}N HSQC spectra recorded at shorter acquisition time (Supplementary Table S3) shows complete crosspeak dispersion similar to that of the longer acquisition time spectrum. Maintenance of spectral quality during 1 week of sample storage suggests stability of protein conformation at 0.25 mM during this time.

The expected number of ^1H – ^{15}N backbone correlation crosspeaks for this construct (Fig. S1) is 162, including 23 ^1H – ^{15}N correlation crosspeaks predicted to arise from the N-terminal cleavable (His) $_6$ -fusion tag. The 156 crosspeaks detected in the spectrum likely include all or most core protein resonances. Two sets of crosspeaks of different intensity distributions were present in the spectrum. The predominant set of ~ 130 crosspeaks shows moderately sharper linewidths, whereas, the minority set of ~ 26 crosspeaks has broader linewidths of intensity at least 50% lower than the average intensity of the majority set of crosspeaks. These differences in linewidth might reflect different dynamic properties elicited by the respective resonances. The weaker intensity crosspeaks could include some or all ^1H – ^{15}N resonances from the (unstructured) (His) $_6$ tag region, and/or could reflect solvent exchange effects for these $^1\text{H}^{\text{N}}$ resonances. Although protein aggregation was operationally minimized at pH 6.2, a degree of oligomerization producing slower rotational correlation times of minority set of crosspeaks cannot be ruled out. Future completion of NMR assignment will provide further details.

Fig. 4 shows the well-dispersed 2D ^1H – ^{13}C HSQC spectrum of hPDS $_{\text{ST}}$ STAS- Δ IVS. Downfield-shifted ^1H – ^{13}C correlation crosspeaks within the $^1\text{H}^{\alpha}$ region likely indicate signals from residues involved in β -strand formation. The extreme upfield-shifted crosspeaks (below 0.0 ppm) identify residues exhibiting a ring current shift effect, corroborating the 1D ^1H NMR data. These data together suggest that hPDS $_{\text{ST}}$ STAS- Δ IVS adopts a folded conformation in solution.

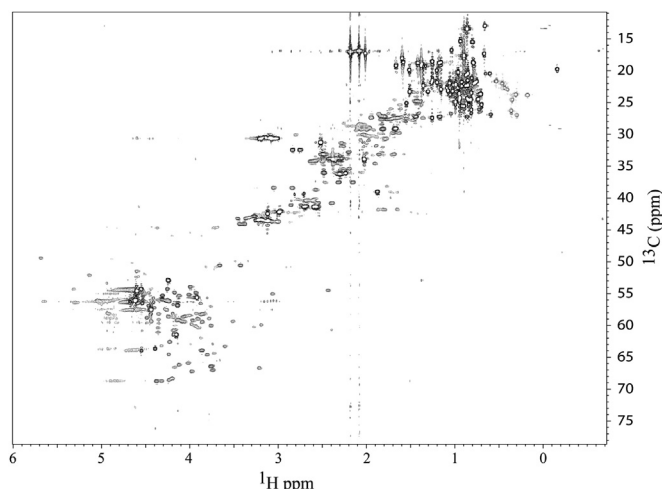


Fig. 4. Heteronuclear 2D ^1H – ^{13}C HSQC spectrum of hPDS $_{\text{ST}}$ STAS- Δ IVS. ^1H – ^{13}C correlation crosspeaks are well-dispersed. Downfield shifted ^1H – ^{13}C correlation crosspeaks within $^1\text{H}^{\alpha}$ region likely indicate signals from residues involved in β -strand formation. The extreme upfield-shifted crosspeaks (below 0.0 ppm) likely identify residues exhibiting ring current shift effect, and corroborate the 1D ^1H NMR data presented here.

3.7. NMR detection of nucleotide binding by hPDS $_{\text{ST}}$ STAS

The nucleotide-binding properties of anti- σ factor antagonist SpolIAA [27], STAS domain of Rv1739c from *M. tuberculosis* [15], and STAS domains from full-length YchM/DauA [12] prompted evaluation of nucleotide binding by hPDS $_{\text{ST}}$ STAS- Δ IVS.

We first tested stability of NMR samples by collecting a series of 2D ^1H – ^{15}N HSQCs (2 h 45 min each) at days 1, 3, 4, and 7 after purification, with storage at 4 $^{\circ}\text{C}$. All 2D ^1H – ^{15}N HSQC spectra revealed similar degrees of dispersion, with minimal precipitation over the 7 d period. These data warranted preparation of $^{13}\text{C}/^{15}\text{N}$ doubly-enriched NMR sample for resonance assignment and structural characterization.

In order to study hPDS $_{\text{ST}}$ STAS- Δ IVS interaction with nucleotide by NMR chemical shift perturbation (CSP), 2D ^1H – ^{15}N HSQC spectra of freshly prepared ^{15}N -STAS- Δ IVS were collected in the presence of sequentially increasing [GDP]. Data revealed GDP-induced NMR chemical shift perturbation (CSP) for select protein residues. NMR titration experiments identified 20 mM GDP as a saturating concentration for hPDS STAS- Δ IVS, as higher concentrations did not significantly increase CSP (not shown). Overlay of 2D ^1H – ^{15}N HSQC spectra of STAS- Δ IVS domain in the absence and presence of increasing [GDP] is shown in Fig. 5A. Sections of the spectrum with residues exhibiting the largest GDP-induced CSPs are enlarged in Fig. 5B–D. GDP-induced conformational perturbations include resonances showing only weighted average peak shifts, whereas other select resonances exhibit peak shifts accompanied by decreased crosspeak intensity. GDP addition to STAS- Δ IVS also elicited emergence of several novel resonances (Fig. 5A and B) probably reflecting nucleotide-induced stabilization of normally undetected, flexible residues, and/or slow conformational exchange of select residues in their local environments. These analyses suggest that GDP binding induces CSP of select protein residues, a process that occurs at faster-intermediate NMR time scales. Taken together, these results strongly suggest that hPDS $_{\text{ST}}$ STAS- Δ IVS binds guanine nucleotide.

3.8. Nucleotide binding by hPDS $_{\text{ST}}$ STAS- Δ IVS by intrinsic fluorescence quench

The presence in hPDS $_{\text{ST}}$ STAS- Δ IVS (Fig. 1) of a single Trp residue, Trp518, and 6 Tyr residues (Tyr530, Tyr536, Tyr556, Tyr691, Tyr698, and Tyr728) allowed assessment of nucleotide binding by intrinsic fluorescence quench. hPDS $_{\text{ST}}$ subjected to 280 nm excitation exhibited a fluorescence emission λ_{max} of 320 nm. Fig. 6A illustrates the gradual quench of intrinsic STAS fluorescence with progressively increasing concentrations of GDP, with maximum observed quench of 14.2%. The observed quench isotherm is compatible with a single nucleotide binding site of $K_{1/2} = 178 \pm 81 \mu\text{M}$ (Fig. 6B). The data suggest a nucleotide interaction surface in STAS domain that directly or indirectly perturbs Trp and/or Tyr residues through conformational effects on adjacent or interposed residues. These results are consistent with the heteronuclear NMR data showing nucleotide binding by hPDS $_{\text{ST}}$ STAS- Δ IVS. Similar future experiments will examine nucleotide binding of full-length wild type and disease-associated mutants, and define the nucleotide binding surface of the protein. Preliminary data (Sharma et al., unpublished) confirm nucleotide binding by the recombinant full-length STAS domain of *A. thaliana* sulfate transporter SULTR1;2, further strengthening the hypothesis that STAS domains are nucleotide binding proteins [4].

The tendency of hPDS $_{\text{ST}}$ STAS- Δ IVS to aggregate was modulated by salt concentration and pH. Buffer conditions of ≥ 500 mM NaCl and pH 6.2 each reduced protein aggregation. At 150 mM NaCl, the protein exhibited a similar monomer-dimer equilibrium at both pH 6.2 and pH 7.9. SLC26A/SulP proteins in solution are known to exhibit dimeric and higher order oligomeric structures [2,12,28]. Rat

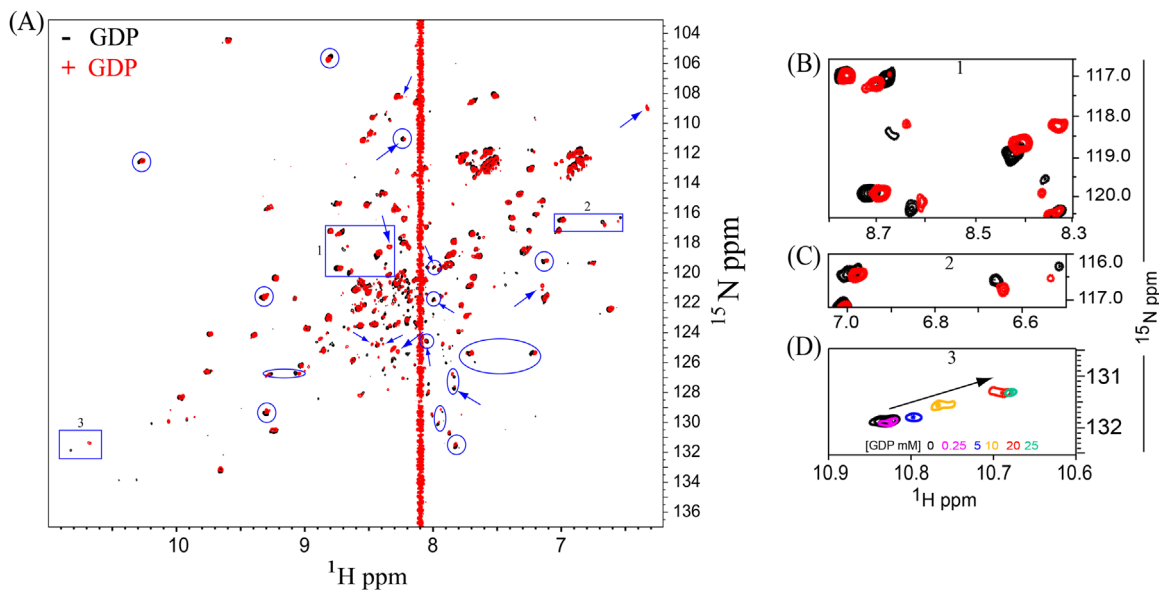


Fig. 5. NMR detection of GDP interactions with hPDS_{ST} STAS-ΔIVS. (A) Superposition of 2D ¹H-¹⁵N HSQC spectra of hPDS STAS in absence (black contours) and presence of 20 mM GDP (red contours). Chemically perturbed ¹H-¹⁵N correlation resonances are indicated by circles, ellipses, and boxes. Arrows show emergent resonances in GDP-saturated STAS spectra. Arrowed circles denote resonances showing CSP with decreased intensity. The guanine proton peak is at ~8.09 ppm. (B and C) Magnified regions of boxed portions of 2D ¹H-¹⁵N HSQC of STAS-ΔIVS in absence (black) and presence of (saturating) 20 mM GDP (red). (D) Tryptophan side-chain ¹H-¹⁵N correlation crosspeaks showing graded perturbation upon titration with increasing [GDP] (scale below panel).

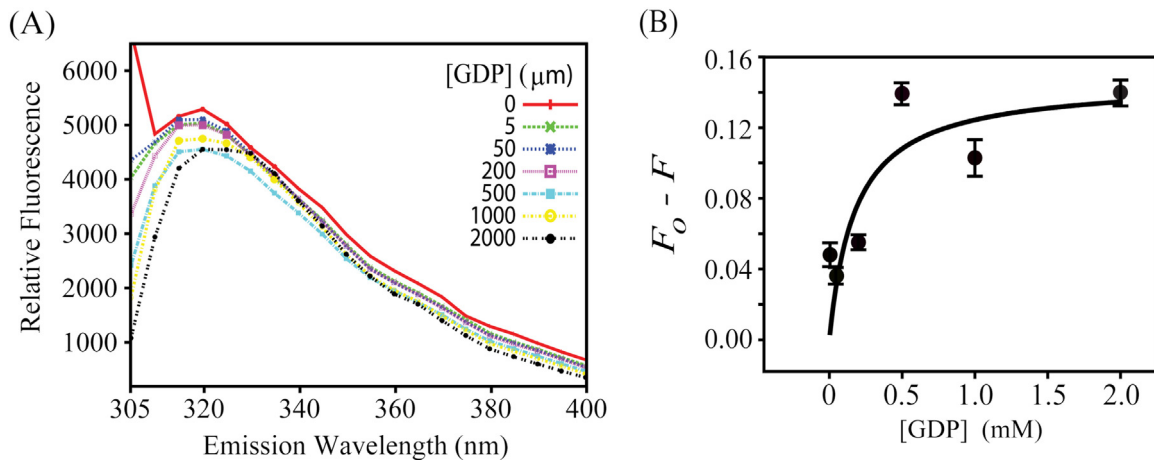


Fig. 6. Intrinsic tryptophan/tyrosine fluorescence quench (A) Intrinsic fluorescence quench of hPDS_{ST} STAS-ΔIVS (20–23 μM) by indicated [GDP]. (B) GDP concentration dependence for quench of hPDS_{ST} STAS peak intrinsic fluorescence at 320 nm, with $K_{1/2} = 178 \pm 81 \mu\text{M}$. Values are mean \pm S.E. ($n=3$).

Prestin STAS-ΔIVS exhibited dimers and tetramers in solution with a tendency toward formation of higher molecular weight aggregates [29]. Recombinant mouse Slc26a9 STAS also exhibited a monomer-dimer equilibrium [30]. Protein aggregation was noted for *E. coli* DauA STAS domain variants harboring mutations at the sites homologous to the disease-associated mutations in SLC26 STAS domains [31]. Future studies will test the role of the three Cys residues of hPDS STAS (aa 565, 662, and 706) in STAS oligomerization. Rat prestin STAS template-based modeling of hPDS STAS-ΔIVS suggests occupancy of flexible loop positions by the three homologous Cys residues (Cys706 is the first residue in helix α 3) with side chain exposure to the solvent environment (Supplementary Fig. S4). Thus, alterations in solvent pH, salt concentration, and redox environment may influence the native cysteine-cysteine equilibrium, including possible inter-monomer cystine linkage.

IVS deletion from the hPDS STAS domain has allowed a reproducible purification protocol, yielding well-folded protein in

quantities suitable for NMR structural studies. Attempts are currently underway to purify full-length hPDS STAS domain polypeptide from soluble fractions in quantity sufficient for solution NMR, using solubility-enhancing fusion tags. However, the present study shows that the IVS region is dispensable for nucleotide binding by hPDS STAS.

4. Conclusions

The class of eukaryotic STAS domains from rodent and human SLC26 transporters has proven difficult to express in a well-folded conformation at the high concentrations required for structural studies. With the exception of rat SLC26A5/prestin, only one ¹H-¹⁵N 2D HSQC spectrum has been reported from a mammalian STAS domain (SLC26A3). However no structural information has been reported for SLC26A3 STAS domain, perhaps reflecting low sample solubility [8], or for STAS domain from any human member. Here we have

presented data on the molecular cloning, expression and purification, and protein folding characterization of hPDS_{ST} STAS-ΔIVS as characterized by CD and by 1D and 2D heteronuclear NMR. Our purification protocol has yielded well-folded U-¹³C/¹⁵N labeled recombinant STAS in the milligram quantities sufficient for future unambiguous NMR residue assignment and elucidation of three dimensional structure of this deafness gene product, SLC26A4/pendrin. We found that hPDS_{ST} STAS-ΔIVS exists in solution in monomer and dimer states, with propensity to higher-order oligomerization. We showed by intrinsic fluorescence quench and by CSP NMR methods that hPDS STAS-ΔIVS can bind GDP. These results are of importance not only for structure/function studies of hPDS STAS, but may in addition be applicable to STAS domains of other human SLC26 anion transporter polypeptides. This information will increase our understanding of a known therapeutic target for treatment of congenital deafness. It will also increase understanding of an underappreciated major pathway for distal nephron chloride reabsorption representing an important target for diuretic development, with implications for enhanced management of fluid overload in settings of trauma and intensive care, and in chronic cardiac, renal, and liver diseases.

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Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Transparency document

Transparency document data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.08.022>.

Appendix B. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.08.022>.

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