Biophysical analysis of the N-terminal domain from the human Protein Phosphatase 1 Nuclear Targeting Subunit PNUTS suggests an extended transcription factor TFIIS-like fold

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Running Title: Characterization of PNUTS N-terminal domain

Abstract

Human Protein Phosphatase 1 Nuclear Targeting Subunit (PNUTS) plays critical roles in DNA repair, cell growth and survival. The N-terminal domain of PNUTS mediates critical interactions with Tox4 and the phosphatase and tensin homolog PTEN, which are essential for the roles of this protein. To characterize this N-terminal domain, we have established its recombinant overproduction in *E. coli* utilizing NusA fusion. Upon removal of the tag, the remaining PNUTS sample is soluble and highly pure. We have characterized the N-terminal domain using circular dichroism and nuclear magnetic resonance and analyzed its sequence using bioinformatics. All data agree in suggesting that the PNUTS N-terminal segment adopts a compact, globular fold rich in α-helical content, where the folded fraction is substantially larger than the previously annotated fold. We conclude that this domain adopts an extended form of the Transcription Factor S-II (TFIIS) leucine/tryptophan conserved (LW)-motif, where additional helices are integrated into the domain architecture. Thermal denaturation yielded a melting temperature of ~49.5^{o}C, confirming the stability of the fold. These findings pave the way for the molecular characterization of functional interactions mediated by the N-terminal region of PNUTS.

Keywords

Recombinant protein overexpression / Secondary structure prediction / Thermal denaturation / Circular Dichroism / Nuclear magnetic resonance

List of Abbreviations

CD: Circular Dichroism

EDTA: ethylenediaminetetraacetic acid

HSQC: Heteronuclear Single Quantum Coherence

NMR: Nuclear Magnetic Resonance

PNUTS: Protein Phosphatase 1-binding Nuclear Targeting Protein

PP1: Protein Phosphatase 1

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TCEP: *tris*-2-carboxyethyl-phosphine

TFIIS: Transcription Factor IIS

TFIIS LW-motif: structural motif of the Transcription Factor IIS with conserved leucine and tryptophan residues

Introduction

Human PNUTS (PP1 Nuclear Targeting Protein) is a Protein Phosphatase 1 (PP1) binding protein with critical functions in the response to cellular stresses, including DNA damage, and the regulation of RNA-polymerase II -mediated gene expression [1-5]. It forms a ternary complex with PP1, Tox4 and WDR82 that targets PP1 to the nucleus [3-10], and further interacts with the tumour suppressor phosphatase and tensin homolog PTEN [11]. Despite its significance to key transcriptional processes, PNUTS is poorly characterized. At the molecular level, PNUTS is a largely unstructured protein that contains two small folded domains, located at each of its termini. The N-terminal domain is predicted to be similar to the N-terminal transcription factor IIS (TFIIS) LW domain (so-called by the presence of invariant leucine and tryptophan residues; [12]) and binds to Tox-4 [6] and PTEN [11]. Such TFIIS LW domains are small four-helix bundles that are present in transcription factors such as MED26 and elongin A [12]. They are part of the larger TFIIS module that engages RNA-polymerase II [13,14] and Tox-4 [6]. The PNUTS C-terminal region contains a zinc finger domain implying a possible interaction with nucleic acids [14], although this domain in PNUTS is not known to bind either RNA or DNA. The polypeptide region between the TFIIS LW and zinc finger domains in PNUTS is highly unstructured and plays a conserved role in binding to PP1 [15-17].

We have established the recombinant overproduction of the N-terminal region of human PNUTS in soluble form and characterized it biophysically. Our analysis suggests that this N-terminal segment contains a larger fold than the currently annotated TFIIS LW-like domain. Knowledge of the correct boundaries of this domain provides now better guidance for molecular studies of PNUTS protein-protein interactions.

Methods

Structure prediction from sequence data

For the initial identification of structural homologs we employed the HHpred online server that uses hidden Markov models for comparative analysis of sequences [18-20]. The server also incorporates secondary structure prediction using the PSIPRED method [21]. In addition, we also used the Network Protein Sequence Analysis secondary structure prediction server (https://npsa-prabi.ibcp.fr) implementing the MLRC [22], DSC [23], and PHD predictive methods [24]. For identification of the putative fold we utilised the intensive search mode of the Phyre2 online server [25]. The latter employs hidden Markov models to generate multiple sequence alignments from protein structures deposited at the Protein Data Bank (www.rcsb.org) [25].

Molecular Biology

Plasmid DNA containing His_6-NusA-3C-His_6-PNUTS (UniProtKB Q96QC0) was purchased from the Medical Research Council Dundee Phosphorylation and Ubiquitilation unit (product DU37545). His_6-NusA-3C-His_6-$PNUTS^{1-158}$ was subcloned from the former using ligation independent cloning into the pOPINB vector (Oxford Protein Production Facility, UK). This vector incorporates an additional N-terminal His_6-tag prior to the insert, resulting in the His_{12}-NusA-3C-His_6-PNUTS protein product. The clone was confirmed by sequencing (GATC-biotech).

Protein expression and purification

Protein expression was in *E. coli* strain BL21*(DE3) (Invitrogen) grown at 37^{o}C in Luria-Bertani medium supplemented with 25µg/ml kanamycin. At an OD_{600} = 0.6, cultures were cooled to 18^{o}C, expression induced with 0.5mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and cells further incubated for 16 hours. Cells were harvested by centrifugation and resuspended in 20mM sodium phosphate pH 7.4, 500mM NaCl, 20mM Imidazole, 3mM β-Mercaptoethanol containing an ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche) and 1mg/ml bovine deoxyribonuclease (Sigma). Cells were lysed using pressure homogenisation. Lysates were clarified by centrifugation and filtered using a 20µm filter prior to fast liquid chromatography. Initial purification was by metal affinity chromatography in a 5mL His-Trap HiPrep column (GE healthcare), with the protein eluted using a linear gradient of imidazole (0-500mM). The sample was then buffer exchanged into 20mM Tris pH 7.4, 150mM NaCl, 3mM β-Mercaptoethanol using a HiPrep 26/10 desalting column and, for tag removal, incubated overnight at 4^{o}C with PreScision protease^{TM} (which was tagged with glutathione S-transferase; GE Healthcare). The cleaved His_{12}-NusA fusion tag was removed by ion exchange capture in a 5ml HiTrap Q HiPrep column (GE Healthcare), with precision protease and His_6-PNUTS eluting in the flow through. This flow through eluate was once again exchanged into 20mM sodium phosphate pH 7.4, 500mM NaCl, 20mM Imidazole, 3mM β-Mercaptoethanol and applied to a 5mL His-Trap HiPrep column(GE healthcare), which captured His_6-PNUTS whilst precision protease did not bind to the column. His_6-PNUTS was then further purified on a 5ml HiTrap S HP column to a purity of >95% as revealed by SDS-PAGE.

For isotopic labelling, bacterial cultures were grown and induced in 2M9 media with 1g of ^{15}N-ammonium chloride (Sigma) added per 1L medium.

Sample preparation for biophysical analysis

Protein samples were buffer exchanged into the respective buffers using a PD-10 desalting column (GE Healthcare) and concentrated using a Millipore 3kDa spin concentrator at 4400rpm. Protein concentration was determined by A_{280} using a Nano-drop 2000 spectrometer (Thermo Scientific).

Nuclear Magnetic Resonance (NMR) spectroscopy

NMR spectroscopy was performed in 20 mM HEPES pH 7.4, 150mM NaCl, 3mM β-Mercaptoethanol with 5% [v/v] $^{2}H_2O$. Data were collected on an AVANCE II+ 800MHz spectrometer (Bruker) equipped with CryoProbe at 298K. For temperature titration, proton shifts were calibrated using trimethylsilyl propanoic acid (TSP) as an external standard. Figures were made using TopSpin 3.1 (Bruker).

Circular Dichroism (CD)

CD data were collected on a Jasco J-1100 spectrometer equipped with a JASCO PTC-348WI temperature control unit. Fresh protein samples were buffer exchanged into 10mM sodium phosphate pH 7.4, 0.5mM *tris*-2-carboxyethyl-phosphine (TCEP) and data collected at 0.5mg/ml in a 0.2mM path length quartz cuvette at a frame rate of 100nm/minute. Prior to deconvolution, control buffer spectra were subtracted and the data

zeroed using the CD signal at λ=260nm. Data were deconvoluted using the Dichroweb server with the CDSSTR method [26,27]. To measure thermal stability, CD spectra were collected in the spectral range λ=180-260 nm and in the temperature range 20-90^{o}C. Temperature was increased at a rate of 1^{o}C per minute and the sample equilibrated for five minutes at each integral degree before the recording of the corresponding spectrum. Data fit was performed using the Boltzman equation in PRISM 7.

Results

Prediction of the existence of a helical domain at the N-terminus of PNUTS

Human PNUTS is a 940-residue long protein with an annotated TFIIS LW domain close to its N-terminus. Its Interpro entry [28] reveals that different domain databases assign different regions to this domain: in Pfam (entry PF08711; [29]) it covers residues Q93-V143 while Smart (SM00509; [30]) and Prosite (PS51319; [31]) allocate it residues K73-Q147 approximately. Thus, there is currently no consensus on the start point of the domain. To identify the boundaries of the PNUTS TFIIS LW fold, we performed a secondary structure prediction from sequence data. The results highlighted two stretches (residues P8-F18 and V27-L56) of high helical propensity prior to the annotated motif (**Fig 1**). In addition, both HHpred and Phyre2 servers identified the protein IWS1 from *E. cuniculi*, of known atomic structure [13], as a distant homolog of the PNUTS N-terminal segment. The structure of IWS1 is that of an extended TFIIS LW fold with an additional N-terminal helical fraction that packs against the core fold forming a helical bundle. All the predicted α-helices in

PNUTS are amphipathic and might, therefore, pack against each other forming a compact helical bundle as that of IWS1. Thus, we considered possible at this stage that the additional N-terminal helices in PNUTS might also contribute to the tertiary fold of its domain.

Recombinant production of the N-terminal domain of PNUTS

We produced the N-terminal fraction of PNUTS (residues 1-158) as a soluble and stable protein product in *E. coli* in the form of a fusion protein of the type His_{12}-NusA-3C-His_6-$PNUTS^{1-158}$ (**Fig 2A**). The His_{12}-NusA tag was cleaved with PreScission protease 3C and removed by ion exchange chromatography profiting from the differential pI values of NusA and $PNUTS^{1-158}$ ($PNUTS^{1-158}$ has pI=9.43 and is positively charged at pH 6.5, while NusA has pI=4.62 being negatively charged at that pH; pI values were calculated using Prot-param [32]). The remaining His_6-$PNUTS^{1-158}$ sample, containing the non-cleavable N-terminal affinity tag, was separated from PreScission protease by nickel affinity chromatography and ion exchange chromatography. This protocol produced a $PNUTS^{1-158}$ protein of high purity at a yield of ~10mg/L *E. coli* culture. The sample migrated in SDS-PAGE at a molecular mass of 19kDa, consistent with the molecular mass calculated from sequence (**Fig 2B**). The identity of the purified product was verified by mass spectrometry.

The N-terminal domain of PNUTS folds into a stable α-helical motif

To test bioinformatics predictions, we analysed the secondary structure composition of PNUTS$^{1-158}$ experimentally using CD. The CD spectrum was characteristic of a helix-rich protein **(Fig 3A)**. We analysed the data using CDSSTR [27] available through Dichroweb (http://dichroweb.cryst.bbk.ac.uk). The spectral deconvolution suggested a protein fold consisting of approx. 56% helical content and a negligible 8% β-strand content. The normalized residual mean square difference of this estimation was 0.016 indicating a close fit of reconstructed and experimental spectra). This estimation of secondary structure content is in good agreement with sequence-based predictions (**Fig 1**) and supports the existence of an extended TFIIS LW fold. A shorter version of the domain fold as annotated in Pfam and Smart/Prosite would have resulted in strongly reduced helical contents of approx. 29% and 37%, respectively.

Next, we sought to evaluate the stability of the PNUTS$^{1-158}$ fold through CD-monitored thermal denaturation. The plot derived from the change in CD signal at λ=208nm in function of temperature follows a regular cooperative sigmoidal profile (**Fig 3B**). The melting temperature derived was Tm=49.5^{o}C, indicating that this is a stable domain.

The N-terminal domain of PNUTS adopts a globular fold

A remaining question was whether the PNUTS$^{1-158}$ fragment forms a compact three-dimensional structure, where the new N-terminal helices contribute to a larger fold. Alternatively, it might be the case that the additional helical elements simply

form flexible tail extension to the TFIIS LW domain. We investigated this question through a preliminary NMR study. The $^{1}H,^{15}N$ Heteronuclear Single Quantum Coherence (HSQC) spectrum of ^{15}N-labelled PNUTS$^{1-158}$ showed high dispersion of well-defined backbone NH resonances with uniform intensity of the signals (**Fig 4**). These characteristics indicate a globular protein fold, supporting the view that a single protein domain spans the whole N-terminal region of PNUTS. In such HSQC spectra, highly dynamic (i.e. disordered) protein regions would result in a group of sharp high-intensity cross-peaks located in a narrow band at 8-8.5 ppm in the ^{1}H dimension; whereas independently folded helical regions detached from the main protein fold would lead to groups of dispersed signals that have distinctly different line-width compared to other signals due to their different dynamics. In addition, independently folded regions are normally connected by unstructured hinge regions, which are commonly identifiable in NMR spectra. Neither of these patterns were observed in the PNUTS$^{1-158}$ spectra, leading us to conclude that it adopts an integrated 3D fold. Signals from the dynamic hexa-histidine tag were not observed due to the fast exchange of the backbone hydrogen with water.

Additional support for a single folded domain comes from the temperature dependence of the $^{1}H,^{15}N$-HSQC spectra (**Fig 4**). Temperature increase resulted in uniform changes in the spectra that reflect a uniform thermal denaturation of a stable domain. Independent helices would have a different stability than a globular domain, manifesting in a selective broadening of a group of resonances upon temperature increase, which was not observed. On increasing the temperature from 25^{o}C to 35^{o}C, the resonances remained of equal intensity, with only resonance shift changes observed. At 45^{o}C, the majority of the resonances showed line broadening and a

uniformly reduced intensity due to the increased exchange with solvent **(Fig 4)**. However, the chemical shift changes were linear in this temperature range, suggesting that the protein fold was uniformly destabilized. At this temperature the chemical shift changes were reversible and the original spectra detected upon reduction of the temperature to 25^{o}C, implying a reversible transitional state. A further temperature increase to 55^{o}C led to irreversible protein denaturation with a complete loss of signals and visible precipitation of the sample. This suggests that the melting of this domain occurs completely within the temperature range 45-55^{o}C, in agreement with the T_m estimation from CD data. In summary, NMR-based findings support our conclusion that PNUTS$^{1-158}$ forms a stable, single domain.

Discussion

Bioinformatics analysis of human PNUTS$^{1-158}$ suggested that this N-terminal segment may contain a larger domain than the currently annotated TFIIS LW motif. To test this prediction, we expressed recombinantly and biophysically characterized the N-terminal region of PNUTS. Using CD and preliminary NMR data, we showed that PNUTS$^{1-158}$ adopts an integrated helical fold, with a stability characterized by a Tm of ~49.5^{o}C. As the N-terminal segment of PNUTS is thought to be involved in multiple protein-protein interactions, the accurate establishment of its domain boundaries is of central importance for future functional studies on PNUTS.

References

1. Xing Z, Lin A, Li C, Liang K, Wang S, Liu Y, Park PK, Qin L, Wei Y, Hawke DH, Hung M, Lin C, Yang L (2014) lncRNA Directs Cooperative Epigenetic Regulation Downstream of Chemokine Signals. Cell 159:1110–1125

2. Kim Y, Watanabe T, Allen PB, Kim Y, Lee S, Greengard P, Nairn AC, Kwon Y (2003) PNUTS , a Protein Phosphatase 1 (PP1) Nuclear Targeting Subunit. J Biol Chem 278:13819–13828

3. Bennett D (2005) Transcriptional control by chromosome-associated protein phosphatase-1. Biochem Soc Trans 33:1444–1446

4. Ciurciu A, Duncalf L, Jonchere V, Lansdale N, Vasieva O, Glenday P, Rudenko A, Vissi E, Cobbe N, Alphey L, Bennett D (2013) PNUTS / PP1 Regulates RNAPII-Mediated Gene Expression and Is Necessary for Developmental Growth. PLOS Genet 9:e1003885

5. Dorn G (2013) miR-34a and the cardiomyopathy of senescence: SALT PNUTS, SALT PNUTS! Cell Metab 17:629-30

6. Lee J, You J, Dobrota E, Skalnik DG (2010) Identification and Characterization of a Novel Human PP1 Phosphatase Complex. J Biol Chem 285:24466–24476

7. Allen PB, Kwon Y, Nairn AC, Greengard P (1998) Isolation and Characterization of PNUTS, a Putative Protein Phosphatase 1 Nuclear Targeting Subunit. J Biol Chem 273:4089–4095

8. Ramakrishnan R, Liu H, Donahue H, Malovannaya A, Qin J, Rice AP (2012) Identification of novel CDK9 and Cyclin T1-associated protein complexes (CCAPs) whose siRNA depletion enhances HIV-1 Tat function. Retrovirology 9:90

9. Kapoor R, Sakshi A, Sanket SP, Binod K, Subbareddy M, Banerjea AC (2015) MicroRNA34a enhances HIV-1 replication by targeting PNUTS/PPP1R10

which negatively regulates HIV-1 transcriptional complex formation. Biochem J 470:293-302

10. Landsverk HB, Mora-Bermúdez F, Landsverk OJ, Hasvold G, Naderi S, Bakke O, Ellenberg J, Collas P, Syljuåsen RG, Küntziger T (2010) The protein phosphtase 1 regulator PNUTS is a new component of the DNA damage response. EMBO Rep 11:868-875
11. Kavela S, Shinde SR, Ratheesh R, Viswakalyan K, Bashyam MD, Gowrishankar S, Vamsy M, Pattnaik S, Rao S, Sastry RA, Srinivasulu M, Chen J, Maddika S (2013) PNUTS Functions as a Proto-Oncogene by Sequestering PTEN. Cancer Res 73:205-214
12. Ling Y, Smith AJ, Morgan GT (2006) A sequence motif conserved in diverse nuclear proteins identifies a protein interaction domain utilised for nuclear targeting by human TFIIS. Nucleic Acids Res 34:2219–2229
13. Diebold M, Koch M, Loeliger E, Cura V, Winston F, Cavarelli J, Romier C (2010) The structure of an Iws1/Spt6 complex reveals an interaction domain conserved in TFIIS, Elongin A. EMBO J 29:3979–3991
14. Klug A, Rhodes D (1987) Zinc fingers: a novel protein fold for nucleic acid recognition. Cold Spring Harbor Symposia on Quantit Biol 52:473–482
15. Choy MS, Hieke M, Kumar GS, Lewis GR, Gonzalez-DeWhitt KR, Kessler RP, Stein BJ, Hessenberger M, Nairn AC, Peti W, Page R (2014) Understanding the antagonism of retinoblastoma protein dephosphorylation by PNUTS provides insights into the PP1 regulatory code. Proc Natl Acad Sci USA 111:4097-4102
16. Bennett D, Lyulcheva E, Alphey L, Hawcroft G. (2006) Towards a comprehensive analysis of the protein phosphatase 1 interactome in Drosophila. J Mol Biol 364:196-212

17. Kim YM, Watanabe T, Allen PB, Kim YM, Lee SJ, Greengard P, Nairn AC, Kwon YG (2003) PNUTS, a protein phosphatase 1 (PP1) nuclear targeting subunit. Characterization of its PP1- and RNA-binding domains and regulation by phosphorylation. J Biol Chem 278:13819-13828

18. Hildebrand A, Remmert M, Biegert A, So J (2009) Fast and accurate automatic structure prediction with HHpred. Proteins 77:128-132

19. Biegert A, Lupas AN (2005) The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Res 33:244–248

20. Söding J, Biegert A, Lupas AN (2005) The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Res 33:W244-248

21. Buchan DW, Minneci F, Nugent TC, Bryson K, Jones DT (2013) Scalable web services for the PSIPRED Protein Analysis Workbench. Nucleic Acids Res 41(W1):W349-W357

22. Guermeur Y, Geourjon C, Gallinari P, Deléage G, Lyon NSDe, Italie A, Pierre U (1999) Improved performance in protein secondary structure prediction by inhomogeneous score combination. Bioinformatics 15:413–421

23. King RD, Sternberg MJE (1996) Identification and application of the concepts important for accurate and reliable protein secondary structure prediction. Protein Sci 5:2298-2310

24. Rost B, Sander C (1993) Prediction of protein secondary structure at better than 70% accuracy. J Mol Biol 232:584-599

25. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE (2015) The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc 10:845–858

26. Whitmore L, Wallace BA (2008) Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases. Biopolymers 89:392–400

27. Sreerama N, Woody RW (2000) Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. Anal Biochem 287:252-260

28. Mitchell A, Chang HY, Daugherty L, Fraser M, Hunter S, Lopez R, McAnulla C, McMenamin C, Nuka G, Pesseat S, Sangrador-Vegas A, Scheremetjew M, Rato C, Yong SY, Bateman A, Punta M, Attwood TK, Sigrist CJ, Redaschi N, Rivoire C, Xenarios I, Kahn D, Guyot D, Bork P, Letunic I, Gough J, Oates M, Haft D, Huang H, Natale DA, Wu CH, Orengo C, Sillitoe I, Mi H, Thomas PD, Finn RD (2015) The InterPro protein families database: the classification resource after 15 years. Nucleic Acids Res 43:D213-21

29. Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, Potter SC, Punta M, Qureshi M, Sangrador-Vegas A, Salazar GA, Tate J, Bateman A (2016) The Pfam protein families database: towards a more sustainable future. Nucleic Acids Res 44:D279-85

30. Letunic I, Doerks T, Bork P (2015) SMART: recent updates, new developments and status in 2015. Nucleic Acids Res 43:D257-260

31. Sigrist CJ, de Castro E, Cerutti L, Cuche BA, Hulo N, Bridge A, Bougueleret L, Xenarios I (2013) New and continuing developments at PROSITE. Nucleic Acids Res 41:D344-347

32. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005) Protein Identification and Analysis Tools on the ExPASy Server. John M Walker (ed): The Proteomics Protocols Handbook, Humana Press pp 571-

607

FIGURE LEGENDS

Figure 1: Structure prediction of the N-terminal domain of human PNUTS

Consensus secondary structure prediction by PSIPRED [21]. The helical content predicted with reasonably high confidence (confidence factor >6) is approx. 49%.

Figure 2: Recombinant production of the N-terminal domain of human PNUTS

A) SDS-PAGE of cell lysate and soluble protein fractions post-clarification by centrifugation; **B)** SDS-PAGE showing the purified His_6-$PNUTS^{1-158}$ protein product at the end of the chromatographic process.

Figure 3: CD characterization of $PNUTS^{1-158}$

A) CD spectrum recorded in 10mM sodium phosphate pH 7.4, 0.5mM TCEP; **B)** CD-monitored thermal denaturation curve showing the change of CD signal at λ=208 nm.

Figure 4: Thermal denaturation of $PNUTS^{1-158}$ monitored by NMR

Superposition of $^{1}H^{15}N$ HSQC spectra of $PNUTS^{1-158}$ at various temperatures in 20mM HEPES pH 7.5, 150mM NaCl, 3mM β-mercaptoethanol measured at 800MHz. The linear change in chemical shifts of cross-peaks upon temperature increase is clearly noticeable.