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Short communication

Mapping the functional synthetase domain of trypanothione synthetase from *Leishmania major*

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Trypanothione synthetase (TryS) is a protein unique to the Kinetoplastida and has been shown to be essential in *Trypanosoma brucei* by RNAi techniques [1,2]. TryS is the sole enzyme responsible for the biosynthesis of trypanothione in the human pathogenic parasites *T. brucei*, *Trypanosoma cruzi* and *Leishmania major* [3–5]. The protein is thought to have two domains: an ATP-dependent synthetase domain that generates the intermediate, glutathionylspermidine, and the final product, trypanothione, from glutathione and spermidine; and an amidase domain, which can hydrolyse glutathionylspermidine and trypanothione to the original substrates. There has only been one previous study that succeeded in resolving these domains from the homologous enzyme glutathionylspermidine synthetase (GspS) from *Escherichia coli* [6]. GspS typically shares about 48% amino acid similarity and 32% identity with the TryS proteins from the various species. To date, the three-dimensional crystal structure for GspS and TryS has not been elucidated and although a short communication outlining the optimized NMR conditions for the N-terminal amidase domain of GspS from *E. coli* was published a number of years ago [7], no subsequent data have been released. Little is known about the active sites of TryS, although site-directed mutagenesis has highlighted an essential Cys residue for the amidase activity of GspS from *Crithidia fasciculata* which is conserved across all species of GspS and TryS [8]. In addition, the important roles of Arg-553 and Arg-613 for synthetase activity were recently identified in TryS from *C. fasciculata* [9]. Our attempts to crystallise the full length TryS from the various species of trypanosomatids have so far been met with limited success, leading us to try and generate the C-terminal synthetase domain of TryS from *L. major*. In an attempt to determine the domain boundary the application of GlobPlot (a web service tool http://globplot.embl.de) was utilized to predict the propensity of the TryS protein sequence to be ordered or disordered and thus identify domains within proteins [10]. This should highlight the inter-domain region and hence be useful in identifying the synthetase domain region. The full length sequence of TryS from *L. major* was analysed using Russell/Linding propensities and SMART/Pfam domain predictions for globular domain hunting. The graph obtained indicates the Pfam predicted amidase (residues 39–173) and synthetase (residues 237–632) domains, an inter-domain region of 64 amino acids (residues174–236), as well as the N- and C-terminal segments (Fig. 1A). The predicted disordered segments by Russell/Linding definition for TryS are at amino acid positions 1–40, 122–133, 190–216, 476–483 and 643–650, indicating high degrees of flexibility in the polypeptide chain in these regions (Fig. 1B). The disordered region, residues 190–216 (capitalised and underlined in Fig. 1B), is contained within the proposed inter-domain linker region.

Our initial approach was to generate the synthetase domain of *Lm*TryS using the alignment generated by GlobPlot of the known synthetase domains (starting from residues 237, highlighted in Fig. 1A by the green hatched region). In order to create this as a His-tagged construct, polymerase chain reaction amplification using *Pfu* polymerase (Promega) and full length *LmTRYS*_pET15b plasmid DNA [5] as template was carried out to generate the construct ΔN236 (ΔN indicates an N-terminal mutant and the number specifies the number of amino acids deleted from the full length sequence) using 5′-**cat atg** gac gtc agc cgt acc cgc ctg gag g-3′ as the sense primer (the *Nde*I site is indicated in bold) and the reverse primer 5′-**gga tcc** tta gtc gag cca gtt cgc ctt gga c-3′ which includes a *Bam*HI site (in bold)

Abbreviations: GspS, glutathionylspermidine synthetase; TryS, trypanothione synthetase; $T[SH]_2$, trypanothione

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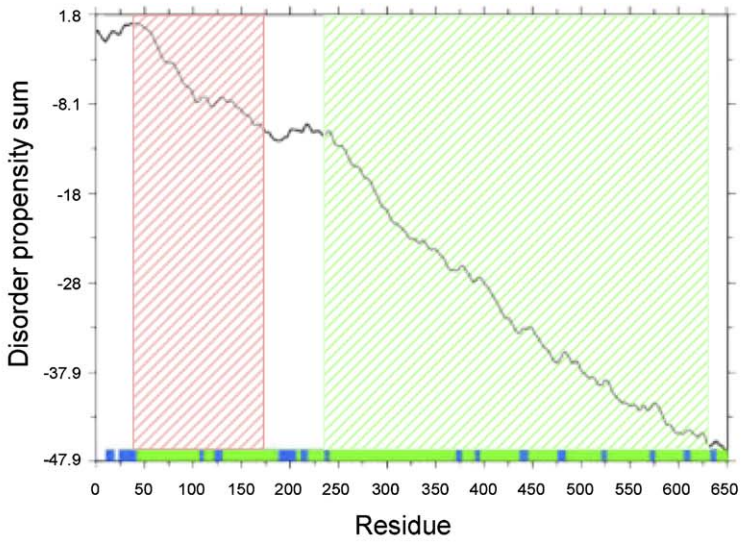
(A)



(B)

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MSSLQRASVS FNKPGHIPFG AVQGYAPGGV PAYSNKHDHY fsgerniedn iffgfkyqcv efarrwllvr kglllpdvnw   80
achifqlkev rdaattesfa vlqvrngttt kpeadallvy pSTDANPVGH VGTitevgdd yvcvadqnyr fhkwesscay  160
klkldhrdgi wtiiddidad eieiplgwlT FPGRANRPEG APPVALHPSL HFKEPPkpyl lrrnflptes kanwldmnnp  240
aerlfveefg mdvsrtrlee kvvsyyesnh efhlrcvayg tqlhaifmea taqviesdek lrlfaipeef wprirhswky  320
qqtyisgrfd fafnnetgev kcfeynadsa stllecgliq qkwaesvgld kqdtrgsgfa vernlkmawa nsgatgrvhf  400
cvdeereeqy talycmqaae avglegklci lfdefrfddn ghvvdsdgvr vrnvwktwmw esaitdyyaa reergENWKP  480
SPKdkvrlcd lllgddweil yfepmwkvip snkailpmiy hnhpehpail kaeyeltdel rkhgyakkpi vgrvgsnvii  560
tsgdgvvhae sggkygkrnm iyqqlfelkk qddyyaiigg wmigdafsgt giredksvit gvdspfaavr iktdklphpv  640
tlKDIDKMAE de                                                                            652
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Fig. 1. GlobPlot analysis of *Lm*TryS. (A) Graphical representation of the Pfam predicted globular amidase and synthetase domains of *Lm*TryS (accession number: AJ311570). *Red* and *green hatched area*, represent the predicted amidase and synthetase domains of *Lm*TryS, respectively. The solid *green* and *blue* bars on the *x*-axis highlight the globular and disordered regions of the protein, respectively. (B) The underlined region highlights the inter-domain linker region of TryS, the predicted disordered segments by Russell/Linding definition are capitalised and in bold, the conserved cysteine and histidine residues within the amidase domain are marked with an arrow and the start sites for the mutated synthetase truncations are in red.

and a stop codon. The subsequent PCR product was then cloned and sequenced in pCR-Blunt II-TOPO (Invitrogen) before subcloning into the *Nde*I/*Bam*HI site of pET15b (Novagen), with an N-terminal hexahis tag. Although soluble protein was obtained for this truncated protein no synthetase activity could be detected.

From this result, it would appear that residues out with this region are essential for trypanothione synthetase activity. With this in mind, a series of truncated fragments of TryS were generated and appropriate primers were synthesised that encompassed the region including the proposed inter-domain region as well as a portion of the amidase domain. Where possible, an existing methionine residue from the sequence was used; otherwise, one was introduced via the *Nde*I site required for insertion into pET15b. The following sense primers were used for the synthesis of each construct: 5′-**cat atg** tcc tcc tgc gcg tac aag ctg aag c-3′ for ΔN155, 5′-**cat atg** gat gac att gac gcg gac gaa atc g-3′ for ΔN174, 5′-**cat atg** ctg cat ccg tca ctg cac ttc aag g-3′ for ΔN205, 5′-**cat atg** aac aac ccg gcg gag cgg cta ttt g-3′ for ΔN250 and the same reverse primer as for ΔN236. A similar cloning strategy was then adopted for each construct as previously outlined for ΔN236.

The resulting constructs (see Fig. 2A for overview) were transformed into BL21Star(DE3)pLysS competent cells and a single colony was used to inoculate 10 ml of LB media with the appropriate antibiotics. Cultures were grown until the OD_{600} reached ∼0.6, cooled to 22 °C and the cultures were then induced overnight with isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.5 mM. Aliquots (1 ml) were taken from each culture and processed to allow the evaluation of both the expression levels by Western blotting and thiol analysis by HPLC.

For Western blotting, a 1 ml aliquot of overnight expressed culture was harvested by centrifugation (6000 × *g*, 5 min, 22 °C) and resuspended in 60 μl of Bugbuster (Novagen) protein extraction reagent containing 1.2 units of benzonase nuclease (Novagen). The cell suspension was then mixed using a rotary mixer for about 20 min at room temperature. Insoluble cell debris was removed by centrifugation (16,000 × *g*, 20 min, 4 °C) and the supernatant was transferred to a fresh tube. Protein concentration was determined by Bradford assay against a BSA standard and typically 5 μg of crude cell lysate was loaded per lane on a NuPAGE Novex 4–12% Bis–Tris gel (Invitrogen) before transferring to polyvinylidene fluoride membrane for subsequent Western analysis (Fig. 2B). Analysis by densitometry show that construct ΔN250, ΔN236 and ΔN205 are expressed at equivalent levels (103, 90 and 94%, respectively) to the full length *Lm*TryS, whereas construct ΔN174 was less well