STUDIES OF PROTEIN COMPLEXES INVOLVED IN THE ADENYLYLATION CASCADE OF THE NITROGEN SIGNALLING PATHWAY IN *Escherichia coli.*

Thesis submitted by
Paula Clancy BSc (JCU), MSc (JCU)
in March 2004.

For the degree of Doctor of Philosophy
in the Department of Biochemistry and Molecular Biology
James Cook University of North Queensland.

I dedicate this work to my father who was always very proud of his girls, and won't be here to see its completion.

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institute of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

……. **……**

Paula Clancy **Mar 2004**

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ABSTRACT

Adenylyl transferase (ATase), the *glnE* gene product from *Escherichia coli*, is a bifunctional enzyme that catalyses the opposing adenylylation and deadenylylation of glutamine synthetase (GS). The overall aim of this thesis was elucidation of the molecular mechanisms of the adenylylation cascade.

A new central domain has been identified using ATase truncation constructs in activity assays and solubility trials. This new regulatory domain is flanked by two flexible Q-linkers, Q1 and Q2. Thus the topology of ATase can be represented as N-Q1-R-Q2-C. The N domain was PII-UMP independent in *in vitro* deadenylylation assays, and had 1000 fold less activity then entire ATase, suggesting PII-UMP binding impacts on the conformation of the deadenylylation active site.

Monoclonal antibodies (mAbs) generated in this work against ATase were characterised using ATase mutants and the truncated proteins. Two mAbs, 5A7 (binds residues 502-548) and 39G11 (binds residues 466-501) both binding in the R domain, blocked the binding of PII, GlnK, PII-UMP and GlnK-UMP to ATase. This is the first report that pinpoints the effector-protein binding sites to within the R domain of ATase.

Both PII and ATase bound α-ketoglutarate (α-kg) in direct binding assays. Several lines of evidence suggested that PII contains the high affinity α-kg binding site and ATase the low affinity site. This study demonstrates for the first time that the two small effector-molecules α-kg and glutamine (gln) probably bind in the last 340 residues of ATase (Q2-C domain), possibly near the adenylylation active site.

The demonstration that the ATase mutant W694G presented a gln independent phenotype suggests that the bulky side chain of Trp 694 must move out of the adenylylation active site, so that GS can dock and be modified. Surface plasmon resonance (SPR) data suggested the binding of gln within Q2-C is transmitted to the R domain as an allosteric inhibitor of PII-UMP binding, and consequently deadenylylation.

A panel of mAbs was also produced against PII and characterised using a series of PII mutants. Two of the PII mAbs 19G4 (binds PII/GlnK) and 24H2 (binds PII) were used further to demonstrate that heterotrimers are formed between PII and GlnK *in vivo* in nitrogen starved cells.

It is well documented that the T-loops of PII and GlnK are probably the principal regions used by these signalling proteins to bind to the various receptor-proteins such as ATase, UTase and NRII. This study suggests PII and GlnK also interact with GS. Using PII mutants carrying specific GlnK residues at positions 43, 52 and 54 in the T-loop this study demonstrated the Asp at position 54 was the critical determinant of PII T-loop binding to GS, whereas the interaction with UTase involved both Asp54 and Thr43.

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Figure 1.1 ***Diagrammatic presentation of the nitrogen-signalling pathway in E. coli.*** Derived from van Heeswijk (1998). Cellular nitrogen level sensed by UTase. In high N conditions UTase deuridylylates the signalling molecules PII-UMP/GlnK-UMP. PII then stimulates ATase to adenylylate GS (inactivation). PII also interacts with NRII stimulating it to phosphorylate NRI. NRI-P activates expression of *glnA* (GS). In low N conditions PII is uridylylated by UTase. PII-UMP then stimulates ATase to deadenylylate GS-AMP (activation) 3

Figure 1.2 ***Structure of Glutamine synthetase.*** Schematic diagram of the GS protein from *Salmonella typhimurium* (GS_{ST}) derived from the 3D X-ray crystal structure (Yamashita *et al.*, 1989). 9

Figure 1.3 ***Sequence alignment of various nucleotidyl transferases using polymerase motif.*** (a) Overlay of polymerase motifs from known 3D structures of nucleotidyl transferases (NT). Deadenylylation domain of ATase ($AT\text{-}N_{440}$) (green) (Xu *et al.*, 2003b), human polymerase β (orange) (Sawaya *et al.*, 1997), kanamycin NT (magenta) (Pedersen *et al.*, 1995) and yeast poly A polymerase (cyan) (Bard *et al.*, 2000; Martin *et al.*, 2000). (b) Sequence alignment derived from structural overlay (Xu *et al.*, 2003b). This sequence alignment differs slightly from the original alignment of Holm and Sander (1995). The UTase protein from *E. coli* has one β polymerase motif and the ATase protein from the same organism has two β polymerase motifs. Highly conserved residues such as the double Asp in the catalytic site of rat DNA polymerase β have been highlighted in red and other residues also mutated in the two ATase active sites are highlighted in black (McLoughlin, 1999). 12

Figure 1.4 ***Analysis of the α-helix in the ATase Q1 linker.*** (a) Top view representation of the helical region of Q1 from residues 448-461. The respective amino acids and their relative positions in the helix are indicated on the helical wheel. The hydrophobic residues are highlighted in red, whilst the hydrophilic residues are black. The hydrophobicity of the residues was assigned according to Engelman *et al.*, (1986). (b) Schematic representation of $AT\text{-}C_{521}$, which includes the Q1 linker. The hydrophobic side of the α-helix in Q1 is associated with a proposed hydrophobic patch in the N-terminal region of the construct i.e. the putative R domain (diagram courtesy of Jaggi, 1998). 13

Figure 1.5 ***Schematic representation of the truncation constructs of ATase.*** N- and C-terminal truncation constructs of ATase produced by Jaggi (1998), Wen (2000), and O Donnell (2000). Truncations of the ATase protein (946 residues long) were designated AT-N or AT-C depending on their location in the linear polypeptide chain and the number of amino acid residues contained in each truncation construct is indicated by a subscripted figure (eg. $AT\text{-}N_{440}$ refers to the N-terminal 440 residues of ATase). Also indicated on the diagram are the positions of the two predicted β-polymerase motifs (BPM1 & BPM2) (Holm and Sander, 1995) and the two Q-linkers (Q1 & Q2) (Wooton and Drummond, 1989). The black segments represent the boundaries between the adjoining constructs. 14

Figure 1.6 ***Structural model proposed for ATase.*** Jaggi (1998) proposed a structural model for ATase based on solubility and activity studies of the ATase truncation constructs (Figure 1.5). The ATase protein probably comprises three domains; the first of which, is the N-terminal domain (residues 1-440) which binds PII and PII-UMP/α-kg and is responsible for the deadenylylation of the inactive GS-AMP protein. The second domain is the central domain (residues 463-604) which probably binds gln, and the third domain is the C-terminal domain (residues 627-946) which is responsible for adenylylation of the active GS protein. There are also two "Q" linker regions: Q1 (residues 441-462) and Q2 (residues 606-627) (Jaggi, 1998). 15

Figure 1.7 ***Structure of N domain of ATase.*** The 3D structure of the AT-N_{440} construct (Figure 1.4) has now been solved with X-ray crystallography (Xu *et al.*, 2003b). The two highly conserved Asp173 and Asp175 residues within the deadenylylation active site (Holm and Sander, 1995), which are important for positioning the Mg^{2+} ion required for deadenylylation activity, and the Asn169 residue, which probably helps position the phosphate involved in the deadenylylation reaction correctly have been highlighted. 16

Figure 1.8 ***Structure of N-terminal domain of NRI.*** Structure of the N-terminal domain of the NRI protein, which contains the highly conserved Asp54 residue (site of reversible phosphorylation). This structure was determined using NMR spectroscopy (Volkman *et al.*, 1995). 18

Figure 1.9 ***Structure of the nitrogen signalling proteins PII and GlnK of E. coli.*** X-ray crystal structure of the PII (pink) (Carr *et al.*, 1996) and GlnK (cyan) (Xu *et al.*, 1998) proteins from *E. coli* have been overlaid to demonstrate their structural similarities. In (a) the monomer is depicted and in (b) the trimer is depicted. The three T-loop residues where they differ have also been highlighted. It must be noted that the position of the T-loops in these three dimensional structures is constrained by the crystal lattice of the protein crystal and only indicative of one of the many potential conformations for the T-loop, which is probably mobile in solution. 22

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Figure 3.2 ***Indirect ELISA for screening mAb binding to PII and mutants.*** Schematic diagram of the principles behind the indirect ELISA used to characterise PII_{wt}, PII mutants and GlnK. 83

Figure 3.3 ***PII/GlnK heterotrimers form in vivo.*** Native gel (see section 2.2.6.2) of extracts from wild type *E. coli* (YMC10) and a PII deficient (RB9060) and GlnK deficient *E. coli* strain (WCH30) probed with mAb 19G4 (PII/GlnK) lanes 1-3, and mAb 24H2 (PII specific) lanes 4-6. van Heeswijk *et al*., (2000) *PNAS* **97**:3942-3947. 90

Figure 3.4 ***Antigenic residues of the PII protein.*** The antigenic residues determined from the comprehensive ELISA screens (Tables 3.2-3.5) have been highlighted in (a) ribbon diagram and (b) a surface diagram of the PII protein (Xu *et al.*, 1998). The three or four residues which showed a reduction in mAb binding are highlighted in red and the two residues, which still bound to all the mAbs but at a lower level are highlighted in blue. 91

Figure 3.5 ***R domain mAb binding to ATase and truncation constructs.*** Western blot of 12% SDS PAGE (see section 2.2.6.5) of whole cell extracts for the ATase protein and various ATase truncation constructs (Table 3.7) using (a) mAb 5A7 as purified ascitic fluid (see section 2.2.9.8) and (b) mAb 39G11 as crude ascitic fluid. The bands indicating the appropriate induced proteins are marked with arrows. 94

Figure 3.6 ***C domain mAbs binding to ATase, truncation constructs and mutants.*** Western blot of 12% SDS PAGE (see section 2.2.6.5) of whole cell extracts for the ATase protein, various ATase truncation constructs (Table 3.7) and ATase mutants using the C domain mAbs (a) 6A3 and (b)27D7 as purified ascitic fluid (see section 2.2.9.8). The bands indicating the appropriate induced proteins are marked with arrows. 96

Figure 3.7 ***N domain mAb binding to ATase, truncation constructs and mutants.*** Western blot of 12% SDS PAGE gel (see section 2.2.6.5) of whole cell extracts for the ATase protein, various ATase truncation constructs and ATase point mutant proteins using the N domain mAb 6B5 as purified ascitic fluid (see section 2.2.9.8). The bands indicating the appropriate induced proteins are marked with arrows. 97

Figure 3.8 ***Binding region of N domain mAb.*** Surface diagram of AT-N_{440} (Xu *et al.*, 2003b) the deadenylylation domain of ATase. The purple region is the first 311 residues of the protein where the 6B5 mAb binds. Also shown on the diagram is the binding cavity for the GS-AMP protein. 98

Figure 3.9 ***Monoclonal antibody binding regions of the ATase protein.*** This diagram shows the regions of the ATase protein where the mAbs are binding derived from the Western blotting data using all the ATase truncation constructs and ATase point mutant proteins (see section 3.3.3). Also shown in the diagram are the putative active sites (Holm and Sander, 1995) and the two putative Q-linkers (Wooton and Drummond, 1989). 99

Figure 4.1 ***Sequence alignment of several representative PII-like proteins.*** Sequences for 14 PII homologue proteins from different species recovered from the SWISS-prot data bank have been aligned. Residues highlighted in red were conserved in all of the 50 sequences retrieved. Residues highlighted in blue represent 1-3 changes out of the 50 sequences retrieved: Leu20 had 1 change, Tyr51 had 2 changes, Lys58 had 1 change, Thr83 had 3 changes, Asp88 had 1 change and Phe92 had 2 changes. Comparative sequence identities with the *E. coli* PII protein are given as a percentage. Secondary structural elements are designated according to Carr *et al.*, (1996). Abbreviations and references are as follows: glnB_ecoli (*E. coli*; Vasudevan *et al.*, 1991), glnB_klepn (*Klebsiella pneumoniae*; Holtel and Merrick, 1988), glnB_haein (*Haemophilus influenzae*; Fleischmann, 1995), glnB_braja (*Bradyrhizobium japonicum*; Martin *et al.*, 1989), glnB_azobr (*Azospirillum brasilense*; De Zamarockzy *et al.*, 1990), glnB_rhime (*Rhizobium meliloti*; Arcondeguy *et al.*, 1996), glnB_rhoru (*Rhodospirillum rubrum*; Johansson and Nordlund, 1996), glnB_rhoca (*Rhodobacter capsulatus*; Kranz *et al.*, 1990), glnK_ecoli (*E. coli* GlnK; van Heeswijk *et al.*, 1995), glnB_rhilv (*Rhizobium leguminosarum*; Colonna-Romano *et al.*, 1987), glnB_synp7 (*Synechococcus* sp. PCC 7942; Tsinoremas *et al.*, 1991), glnB_porpu (*Porphyra purpurea*; Reith and Munholland, 1993), nrgb_bacsu (*Bacillus subtilis*; Wray *et al.*, 1994) and gln1_metmp (*Methanococcus maripaludis*; Kessler *et al.*, 1998). 103

Figure 4.2 ***Location of mutated residues within the 3D model of the PII protein.*** In this diagram the mutated residues have been highlighted in the (a) monomer, (b) trimer and (c) trimer surface of the *E. coli* PII protein. The residues have been assigned to the

important regions of the protein, such that green sidechains are T-loop mutants, blue sidechains are ATP-binding cleft entrance mutants, and red sidechains are ATP-binding cleft mutants. 104

Figure 4.3 ***Adenylylation assays for PII and PII mutants.*** The representative curves (a & b) show adenylylation of the GS protein stimulated by several of the mutants and PII_{wt} protein. In (a) the assay has reached a steady state and in (b) only the first 5min are examined. Standard adenylylation conditions (see section 2.2.10.2.1) were used except that the GS concentration used was 25nM (half the normal concentration). The assays were also run with high α-kg (1mM) and low α-kg (10μM). All assays were performed in duplicate and with PII_{wt} as a reference. The initial rate curves were fitted with a linear regression using Microsoft Excel. For each of the mutants that had activity in the assay the R^2 coefficient for the linear regression was >94%, often 99% (except for T43A, which was 90% and E106A, which was 91%). PII_{wt} (open diamond), PII:Y51S (closed square), PII:G24D (closed triangle), PII:R103D (open triangle), PII:T104A (X), PII:K90N (open circle), no AT (closed diamond) and no PII (open square). The ATase protein has a small amount of activity when there is no PII protein present stimulated by gln alone (using half the normal concentration of GS protein minimised this activity). (c) Initial rates for all the mutant PII proteins expressed as a proportion of wild type activity without α-kg. 113

Figure 4.4 ***Surface Plasmon Resonance sensorgrams for PII and mutants interacting with ATase.*** This figure shows the sensorgrams for PII_{wt} (15μM) and several mutants interacting with ligated ATase in SPR using the Biacore X (see section 4.2.4). A CM5 dextran chip was ligated with approximately 86 $fmolmm^{-3}$ purified ATase using amine coupling. For a negative control the various small effector mixes with no protein were run across the ligated chip and the resulting curves subtracted from the runs where protein had been included. The subtracted curves were used for the analyses. Protein alone (black dashed), protein+1mM ATP+2mM Mg^{2+} (red dashed), protein+1mM ATP+2mM Mg^{2+}+2uM α-kg (solid black) and protein+1mM ATP+2mM Mg^{2+}+1mM α-kg (solid red). 115

Figure 4.5 ***Comparative adenylylation rates and direct binding of PII and several PII mutants to ATase.*** This curve shows the amount of PII_{wt} or PII mutant protein bound to the ligated ATase protein 1min post addition of analyte protein (RU) derived from Figure 4.4 presented as a proportion of PII_{wt} binding with no α-kg (solid colour). Also included in this curve are the adenylylation rates for these conditions (see section 4.2.3) presented as a proportion of PII_{wt} activity with no α-kg (diagonal stripe). All assays were performed in duplicate and with PII_{wt} protein as a reference. The initial rate curves were fitted with a linear regression using Microsoft Excel. For each of the mutant proteins that had activity in the assay the R^2 coefficient for the linear regression was >94%, often 99%. ATase has a small amount of adenylylation activity when there is no PII present stimulated by gln alone, this activity has been subtracted from the adenylylation rates. In both assays high α-kg was 1mM, but in SPR and adenylylation low α-kg was 2μM and 10μM, respectively. 116

Figure 4.6 ***3D model of PII complexed to ATP.*** (Xu *et al.*, 2001) In this (a) stick diagram and (b) surface diagram of the PII trimer (*E. coli*) the R103D residue has been highlighted in red and the complexed ATP molecule in cyan. Note the exposed section of the Asp at position 103 is adjacent to the exposed portion of the ATP molecule on the surface of the protein. In this crystal of PII the T-loop was disordered, so the structure of that region of the protein could not be resolved. 117

Figure 4.7 ***Uridylylation assays for PII and PII mutants.*** The representative curves (a & b) show uridylylation of several of the PII mutants and PII_{wt} (*E. coli*). In (a) the assay has reached a steady state and in (b) only the first 5min are examined. Standard uridylylation conditions were used (see section 4.2.5). All assays were performed in duplicate and with PII_{wt} as a reference. The initial rate curves have been fitted with a linear regression using Microsoft Excel. For each of the PII mutants that showed activity in the assay the R^2 coefficient for the linear regression was >94%, often 99% (except for R103D, which was 90%). PII_{wt} (open diamond), PII:G24AT26A (closed square), PII:G24D (closed triangle), PII:Y51F (asterisk), PII:T104A (X), PII:E106A (closed circle). (c) Initial rates for all the PII mutants expressed as a proportion of wild type activity. 118

Figure 5.1 ***Structure of the nitrogen signalling proteins PII and GlnK of E. coli.*** X-ray crystal structure of the PII (pink) (Carr *et al.*, 1996) and GlnK (cyan) (Xu *et al.*, 1998) proteins from *E. coli* have been overlaid to demonstrate their structural similarities. In (a) the monomer is depicted and in (b) the trimer is depicted. The three residues that differ in the T-loop have also been highlighted. (c) The 112 residues from the monomers of both proteins have been aligned. Non-conserved residues are highlighted in blue, and the highly conserved T-loop is highlighted in red. The three non-conserved residues within the T-loop: T43A, M52S and D54N are highlighted in purple. 125

Figure 5.2 ***Regulation of PII conformation.*** The main C signal is α-kg. Trimeric PII has three α-kg binding sites and three uridylylation sites. The binding of α-kg influences the ability of PII to interact with ATase and NRII, both of which are involved in N regulation. At low α-kg concentrations, the conformation of PII is such that it is able to interact with ATase and NRII. At high α-kg concentrations, the conformation of PII is such that it cannot interact with ATase or NRII. Additionally, uridylylation reduces the negative co-operativity in α-kg binding. Ovals, circles and triangles are used to represent the three different conformations of PII. Small black dots represent bound molecules of α-kg (Diagram reproduced from Ninfa and Atkinson, 2000). 126

Figure 5.3 ***Adenylylation assay using the effector-proteins PII and GlnK.*** This assay shows the improvement in activity of the ATase protein with increasing concentrations of the GlnK protein by measuring the production of γ-glutamyl hydroxamate by GS. Both (a) standard assay conditions (see section 5.2.3) & (b) standard assay conditions + 10μM α-kg were used. No AT (♦), 0.025μM PII (□), no PII (▲), 0.025μM GlnK (●), 0.125μM GlnK (Δ), 0.25μM GlnK (○). For (b) only 0.025μM PII (no α-kg) (■). All assays were performed in duplicate and with PII_{wt} as a reference. Error bars have not been shown on the curves as they hinder visual inspection. The standard error range for all the curves is generally <0.4. 134

Figure 5.4 ***Deadenylylation assay using the effector-proteins PII-UMP and GlnK-UMP.*** This assay (see section 5.2.3) shows the improvement in deadenylylation activity of the ATase protein with increasing concentrations of the GlnK-UMP effector-protein by measuring the production of γ-glutamyl hydroxamate by GS-AMP. No AT (◊), 0.025μM PII-UMP (■), 0.025μM GlnK-UMP (▲), 1μM GlnK-UMP (●), 2uM GlnK-UMP (♦). All assays were performed in duplicate and with PII_{wt} as a reference. Error bars have not been shown on the curves as they hinder visual inspection. The standard error range for all the curves is generally <0.4. 135

Figure 5.5 ***Glutamine effect on PII-UMP and GlnK-UMP stimulation in deadenylylation with no α-kg present.*** This deadenylylation assay (see section 5.2.3) measured the amount of γ-glutamyl hydroxamate produced by GS-AMP, when

the assay used twice as much GS-AMP and uridylylated effector-proteins as standard conditions (see section 5.2.3), no α-kg and with/without gln (20mM). The initial rate curves have been fitted with a linear regression using Microsoft Excel. 50nM PII-UMP, no kg (◊), 50nM PII-UMP, no kg, gln (20mM) (■), 4μM GlnK-UMP, no kg (Δ), 4μM GlnK-UMP, no kg, gln (20mM) (○). All assays were performed in duplicate and with PII_{wt} as a reference. Error bars have not been shown on the curves as they hinder visual inspection. The standard error range for all the curves is generally <0.4. 136

Figure 5.6 ***PII mediated adenylylation effector-protein inhibition assay.*** This curve shows the changes in activity of ATase stimulated by PII with additional effector-proteins added to the adenylylation assay. Activity is measured by the production of γ-glutamyl hydroxamate by GS. Standard assay conditions (see section 5.2.3) were used. PII (25nM) only (♦), PII (25nM + PII-UMP (25nM) (□), PII (25nM + PII-UMP (500nM) (▲), PII (25nM) + GlnK (25nM) (○), PII (25nM) + GlnK-UMP (25nM) (Δ). All assays were performed in duplicate and with PII_{wt} as a reference. Error bars have not been shown on the curves as they hinder visual inspection. The standard error range for all the curves is generally <0.4. 137

Figure 5.7 ***PII-UMP mediated deadenylylation effector-protein inhibition assay.*** This assay shows the changes in activity of the ATase protein stimulated by the PII-UMP protein with additional effector-proteins added to the deadenylylation assay. Activity is measured by the production of γ-glutamyl hydroxamate by GS-AMP. Standard assay conditions (see section 5.2.3) were used. PII-UMP (25nM) only (◊), PII-UMP (25nM) + PII (25nM) (■), PII-UMP (25nM) + PII (125nM) (▲), PII-UMP (25nM) + PII (5μM) (●), PII-UMP (25nM) + GlnK-UMP (250nM) (♦). All assays were performed in duplicate and with PII_{wt} as a reference. Error bars have not been shown on the curves as they hinder visual inspection. The standard error range for all the curves is generally <0.4. 138

Figure 5.8 ***ATP binding to PII and GlnK.*** PII and GlnK (10μM) were assessed for ATP binding (see section 5.2.4) using radio-labelled ^{14}C ATP with and without α-kg (1mM). PII (teal), GlnK (pink), PII+1mM kg (blue) and GlnK+1mM kg (red). 139

Figure 5.9 ***α-Ketoglutarate binding to PII and GlnK.*** PII and GlnK (10μM) were assessed for α-kg binding (see section 5.2.4) using radio-labelled ^{14}C α-kg in the presence of ATP (2mM). PII (blue) and GlnK (red). 140

Figure 5.10 ***Uridylylation assay for purified PII and GlnK.*** These curves show the steady state assay (see section 2.2.10.1.1) and the first 5min of the assay in the inset (see section 2.2.10.1.2). The initial rate curves were fitted with a linear regression using Microsoft Excel. The rates of incorporation of UTP^3H for PII and GlnK were 0.476 $\mu Mmin^{-1}$ and 0.7899 $\mu Mmin^{-1}$, respectively. 141

Figure 5.11 ***Comparative adenylylation rates for PII, GlnK and PII swapped mutants.*** The assay was performed with standard conditions (see section 5.2.3) (red) and standard conditions+10uM α-kg (blue). The activity of ATase was measured by the production of γ-glutamyl hydroxamate by GS. All assays were performed in duplicate and with PII_{wt} protein as a reference. The initial rate curves were fitted with a linear regression using Microsoft Excel. The initial rates for all the mutants have been expressed as a proportion of PII_{wt} protein standard activity. 142

Figure 5.12 ***Comparative deadenylylation rates for uridylylated PII, GlnK and swapped PII T-loop mutants.*** Standard assay conditions were used (see section

5.2.3) (red). The assay was also run with PII-UMP, GlnK-UMP and uridylylated mutant proteins at 5x standard concentration (blue). The activity of ATase was measured by the production of γ-glutamyl hydroxamate by GS-AMP. All assays were performed in duplicate and with PII_{wt} protein as a reference. The initial rate curves were fitted with a linear regression using Microsoft Excel. The initial rates for all the proteins have been expressed as a proportion of their individual standard activity. 143

Figure 5.13 ***Uridylylation of partly purified PII, GlnK and swapped PII T-loop mutants.*** This non-denaturing PAGE gel (9% acrylamide) shows the degree of uridylylation of all the proteins after 20 min in the standard uridylylation reaction (see section 5.2.6). The first lane for each protein is the protein prior to uridylylation. The second lane for each protein is the uridylylated protein. The lower band is fully uridylylated protein and the bands in between are partly uridylylated protein. 144

Figure 6.1 ***Schematic representation of the truncation constructs of ATase.*** (a) Truncations of the ATase protein (946 residues long) were designated AT-N or AT-C depending on their location in the linear polypeptide chain and the number of amino acid residues contained in each truncation construct is indicated by a subscripted figure (eg. $AT\text{-}N_{440}$ refers to the N-terminal 440 residues of ATase). Also indicated on the diagram are the positions of the two predicted β-polymerase motifs (BPM1 & BPM2) (Holm and Sander, 1995), the two Q-linkers (Q1 & Q2) (Wooton and Drummond, 1989), and the binding regions of the ATase mAbs (see Chapter 3). The solubility of the constructs is shown in pink. Soluble (S), partly soluble (PS), Insoluble (I), thermal induction ($_T$), and IPTG induction at low temperature ($_l$). (b) Western blot analysis of 12% SDS PAGE gel (see section 2.2.6.5) of whole cell extracts for the various truncation constructs using a purified mix of $AT\text{-}N_{548}$ and $AT\text{-}C_{522}$ polyclonal Ab ascitic fluid for detection (see section 6.2.4). The bands indicating the appropriate induced proteins are marked with arrows. 156

Figure 6.2 ***Generation of the R domain truncation construct.*** (a) PCR product (see section 6.2.2) and (b) ligated plasmid (see section 6.2.2) visualised by agarose gel electrophoresis (see section 2.2.3.1). (c) BLAST search of the NCBI database using sequence derived from R domain vector (see section 6.2.2). Abbreviations and references are as follows: sf 2457T (*Shigella flexneri* 2a; Wei *et al.*, 2003), ec CFT073 (*E. coli*; Welch *et al.*, 2002), ec K12 (*E. coli*; Blattner *et al.*, 1997), sf 301 (*S. flexneri* 2a; Jin *et al.*, 2002), and ec (*E. coli*; van Heeswijk *et al.*, 1993). 157

Figure 6.3 ***Over-expression and solubility test for the R domain construct.*** (a) Coomassie stained 15% PAGE gel showing expression of IPTG induced AT:RQ2 in BL21 (DE3) RecA cells (2.1.4) (see section 6.2.3). (b) Solubility analysis of the constantly expressed truncation construct AT:RQ2 at 18°C, 30°C and 37°C. Western blot of 15% SDS PAGE gel (see section 2.2.6.5) of whole cell extracts (wc) and cell-free lysates (sn) using purified mAb 5A7 (see section 2.2.9.4.2). 159

Figure 6.4 ***Adenylylation assays using ATase and C domain truncation constructs.*** These assays show the changes in activity of (a) ATase (purified), (b) $AT\text{-}C_{518}$ (purified), (c) $AT\text{-}C_{396}$ (purified), (d) $AT\text{-}C_{340}$ (cell lysate) and (e) AT:ΔR (cell lysate) (see section 6.2.1) under various conditions. Activity was assessed by determining the adenylylation state of GS by measuring the production of γ-glutamyl hydroxamate with various combinations of effector-molecules present in the assay. Standard assay conditions were used (see section 6.2.5). No AT/construct+PII+gln (♦), AT/construct+PII+gln (□), AT/construct-PII+gln (Δ), AT/construct+PII-gln (○), and AT/construct-PII-gln (*). All assays were performed in duplicate and with AT_{wt} as a reference. Error bars have not been shown on the curves as they hinder visual

inspection. The standard error range for all the curves is generally <0.4. (f) The first 5min were fitted with a linear regression using Microsoft Excel. The R^2 coefficients for these curves are usually >0.9. The initial rates for all the proteins have been expressed as a proportion of their standard activity. 161

Figure 6.5 ***Deadenylylation assays using ATase and the AT-N_{440} truncation construct.*** These assays show the changes in activity of (a) ATase (purified) and (b) N-terminal truncation construct AT-N_{440} (purified) (see section 6.2.1) under various conditions. Activity was assessed by determining the deadenylylation state of GS-AMP by measuring the production of γ-glutamyl hydroxamate with various combinations of effector-molecules present in the assay. Standard assay conditions were used (see section 6.2.5). No AT/construct+PII-UMP+α-kg (♦), AT/construct+PII-UMP+α-kg (□), AT/construct-PII-UMP+α-kg (Δ), and AT/construct+PII-UMP-α-kg (○). All assays were performed in duplicate and with AT_{wt} as a reference. Error bars have not been shown on the curves as they hinder visual inspection. The standard error range for all the curves is generally <0.4. (c) The first 5 min were fitted with a linear regression using Microsoft Excel. The R^2 coefficients for these curves are usually >0.9. The initial rates for all the proteins have been expressed as a proportion of their standard activity. 163

Figure 6.6 ***Inhibition of adenylylation and deadenylylation activity in ATase by N domain mAb 6B5.*** These assays show the changes in activity of ATase by measuring the production of γ-glutamyl hydroxamate by GS/GS-AMP when the 6B5 mAb is present in the (a) adenylylation and (b) deadenylylation assays. Standard assay conditions were used (see section 6.2.5). The purified mAb (see section 6.2.4) was preincubated with ATase (1:1) for 30min at room temperature. No AT+PII-UMP+α-kg (♦), AT+PII-UMP+α-kg (□), and AT+PII-UMP+α-kg+6B5 (○). All assays were performed in duplicate and with AT_{wt} as a reference. Error bars have not been shown on the curves as they hinder visual inspection. The standard error range for all the curves is generally <0.4. 166

Figure 6.7 ***Inhibition of adenylylation activity in ATase and AT-C_{518} by R domain mAbs 5A7 and 39G11.*** These assays show the changes in activity of (a) ATase and (b) the C-terminal truncation construct AT-C_{518} in adenylylation by measuring the production of γ-glutamyl hydroxamate by GS with R domain mAbs 5A7 and 39G11 present. Standard assay conditions (see section 6.2.5) were used and the mAbs (see section 6.2.4) were preincubated with AT/AT-C_{518} (1:1) for 30min at room temperature. No AT/AT-C_{518}+PII+gln (♦), AT/AT-C_{518}+PII+gln (□), AT/AT-C_{518}-PII+gln (Δ), AT/AT-C_{518}+PII+gln+5A7 (◊), and AT/AT-C_{518}+PII+gln+39G11 (-). All assays were performed in duplicate and with AT_{wt} as a reference. Error bars have not been shown on the curves as they hinder visual inspection. The standard error range for all the curves is generally <0.4. 167

Figure 6.8 ***Inhibition of deadenylylation activity in ATase by R domain mAb 5A7.*** This assay shows the change in activity of ATase in deadenylylation by measuring the production of γ-glutamyl hydroxamate by GS-AMP with R domain mAb 5A7 present. Standard assay conditions (see section 6.2.5) were used and the mAb (see section 6.2.4) was preincubated with AT (1:1) for 30min at room temperature. No AT+PII+gln (♦), AT+PII-UMP+α-kg (□), AT-PII-UMP+α-kg (Δ), and AT+PII-UMP+α-kg+5A7 (◊). All assays were performed in duplicate and with AT_{wt} as a reference. Error bars have not been shown on the curves as they hinder visual inspection. The standard error range for all the curves is generally <0.4. 168

Figure 6.9 ***Deadenylylation activity of ATase in the presence of C domain mAbs 6A3 and 27D7.*** These assays show the changes in activity of ATase in deadenylylation by measuring the production of γ-glutamyl hydroxamate by GS-AMP with C domain mAbs 6A3 and 27D7 present. Standard assay conditions (see section 6.2.5) were used and the mAbs (see section 6.2.4) were preincubated with AT (1:1) for 30min at room temperature. No AT+PII-UMP+α-kg (♦), AT+PII-UMP+α-kg (□), AT+PII-UMP+α-kg+6A3 (+), and AT+PII-UMP+α-kg+27D7 (*). All assays were performed in duplicate and with AT_{wt} as a reference. Error bars have not been shown on the curves as they hinder visual inspection. The standard error range for all the curves is generally <0.4. 169

Figure 6.10 ***Adenylylation activity of ATase and AT-C_{518} in the presence of C domain mAbs 6A3 and 27D7.*** These assays show the changes in activity of (a) ATase and (b) the C-terminal truncation construct AT-C_{518} in adenylylation by measuring the production of γ-glutamyl hydroxamate by GS with C domain mAbs 6A3 and 27D7 present. Standard assay conditions (see section 6.2.5) were used and the mAbs (see section 6.2.4) were preincubated with AT/AT-C_{518} (1:1) for 30min at room temperature. No AT/AT-C_{518}+PII+gln (♦), AT/AT-C_{518}+PII+gln (□), AT/AT-C_{518}-PII+gln (Δ), AT/AT-C_{518}+PII+gln+6A3 (+), and AT/AT-C_{518}+PII+gln+27D7 (*). All assays were performed in duplicate and with AT_{wt} as a reference. Error bars have not been shown on the curves as they hinder visual inspection. The standard error range for all the curves is generally <0.6. 170

Figure 6.11 ***Inhibition of C-terminal truncation construct activity by C domain mAbs 6A3 and 27D7 in adenylylation with no glutamine.*** These assays show the changes in activity of the C-terminal truncation constructs (a) AT-C_{518} (purified; see section 6.2.1), (b) AT-C_{396} (purified; see section 6.2.1), and (c) AT-C_{340} (cell lysate; see section 6.2.1) in adenylylation with no gln. Activity was measured by the production of γ-glutamyl hydroxamate by GS with C domain mAbs 6A3 and 27D7 present. Standard assay conditions (see section 6.2.5) were used and the mAbs (see section 6.2.4) were preincubated with the constructs (1:1) for 30min at room temperature. No construct+PII+gln (♦), construct+PII+gln (□), construct+PII-gln (Δ), construct+PII-gln+6A3 (○), and construct+PII-gln+27D7 (*). All assays were performed in duplicate and with AT_{wt} as a reference. Error bars have not been shown on the curves as they hinder visual inspection. The standard error range for all the curves is generally <0.4. 171

Figure 6.12 ***Schematic representation of the different conformations of ATase in adenylylation and deadenylylation.*** The activity results from the adenylylation and deadenylylation assays shown in Figures 6.4 and 6.5 have been summarised in this diagram. Uncomplexed ATase has a "closed" conformation (Jaggi, 1998) and has minimal activity in either assay. Removal of the N or R domains gives rise to polypeptides with similar adenylylation activity to PII complexed ATase and removal of the R+C domain gives rise to a polypeptide, which has activity independent of PII-UMP in deadenylylation. Addition of PII to the adenylylation assay or PII-UMP to the deadenylylation assay causes a shift in the position of the N domain relative to the C domain and ATase adopts the "open" conformation (Jaggi, 1998). The complexed ATase is then capable of adenylylating GS or deadenylylating GS-AMP, depending on the other effectors present in the assay. The adenylylation active site is shown in white and is accessible to GS in all the conformations except the uncomplexed "closed" conformation and the deadenylylation active site shown in cyan is accessible to GS-AMP in all conformations except the uncomplexed "closed" conformation. 175

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ABBREVIATIONS

Ab	Antibody
ABTS	2,2'-azino-bis 3-ethylbenzthiazoline 6-sulphonic acid
ADP	Adenosine diphosphate
Ag	Antigen
Ala	Alanine residue
AMP	Adenosine monophosphate
Arg	Arginine residue
Asn	Asparagine
Asp	Aspartate residue
ATase	Adenylyltransferase
ATP	Adenosine triphosphate
BCIP	15-bromo-4-chloro-3-indolyl phosphate p-toluidine salt
bp	Base pair
C	Carbon
^{o}C	Degrees Celsius
cm^2	Centimetres squared
CPU	Central processing unit
CTAB	Cetyltrimethyl ammonium bromide
CTP	Cytidine triphosphate
d	Day
DEAE	Diethylaminoethyl
DMEM	Dulbeccos minimal essential media
DMF	Dimethylformamide
DMSO	Dimethylsuphoxide
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoassay
Ep	Epitope
g	Gram
GDH	Glutamate dehydrogenase
gln	Glutamine
Gln	Glutamine residue
glu	Glutamate
Glu	Glutamate residue
Gly	Glycine residue
GOGAT	Gltuamine amide-2-oxoglutarate amino transferase
GS	Glutamine synthetase
GS_{MT}	Glutamine synthetase from *Mycobacterium tuberculosis*
GS_{ST}	Glutamine synthetase from *Salmonella typhimurium*
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine residue
HRPO	Horseraddish peroxidase
IgG	Immunoglobulin G

IgM	Immunoglobulin M
Ile	Isoleucine residue
IP	Intraperitoneally
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IV	Intravenously
K_d	Disassociation constant
K_m	Michaelis constant
kD	Kilodalton
α-kg	α-Ketoglutarate
L	Litre
λ	Wavelength
LB	Luria Bertani broth
Leu	Leucine residue
Lys	Lysine residue
M	Molar
mAb	Monoclonal antibody
Met	Methionine residue
Mg^{2+}	Magnesium ion
min	Minute
mg	Miligram
mL	Mililitre
mL^{-1}	Per mililitre
mM	Milimolar
Mn^{2+}	Manganese ion
N	Nitrogen
$NADP^+$	Nicotinamide adenine dinucleotide phosphate, oxidised form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NBT	Nitrobluetetrazolium
NH_3	Ammonia
nm	Nanometre
NMR	Nuclear magnetic resonance
NP40	Nonidet P40
OD_{405}	Optical density measured at wavelength 405 nanometres
OD_{492}	Optical density measured at wavelength 492 nanometres
OD_{595}	Optical density measured at wavelength 595 nanometres
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethyleneglycol
Phe	Phenylalanine residue
PMSF	Phenylmethylsulphonyl fluoride
Pro	Proline residue
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodiumdodecylsulphate
Ser	Serine residue
SPR	Surface plasmon resonance
TCA	Trichloracetic acid
TEMED	N,N,N,N-tetramethylethylenediamine
Thr	Threonine residue

Tyr	Tyrosine residue
μCi	Microcuries
μg	Microgram
μL	Microlitre
μm	Micrometre
μM	Micromolar
UMP	Uridine monosphate
UTase	Uridylyltransferase-uridylyl removing enzyme
UTP	Uridine triphosphate
UV	Ultraviolet
V	Volts
Val	Valine residue
v/v	Volume per volume
well^{-1}	Per well
w/v	Weight per volume
3D	Three dimensional

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