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The two opposing activities of adenylyl transferase reside in distinct homologous domains, with intramolecular signal transduction

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Adenylyl transferase (ATase) is the bifunctional effector enzyme in the nitrogen assimilation cascade that controls the activity of glutamine synthetase (GS) in *Escherichia coli*. This study addresses the question of whether the two antagonistic activities of ATase (adenylylation and deadenylylation) occur at the same or at different active sites. The 945 amino acid residue ATase has been truncated in two ways, so as to produce two homologous polypeptides corresponding to amino acids 1–423 (AT-N) and 425–945 (AT-C). We demonstrate that ATase has two active sites; AT-N carries a deadenylylation activity and AT-C carries an adenylylation activity. Glutamine activates the adenylylation reaction of the AT-C domain, whereas α-ketoglutarate activates the deadenylylation reaction catalysed by the AT-N domain. With respect to the regulation by the nitrogen status monitor PII, however, the adenylylation domain appears to be dependent on the deadenylylation domain: the deadenylylation activity of AT-N depends on PII-UMP and is inhibited by PII. The adenylylation activity of AT-C is independent of PII (or PII-UMP), whereas in the intact enzyme PII is required for this activity. The implications of this intramolecular signal transduction for the prevention of futile cycling are discussed.

Keywords: *Escherichia coli* adenylyl transferase/flexible linker/intramolecular signal transduction/nucleotidyl transferase/PII protein

Introduction

Bacteria use sophisticated metabolic control to survive in a nutrient-depleted or rich environment without wasting valuable energy resources. In enteric bacteria and related organisms, glutamine synthetase (GS) is one of the key enzymes involved in nitrogen assimilation and it is subject to very tight regulatory control. In addition to feedback inhibition by a range of end products of nitrogen metabolism (e.g. CTP, ATP, histidine, etc.), a series of proteins have evolved that sense the nitrogen level and regulate the transcription and enzymatic activity of GS (reviewed by Magasanik, 1993).

The regulation of enzymatic activity of GS involves principally two bifunctional enzymes, uridylyl transferase (UTase) and adenylyl transferase (ATase), and the signal-transducing protein PII. When the level of nitrogen is low, UTase (the sensor enzyme) converts PII to PII-UMP by the covalent attachment of the UMP moiety from UTP to its tyrosine residue at position 51 (Son and Rhee, 1987; Jaggi *et al.*, 1996). PII is a trimer (Vasudevan *et al.*, 1994), and its three-dimensional structure has been determined to a resolution of 1.9 Å (Cheah *et al.*, 1994; Carr *et al.*, 1996). PII-UMP interacts with ATase to stimulate the deadenylylation activity of the latter so that catalytically inactive GS-AMP can be converted by phosphorolytic cleavage to active GS (Figure 1) which catalyses the ATP-dependent condensation of ammonia with glutamate to form glutamine (Anderson and Stadtman, 1970).

In conditions of nitrogen excess, the uridylyl-removing activity of UTase is stimulated so that PII-UMP is converted to PII by hydrolysis of the phosphodiester bond between the phenoxyl group of Tyr51 and the UMP moiety. The unmodified PII interacts with ATase to stimulate the adenylylation activity of the latter, which converts GS to inactive GS-AMP (Figure 1). In addition to controlling GS activity by covalent modification, UTase and PII, together with the two-component regulatory system comprising NR_I(NtrC) and NR_{II}(NtrB), modulate the level of transcription of the GS structural gene (reviewed by Magasanik, 1993).

Together, UTase, PII, ATase and GS constitute an example of the regulatory cascades in which enzymes at one level modulate and demodulate the activities of enzymes at the subsequent level. Such cascades have distinct control potential, including signal amplification (Goldbeter and Koshland, 1984) and increased controllability (Kahn and Westerhoff, 1991; Westerhoff *et al.*, 1991). An intriguing feature with distinct implications for control is that, like UTase, ATase is ambivalent in its activities: depending on the molecular signals that ATase receives from its environment, it catalyses adenylylation or deadenylylation of GS. These two reactions are not each other's microscopic reversal, and must, therefore, involve different catalytic mechanisms. This poses the intriguing question of whether the two activities are alternative activities of a single active site of the ATase or reside at two different active sites which should then be coupled somehow to prevent too much futile cycling.

The work in this report describes the dissection of the bifunctional enzyme ATase into two polypeptides by gene manipulation and overexpression. ATase is encoded by the *glnE* gene located at 68.9 minutes on the *Escherichia coli* genome and is constitutively expressed (Muse and Bender, 1992; van Heeswijk *et al.*, 1993). The adenylyl-

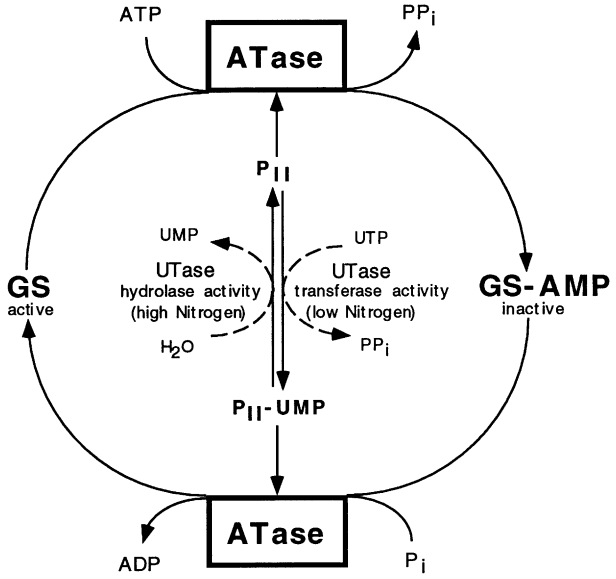

Fig. 1. The signalling cascade that controls the regulation of glutamine synthetase activity. Depending on the cellular nitrogen status, the sensor enzyme uridylyl transferase (UTase) maintains the signal transduction protein, either as PII or as PII-UMP. The adenylylation and deadenylylation activities of adenylyl transferase (ATase) are regulated by PII and PII-UMP, respectively. The transcriptional regulation of the GS level through the two-component system (NR_I/NR_{II}) is not shown.

ation and deadenylylation activities of ATase are associated with a single polypeptide chain of mol. wt ~115 kDa (Caban and Ginsburg, 1976). A 70 kDa fragment derived from ATase was still capable of converting GS to GS-AMP in the presence of glutamine and ATP (Hennig and Ginsburg, 1971). More recently, the nucleotide sequence determination of *glnE* and analysis of the resulting predicted protein sequence indicated that the N-terminal region of ATase is highly homologous to its C-terminal region (van Heeswijk *et al.*, 1993). We hypothesized that the ATase consists of two homologous catalytic domains. This study tests this hypothesis. In line with our ultimate goal of obtaining an atomic level of understanding of signal transduction mechanisms that control the activity of GS, we have produced two non-overlapping truncated polypeptides, AT-N and AT-C, corresponding to the N- and C-termini of ATase. We demonstrate that both truncated polypeptides are catalytically active. Most notably, the two activities are distinct: one domain catalyses the adenylylation and the other the deadenylylation. Evidence for regulatory coupling of the two activities is presented.

Results

Expression and partial purification of ATase, AT-N and AT-C

The analysis of the predicted primary sequence of ATase revealed that there are two highly homologous domains within the single polypeptide (van Heeswijk *et al.*, 1993). In order to examine whether these separate domains have catalytic activity, we used PCR to amplify the 5′ region of *glnE* encoding AT-N. The nucleotide sequence of the amplified DNA was identical to that reported by van Heeswijk *et al.* (1993), starting at position 1387 (the position of the ATG start codon) and ending at position

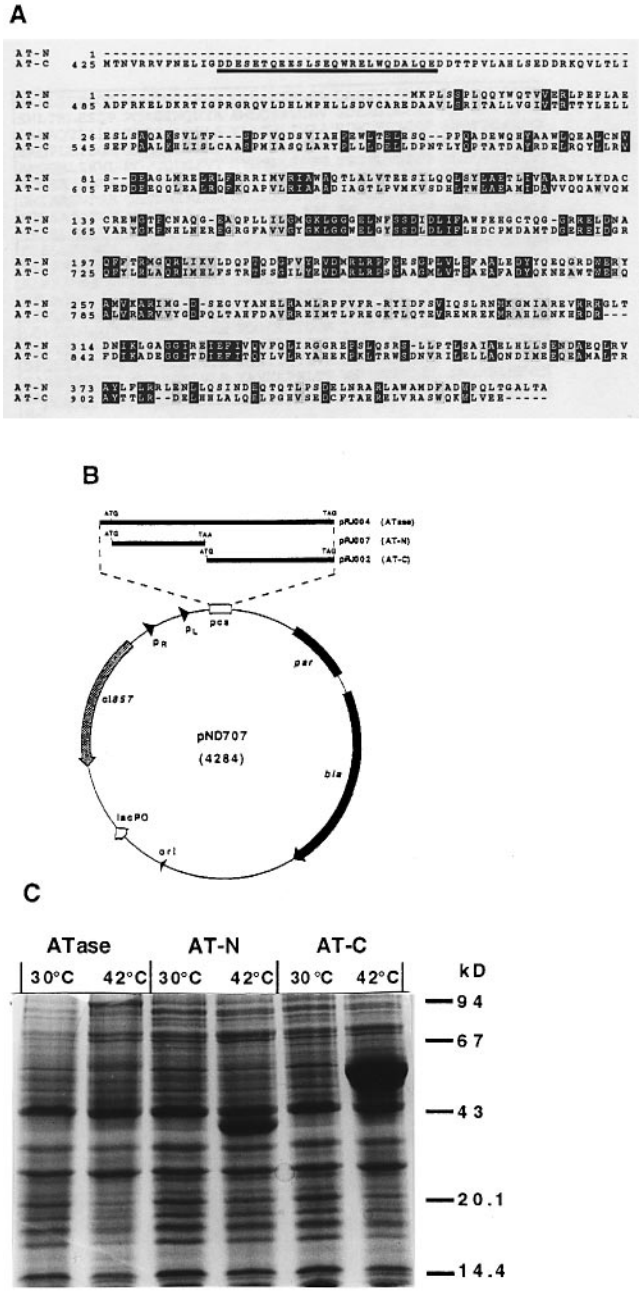

Fig. 2. Primary amino acid sequence, physical map and overexpression of ATase and its two domains AT-N and AT-C. (**A**) Amino acid sequence alignment of the AT-N and AT-C domains of ATase where identical residues are in dark boxes and homologous residues are in light-shaded boxes. The numbering of the residues reflects their position in the 945 amino acid ATase. The proposed 'Q-linker' sequence (see text) in the N-terminal region of AT-C is underlined. (**B**) Expression vectors pRJ004, pRJ007 and pRJ002 for ATase, AT-N and AT-C respectively. The genes for ATase and its two domains were cloned into the polyclonal site of vector pND707 downstream of the λ tandem promoters P_R and P_L. (**C**) Overexpression of ATase, AT-N and AT-C. Coomassie brilliant blue-stained SDS–polyacrylamide gel (10%) of indicated samples of whole cell lysates withdrawn before induction, when cells were grown to an OD_{595} of 0.5 at 30°C and after temperature-upshift induction and growth at 42°C for 4 h. The cells were harvested and suspended in SDS gel loading buffer to an OD_{595} of 10.

2655. A 'TAA' translation stop codon was introduced after position 2655. The fragment is predicted to encode a polypeptide of 423 amino acids (Figure 2A). Figure 2C

shows the overexpression of the AT-N domain at 42°C. Plasmid pRJ007 (Figure 2B) directs the synthesis of a polypeptide of ~48 kDa (predicted M_r 48 300 Da).

The construction of AT-C utilized a convenient *Nde*I site at position 2656 (van Heeswijk *et al.*, 1993) of the *glnE* open reading frame. The ATG codon within the *Nde*I recognition sequence (CATATG) was in-frame with the amino acid sequence encoding the ATase. The expression plasmid pRJ002 (Figure 2B) predicted to encode AT-C (521 amino acids; M_r 58 200 Da), expressed AT-C to a high level (Figure 2C). The difference in the level of expression of AT-N and AT-C may reflect a different efficiency of translation since the level of transcription should be identical in the two constructs. RNA fold analysis of the 5′ end of the nucleotides encoding AT-N and AT-C indicated that the former may have more secondary structure (data not shown).

Expression plasmid pRJ004 encoded the entire ATase (Figure 2C). The low level of expression from this construct may be due to the presence of a long 5′-untranslated sequence and utilization of the *glnE* ribosome-binding site rather than that provided by pND707 (Love *et al.*, 1996). In order to increase its expression, the 5′-untranslated region of *glnE* was removed by cloning a 2353 bp *Sal*I–*Sph*I fragment (from pMR207) containing the coding region of the C-terminal part of AT-N and the entire AT-C into the same sites in pRJ007, to give plasmid pRJ009. The level of expression of ATase in the new construct was comparable with that of AT-N directed by pRJ007 (data not shown). The three polypeptides were partially purified as described in Materials and methods, and hardly any ATase is detectable in the preparations of AT-N and AT-C by Western blot analysis using ATase antiserum (data not shown), despite the fact that the proteins were overexpressed and purified from the *glnE*$^+$ *E.coli* strain TG1.

AT-N and AT-C have different and independent catalytic activities

Domains AT-N and AT-C were both subjected to the adenylylation and deadenylylation assay conditions. The deadenylylation activity of ATase, AT-N and AT-C was examined by their effect on GS activity in an assay based on the formation of γ-glutamyl hydroxamate (γ-GH) by unadenylylated GS but not by adenylylated GS (Stadtman *et al.*, 1979; van Heeswijk *et al.*, 1996). As expected, the intact ATase (Figure 3A and B), exhibited deadenylylation activity. This control shows that the partially purified enzyme has converted the substrate GS-AMP to active GS, as indicated by an increase in the γ-glutamyl transfer activity. The product of this reaction accumulates over time, as indicated by the saturation curve connected by closed squares in Figure 3A and B. Under the conditions of the assay, the deadenylylation of GS-AMP increases in a linear fashion in the first 10 min and reaches saturation after that time period. The AT-N domain exhibited a molar deadenylylation activity similar to or higher than that of the complete ATase (Figure 3A). Reducing the concentration of AT-N diminished the rate of γ-GH formation. The AT-C domain, on the other hand, did not show appreciable γ-glutamyl transfer activity (Figure 3B). The amount of γ-GH remained at background level during the period of the assay even for AT-C concentrations double that of the positive control of complete ATase.

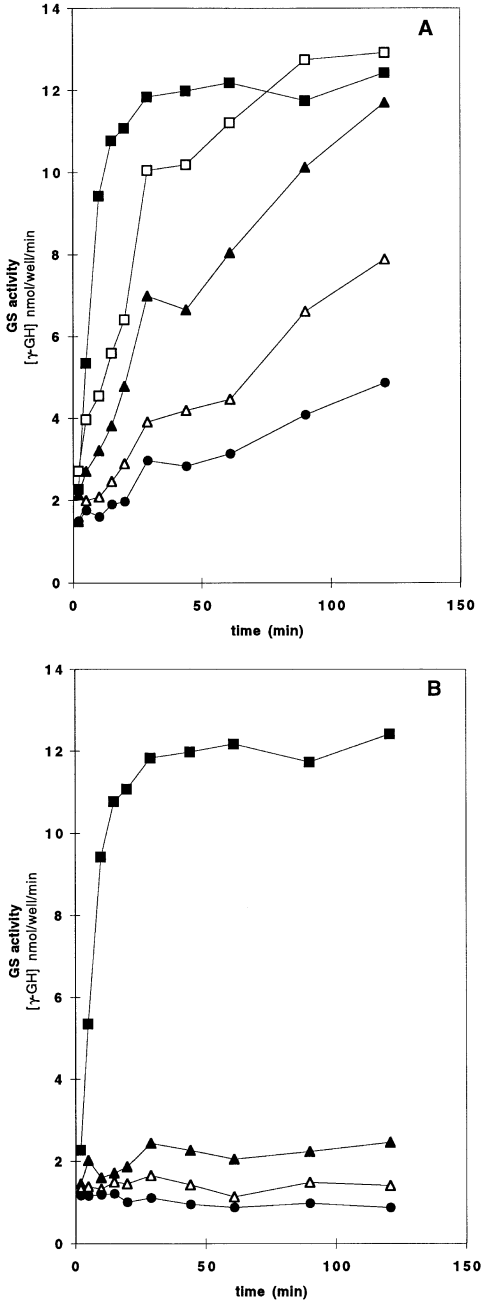

Fig. 3. AT-N is the deadenylylation domain. The deadenylylation with time of GS-AMP (n = 11) by the AT-N domain or by the complete ATase is indicated by an increasing production rate of γ-glutamyl hydroxamate by activated GS. The γ-glutamyl transferase assay was carried out as described in Materials and methods. The complete ATase enzyme (21.8 nM) was run as a positive control with PII-UMP at a concentration of 26 nM (A and B, ■). (**A**) With PII-UMP kept at excess concentrations, AT-N was added to the assay at the following concentrations: □ 3.4 nM, ▲ 1.7 nM, △ 0.85 nM, ● 0.425 nM. (**B**) The AT-C domain shows no activity under the same deadenylylation conditions. PII-UMP was kept at excess molar concentrations relative to AT-C, and AT-C was added to the assay at the following concentrations: ▲ 70.2 nM, △ 35.1 nM, ● 17.6 nM.

Conversely, the same three proteins were examined for adenylylation activity (Figure 4A and B) using GS as the substrate. In this assay, the adenylylation reaction will inactivate the enzyme and render it incapable of carrying

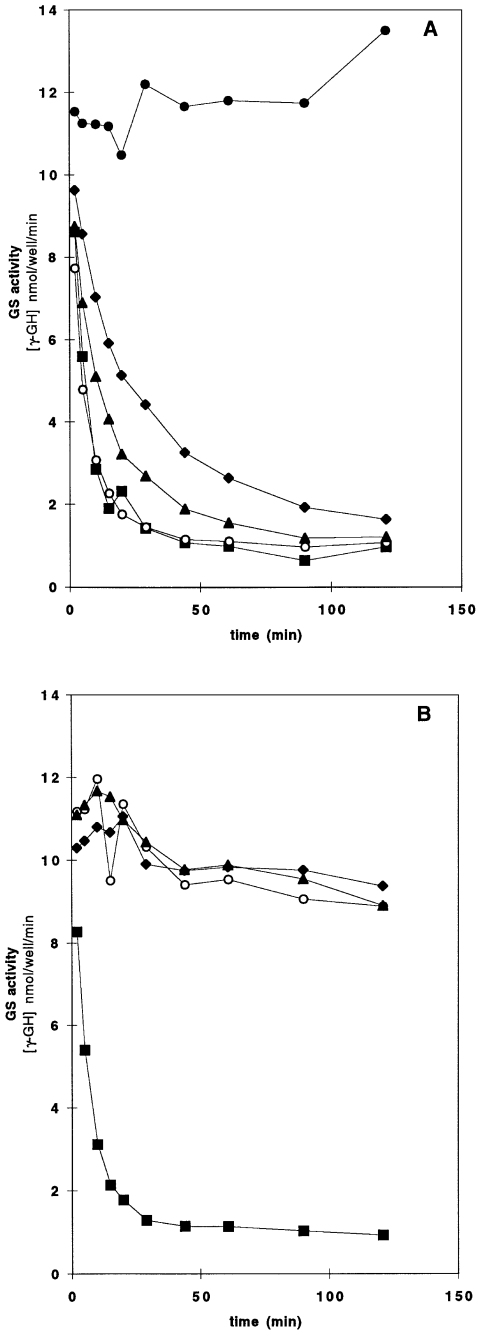

Fig. 4. AT-C is the adenylylation domain. Adenylylation of GS by the AT-C domain or the intact ATase is indicated by a decreasing production rate of γ-glutamyl hydroxamate by inactivated GS. The γ-glutamyl transferase assay was carried out as described in Materials and methods. The complete ATase was run as a positive control at a concentration of 21.8 nM with the activator protein PII at 27 nM (A and B, ■). (**A**) With PII kept at excess concentrations, the assay was carried out with AT-C at various concentrations: ○ 2.2 nM, ▲ 1.1 nM, ◆ 0.53 nM, ● 0 nM. (**B**) The AT-N domain shows no activity under the same adenylylation conditions. PII was kept at excess concentrations and AT-N was added to the assay at the following concentrations: ○ 3.4 nM, ▲ 1.7 nM, ◆ 0.85 nM.

out the γ-glutamyl transfer reaction. As expected, the ATase control converted GS to GS-AMP so that the rate of γ-GH synthesis decreased with the duration of the ATase assay. The AT-N domain did not adenylylate GS and the rate of γ-GH synthesis was not affected (Figure 4B). Varying the concentration of AT-N (within a 4-fold range) did not change the level of γ-GH produced. AT-C, on the other hand, was very active in adenylylating GS at a concentration around two orders of magnitude lower than that of the intact ATase in the positive control (Figure 4A). Taking the results in Figures 3 and 4 together, it is clear that the preparations of AT-N and AT-C did not have appreciable quantities of contaminating ATase since AT-N was active in the deadenylylation reaction in the range of 3.4–0.425 nM and inactive in the adenylylation assay in a similar range. AT-C, on the other hand, did not exhibit significant deadenylylation activity in the concentration range of 70.2–17.6 nM but was very active in adenylylating GS in the concentration range of 2.2 down to 0.53 nM.

Regulation of AT-N and AT-C

ATase catalyses both the adenylylation and the deadenylylation reaction using GS or GS-AMP as substrate, respectively, in response to cellular factors representing the nitrogen status in the cell. The deadenylylation activity of ATase is regulated by PII-UMP and α-ketoglutarate (Adler *et al.*, 1975; Engleman and Francis, 1978). Figure 5A shows that the AT-N domain is regulated similarly, both PII-UMP and α-ketoglutarate are regulatory ligands for the AT-N-catalysed reaction. No deadenylylation activity of AT-N was observed when glutamine was added to the assay instead of α-ketoglutarate. Unmodified PII did not induce any deadenylylation activity in the AT-N domain either with α-ketoglutarate or with glutamine (Figure 5A). Varying the glutamine concentration did not affect the deadenylylation activity of AT-N when PII-UMP and α-ketoglutarate were present (Figure 5B).

For adenylylation, ATase requires PII and glutamine (Engleman and Francis, 1978; Rhee *et al.*, 1978). Indeed, the results in Figure 6A show that varying the PII concentration from 54 nM (where PII is in excess) through to 2.7 nM led to a reduction in adenylylation by ATase. When no PII was added, under the conditions of the assay where phosphate is present to reduce the PII-independent adenylylation of GS, ATase did not exhibit any appreciable adenylylation activity (as indicated by the GS activity remaining high). AT-C, on the other hand, was very active in adenylylating GS independently of PII concentration, in the range from 54 to 6.8 nM. In fact, the adenylylation activity of AT-C was just as potent without any PII added to the reaction mixture (Figure 6A). We then examined the role of glutamine in the adenylylation activity of AT-C. The result in Figure 6B shows that the adenylylation activity of AT-C depended on the concentration of glutamine. The adenylylation activity of AT-C was reduced when the glutamine concentration was decreased from 5 to 0.25 mM, and was completely abolished when glutamine was absent from the adenylylation assay.

The results in this work imply that a binding site for PII-UMP is located within AT-N. From the results in Figure 6A as well as previous demonstrations, we know that PII modulates the adenylylation activity of ATase. By contrast, adenylylation by AT-C is PII independent. Two explanations could account for these observations. One is that the PII-binding site is located close to the hinge region between the two domains and that the truncation may have destroyed this binding site. The other possibility is that the binding sites for both PII and PII-UMP are

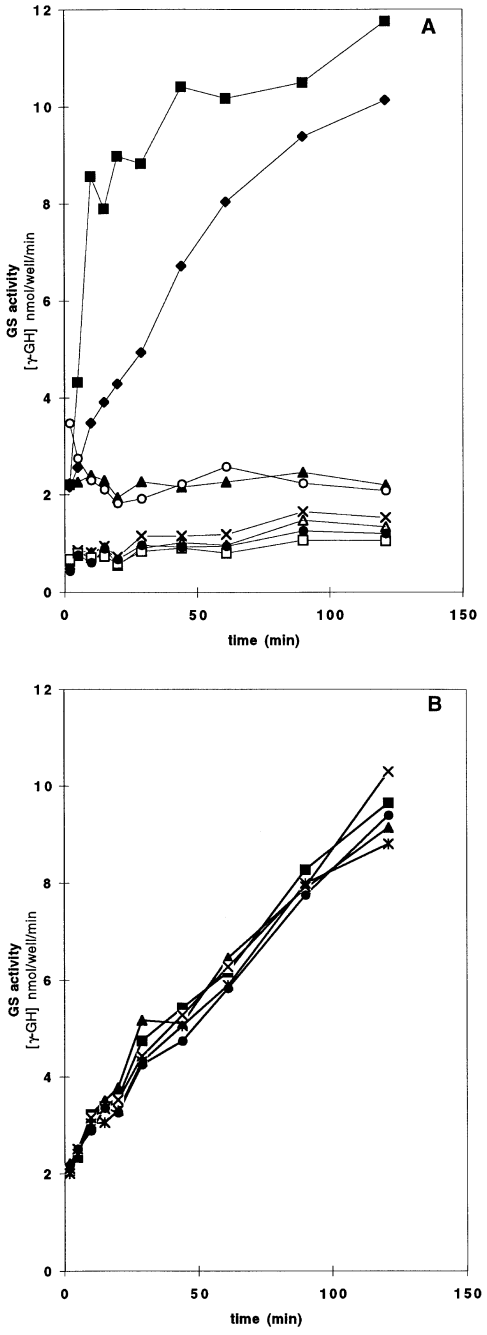

Fig. 5. Regulation of AT-N by PII-UMP and α-ketoglutarate. Deadenylylation of GS-AMP ($n = 11$) by AT-N depends on the presence of the uridylylated signal protein PII-UMP and α-ketoglutarate as indicated by an increasing production rate of γ-glutamyl hydroxamate by activated GS. The deadenylylation reaction does not proceed if the AT-N domain is used with any combination of the factors for the adenylylation reaction. Glutamine does not inhibit the deadenylylation activity of AT-N in the presence of PII-UMP and α-ketoglutarate. (**A**) The deadenylylation assay was carried out with the intact ATase (21.8 nM) with PII-UMP (26 nM) as the positive control (■) and with AT-N (3.4 nM) and various combinations of: PII (670 nM), PII-UMP (653 nM), α-ketoglutarate (20 mM) and glutamine (1 mM) as indicated: ◆ AT-N, PII-UMP, α-ketoglutarate; ▲ AT-N, α-ketoglutarate;× AT-N, PII-UMP; △ AT-N; ○ AT-N, PII, α-ketoglutarate; □ AT-N, PII, glutamine; ● AT-N, PII-UMP, glutamine. (**B**) The deadenylylation assay was carried out with AT-N (320 nM) and with PII-UMP and α-ketoglutarate present at the concentrations described in (A) but with the following concentrations of glutamine added: ● 10 mM, ✳ 4 mM, × 2 mM, ▲ 1 mM, ■ 0 mM.

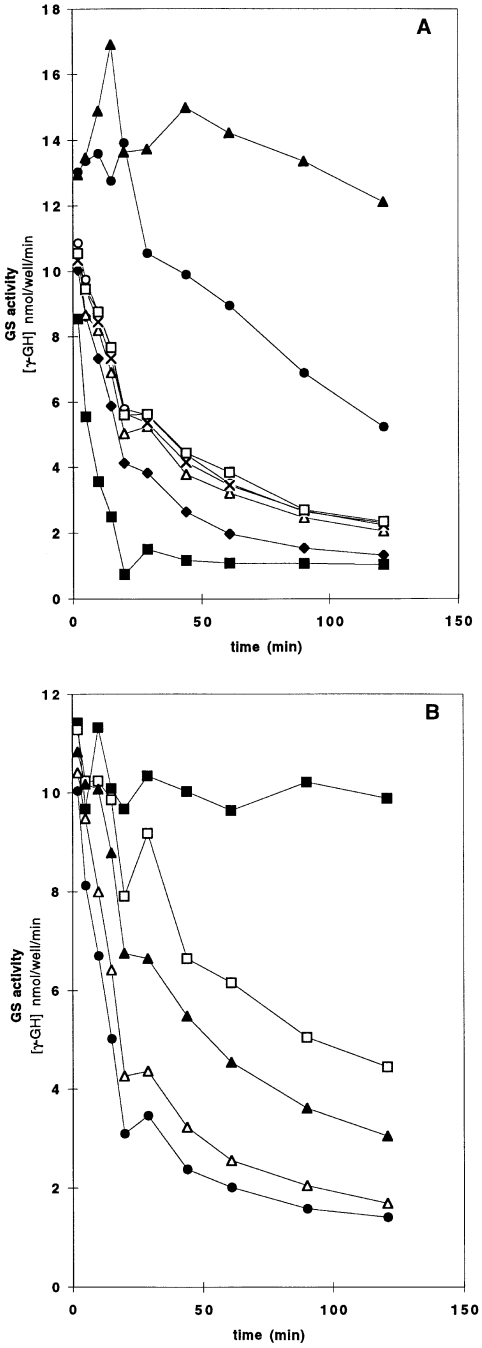

Fig. 6. Regulation of AT-C is independent of PII but requires glutamine. (**A**) The AT-C subdomain adenylylates and thus inactivates GS independently of PII but requires glutamine for activity. The complete ATase requires the presence of both PII and glutamine for its adenylylation activity. The deactivation of GS was monitored by measuring the decreasing production rate of γ-glutamyl hydroxamate as described in Materials and methods. Partially purified AT-C (56.8 nM) was used in the assay in the presence of 1 mM glutamine and with the following PII concentrations: □ 54 nM, × 27 nM, ○ 6.8 nM and △ 0 nM. Partially purified complete ATase (21.8 nM) was added to the assay in the presence of 1 mM glutamine and with the following PII concentrations: ■ 54 nM, ◆ 6.8 nM, ● 2.7 nM and ▲ 0 nM. (**B**) The rate of the adenylylation reaction catalysed by AT-C depends on the concentration of glutamine only. The assay was carried out with partially purified AT-C (56.8 nM) together with the following concentrations of glutamine: ● 5 mM, △ 2 mM, ▲ 0.5 mM, □ 0.25 mM and ■ 0 mM.

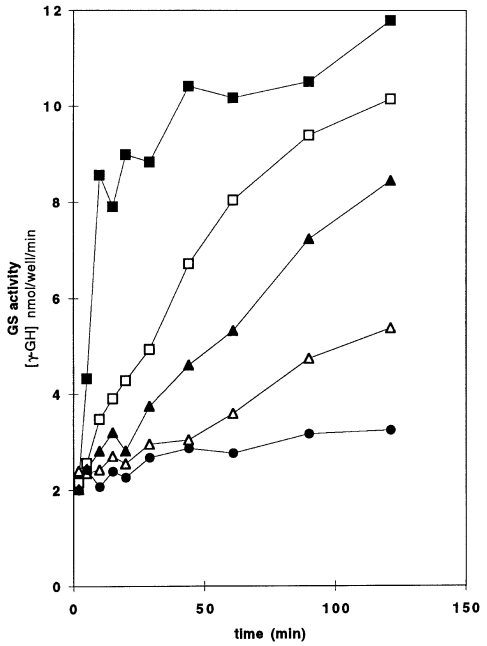

Fig. 7. Interdependent binding of PII and PII-UMP to AT-N. The PII-UMP-modulated deadenylylation of GS catalysed by AT-N is inhibited by the unmodified signal protein PII. The deadenylylation assay was carried out with AT-N (3.4 nM) and PII-UMP (653 nM), and the activation of GS was indicated by an increase in the production rate of γ-glutamyl hydroxamate as described in Materials and methods. PII was added to the assay at the following concentrations: □ 0 nM, ▲ 670 nM, △ 2.68 μM, ● 8 μM; complete ATase (21.8 nM) together with PII-UMP (26 nM) was run as a control (■)

located in AT-N. In order to test this, the deadenylylation of GS-AMP by AT-N was carried out in the presence of PII-UMP and various amounts of PII. Figure 7 shows that the deadenylylation of AT-N in the presence of PII-UMP is decreased by increasing the amount of PII in the reaction. This indicates that PII and PII-UMP can bind at the same site or that the binding site for PII in AT-N is not the same as the one for PII-UMP, but binding of PII has a negative influence on the binding of PII-UMP.

Discussion

The possibility that the activities of ATase may be located in two separate active centres has been alluded to in earlier works. One of the earliest observations was the absence of product inhibition, i.e. unadenylylated GS did not inhibit the deadenylylation reaction of GS-AMP (Wulff *et al.*, 1967). Also, a mutant ATase protein isolated from *Klebsiella aerogenes* displayed only deadenylylation activity (Foor *et al.*, 1975). Furthermore, a proteolytic fragment derived from ATase was shown to have only adenylylation activity (and no deadenylylation activity) in the presence of glutamine, and adenylylated GS did not inhibit the adenylylation reaction (Hennig and Ginsburg, 1971).

The nucleotide sequence of *glnE* that was used to deduce the amino acid sequence of ATase revealed a duplicated homologous sequence with close to 25% identity when the N-terminal region spanning amino acids 1–411 was aligned with the C-terminal region from 523 to 945 (van Heeswijk *et al.*, 1993). In a more recent study, Holm and Sander (1995) found that nucleotidyl transferases such as ATase and UTase are related to DNA polymerase β. Using an amino acid sequence signature for the polymerase β catalytic motif to search the protein database, two catalytic motifs were detected in ATase, one from amino acid 117 to 184 and the second from 643 to 711. Thus, both the N- and C-terminal regions of ATase are each predicted to contain a catalytic motif. On the other hand, only one catalytic motif was detected in UTase although this protein also has two catalytic activities (uridylylation and deuridylylation) and, by analogy to ATase, probably two active sites.

The *glnE* gene was manipulated to produce two truncated polypeptides that do not overlap. In fact, the 945 amino acid ATase was split into two parts, i.e. AT-N running from amino acid 1 to 423, and AT-C running from 425 to 945. Both parts were then shown to have an ATase activity. This brings us to the first conclusion of this study: ATase does not have a single catalytic site, but two (or more). Together with the sequence homology within the molecule, this suggests a model in which the protein is an intramolecular ATase dimer consisting of two domains, each of which has ATase activity. In view of the bifunctional catalytic activity of the ATase, the immediate question then was whether each of the two ATase domains exhibits both activities or if the two domains had specialized, one catalysing the adenylylation reaction, the other carrying out the deadenylylation reaction. The experiments (Figures 3 and 4) demonstrated that the two domains were specialized: a partially purified sample of AT-N contained only the deadenylylation activity and AT-C catalysed only the adenylylation reaction (Figures 3 and 4) whereas ATase catalysed both reactions. The absence of 'background' deadenylylation activity of AT-C (Figure 3B) and the negligible 'background' adenylylation activity of AT-N (Figure 4B) show that the (very) low level of ATase in the partially purified preparations did not influence the analysis. The dissection of ATase into two polypeptides that could carry out enzymatic reactions at respectable rates suggests that the truncation has been made at or near a hinge region that connects the two domains.

The deadenylylation reaction catalysed by intact ATase requires three activators, PII-UMP, ATP and α-ketoglutarate. The deadenylylation by AT-N had similar requirements, and both PII-UMP and α-ketoglutarate are required for activity (Figure 5A). We wanted to investigate if α-ketoglutarate activated AT-N directly to stimulate its deadenylylation activity in the light of the recent finding that the signal transduction protein PII is only able to carry out its function when bound to effector molecules such as α-ketoglutarate (Kamberov *et al.*, 1995). The data presented in Figure 5A suggest that there is no α-ketoglutarate-binding site in AT-N since the deadenylylation reactions containing AT-N and α-ketoglutarate, as well as that with AT-N, PII and α-ketoglutarate, show a higher than background level of γ-GH formed in a time-independent manner. If the increased activity was due to deadenylylation stimulated by α-ketoglutarate-bound AT-N, then the GS activity should increase over the period of the assay. Our interpretation is that at 2 mM, α-ketoglutarate may be an allosteric activator of unadenylylated GS. It activates the single active subunit of

GS-AMP (n = 11) that is used in the assay. Further evidence for this comes from our unpublished observation that the rate of γ-GH synthesis at the start of a standard adenylylation assay with AT-C increased 100% when α-ketoglutarate was present together with glutamine. Since adenylylation inactivates GS, the increased γ-GH makes no physiological sense and can only be explained in terms of direct allosteric activation of GS by α-ketoglutarate. Thus, the activation of the deadenylylation reaction by α-ketoglutarate may be effected through PII-UMP.

The adenylylation reaction catalysed by ATase requires GS and ATP as substrates, while PII and glutamine are required as activators of the reaction. Results in Figure 6A show that AT-C catalyses the adenylylation reaction but, unlike the intact ATase, the adenylylation reaction is completely independent of PII. Glutamine, on the other hand, is essential for the adenylylation reaction catalysed by AT-C. A small proteolytic fragment that was purified in a previous study and was shown to contain the active site for adenylylation but had no deadenylylation activity was also noted to be activated by glutamine (Hennig and Ginsburg, 1971). Thus, the glutamine-binding site is located within AT-C. However, the deadenylylation activity of AT-N is abolished in the presence of PII-UMP and glutamine when α-ketoglutarate is absent (Figure 5A). This is not due to negative regulation by glutamine binding to AT-N or PII-UMP since varying the glutamine concentration from 0 to 10 mM in standard deadenylylation assays with AT-N, PII-UMP and α-ketoglutarate did not alter the rate of deadenylylation of GS-AMP (Figure 5B). Therefore, glutamine only binds at AT-C.

The distribution of the binding sites for the regulatory proteins PII and PII-UMP does not follow the pattern of clear separation into two independent domains observed for the adenylylation and deadenylylation active sites. PII-UMP is required for the deadenylylation reaction by AT-N, as is the case with intact ATase. Therefore, the PII-UMP-binding site is located within AT-N. However, a more complex picture emerges for the regulation of the adenylylation activity of ATase which requires PII. The competition by PII for PII-UMP-binding sites in the deadenylylation reaction (Figure 7) implies that the PII-binding site is also located within AT-N.

This leads to a rather surprising mapping of function to the structure of ATase. The two similar but chemically distinct reactions of adenylylation and deadenylylation are catalysed at distinct sites and even distinct though homologous subunits. The homology suggests that this situation has been different in the evolutionary past. Apparently, there is an advantage in almost but not quite separating the two activities physically. That the two activities have not become affiliated with distinct genes and polypeptides may reflect the requirement for coherent regulation of the two activities. If the two activities of the constitutively expressed ATase (van Heeswijk *et al.*, 1993) were to operate simultaneously, this would lead to futile cycling and loss of ATP hydrolytic free energy. Even though such cycling can be advantageous for regulation (Cárdenas and Cornish-Bowden, 1989; Small and Fell, 1990), it should be under control so as to optimize regulation benefit to free energy cost (Goldbeter and Koshland, 1987). The attunement of the two opposing activities in the two distinct domains appears to be

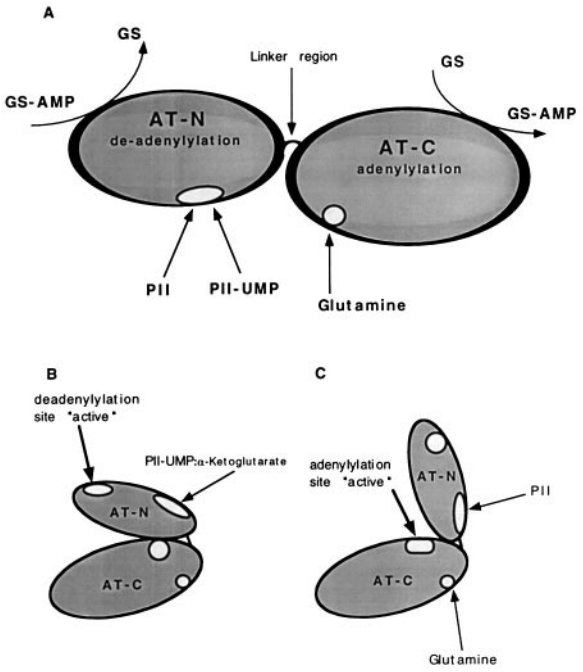

Fig. 8. Proposed model for the bifunctional ATase enzyme. (**A**) Proposed functional model of ATase. The AT-N domain catalyses the deadenylylation of GS. It also contains the interdependent binding sites for PII and PII-UMP, the two forms of the signalling protein required for the adenylylation and deadenylylation reaction respectively. The AT-C domain catalyses the adenylylation of GS and contains the binding site for glutamine. The adenylylation activity of AT-C is regulated through modulation by the AT-N domain. (**B**) Proposed model for the deadenylylation function of ATase. The active site for the deadenylylation of GS, located on the AT-N domain, is regulated by PII-UMP:α-ketoglutarate which binds to a site on the same domain. The site for the adenylylation function on the AT-C domain is inhibited by the AT-N domain. (**C**) Proposed model for the adenylylation function of ATase. Binding of PII to a site on the AT-N domain induces a conformational change in ATase which leads to a change in the relative positions of its two domains. The adenylylation site located on the AT-C domain becomes de-inhibited by a shift in the position of AT-N, and adenylylation of GS can proceed in the presence of glutamine.

accomplished by an intramolecular signal transduction: the uridylylation state of PII appears to be read at the AT-N domain. From here, the activity of this domain, and that of the other domain, are both regulated in a concerted manner. In the latter regulation, a signal has to be transferred between the two domains. The mechanism for this signal transduction is an intriguing question.

Based on the results illustrated in Figures 3–7, we are in a position to propose a structural and functional model for ATase (Figure 8), which encompasses these intriguing regulatory characteristics. Firstly, the enzyme is composed of two functional domains. As shown in Figure 8A, the site of the deadenylylation activity is located within the AT-N domain (48 kDa) which also contains the binding site(s) for PII and PII-UMP. The slightly larger AT-C domain (58 kDa) catalyses the adenylylation reaction and contains the binding site for glutamine. In order to explain how the binding of the signal-transducing protein PII or PII-UMP to the N-terminal domain of intact ATase regulates its two opposing enzymatic activities without energy-expensive futile cycling, we offer the following speculation. The binding of PII-UMP together with the activators α-ketoglutarate and ATP to AT-N is necessary

for the deadenylylation activity of AT-N. The deadenylylation activity of ATase will not occur without the binding of the PII-UMP–ligand complex. Once PII-UMP–ligand complex binds ATase, the enzyme assumes a 'closed' conformation such that, in addition to carrying out the deadenylylation reaction, the AT-N domain will also inhibit the activity of AT-C by blocking the active site for adenylylation (Figure 8B). During the adenylylation reaction, the binding of PII causes ATase to assume an 'open' conformation to reveal the active site for adenylylation which is activated by binding of glutamine within the AT-C domain, whilst at the same time locking the deadenylylation site in AT-N. It has been shown that PII and glutamine can inhibit the deadenylylation activity of ATase synergistically and, conversely, that PII-UMP and α-ketoglutarate can also inhibit the adenylylation reaction of ATase synergistically (Engelman and Francis, 1978). Our data suggest that the binding events of PII and PII-UMP to the N-terminal domain are transmitted to the C-terminal domain, and the glutamine-binding event in the C-terminal domain is transmitted back to the N-terminal domain. There are two possible ways that the transduction of signal between the two domains can occur: either via a dynamic linker that connects the domains or by non-covalent interactions between the two domains.

Close examination of the linker region where there is no homology between AT-N and AT-C (residues 425–509) revealed the presence of a 'Q-linker' (see underlined sequence in Figure 2A) based on the criteria outlined by Wooton and Drummond (1989): the amino acids from position 438 to 462 are hydrophylic and rich in glutamate, glutamine and serine (collectively 56% of the 25 amino acids) and there is a periodic occurrence of a hydrophobic amino acid at every fourth residue in the C-terminal 15 residues. These types of linkers have been found in interdomain regions of several bacterial transcription factors and signal transduction proteins such as the nitrogen regulatory protein NtrC. Interestingly, in NtrC, the Q-linker sequence between the receiver domain and the central and C-terminal domains was shown to tolerate insertions of four or eight amino acids without any significant effect on regulation by NtrB or its transcription enhancement function. However, when the N-terminal domain and the central and C-terminal domains of NtrC were expressed separately, the trancription activation function was lost. From this, it was surmised that the Q-linker serves to tether the functionally diverse domains and the signal transduction probably occurs by interactions between the domains (Wooton and Drummond, 1989).

It is likely that the proposed Q-linker of ATase also tethers the two domains, since the two opposing activities are still intact in the respective domains when they are separated. However, the loss of control of adenylylation activity by PII in AT-C suggests that the tethering increases 'molecular crowding' so that the deadenylylation domain can interact non-covalently with the other domain to prevent futile cycling. This view is consistent with the model that we propose, and the 'open' complex probably still may involve interaction between the two domains but the total contact surface may be smaller.

In order to demonstrate any interaction between the two domains, we measured the adenylylation activity of the C-terminal domain in the presence of a molar equivalent or a 10-fold molar excess of the N-terminal domain both with and without PII or PII-UMP. The results (data not shown) only indicate a small decrease in maximal rate when a 10-fold molar excess of the N-terminal domain was present with or without PII-UMP. In the presence of PII, the inhibition of the adenylylation activity by the N-terminal domain was lower, which supports the notion of change in contact surface area between the two domains in the 'closed' and 'open' form (Figure 8B). The important point here is that the interaction between the separated domains is only weak and, as mentioned before, the Q-linker tethers them to harness the weak interaction for signal transduction. It is also conceivable that, like the Q-linker in NtrC, the ATase linker may also tolerate insertion of additional residues in the linker. Experiments along these lines currently are being carried out in order to understand fully the mechanism of the intramolecular signalling that controls the two opposing activities that are present in the ATase polypeptide and the overall signal transduction events that control the activity of GS. We have also initiated structural studies of the intact ATase, its domains and their complex.

Materials and methods

Bacterial strains, media and growth conditions

The *E.coli* K12 derivatives used in this study are listed in Table I. ATase was produced in AN1459, AT-N and AT-C in TG1, PII in RB9040, UTase in AN1459, unadenylylated GS in RB9017 and GS with an adenylylation state of ~11 (n = 11) in DH5α. All strains were grown in Luria Bertani medium at pH 7.0 with ampicillin (100 μg/ml) where appropriate. For the production of GS (n = 11) in DH5α, the broth was supplemented with NH_4Cl (0.5%). Cells bearing the derivatives of the plasmids pND707, pPL450 and of the phagemid pMA200U were grown at 30°C (Jaggi *et al.*, 1996). All other cells were routinely grown at 37°C.

DNA manipulations

Standard methods were used for *in vitro* manipulations essentially as described in Sambrook *et al.* (1989). Rapid small-scale plasmid preparations were carried out as described by Del Sal *et al.* (1988). DNA sequencing was carried out by Sanger's dideoxy chain termination method (Sanger *et al.*, 1977) using manual sequencing (Sequigen, BioRad) or automated sequencing on an ABI 373. The following sequencing primers were used: primer 9, 5′-GGCAGCATTCAAAG-CAG-3′ (Elvin *et al.*, 1990); AT-NFP2, 5′-TGCGATGCTGCGCC-CGTTTG-3′; AT-NRP1, 5′-AGCCGCTGCCCCATGCGGGT-3′; and M13 universal primer, 5′-GTAAAACGACGGCCAGT-3′. Plasmids used in this work are listed in Table II.

Plasmid constructions

pRJ 007 (Figure 2B). The DNA encoding the N-terminal domain of ATase was obtained by PCR using two oligonucleotides primers: ATFP, 5′-AATCGCATATGAAGCCGCTC-3′; and ATRP, 5′-AACTGCAGTT-ATGCGGTCAGCGCCCCGGTCAG-3′. The forward primer (ATFP) incorporates an *Nde*I restriction enzyme recognition sequence (underlined) which contains the ATG start codon for AT-N. The reverse primer was designed to introduce a stop codon after the codon for amino acid 423. The template for the PCR was the *glnE*-containing plasmid pMR207 (van Heeswijk *et al.*, 1993). The AT-N-encoding fragment was amplified with Expand polymerase (Boehringer Mannheim). The PCR product was cloned into a pGEM T vector (Promega) and a 1.2 kb *Nde*I–*Kpn*I fragment was isolated and cloned into similarly cut plasmid pND707 (Love *et al.*, 1996). The resulting expression plasmid is designated pRJ 007.

pRJ002 (Figure 2B). The DNA encoding the C-terminal domain of ATase was obtained by double digestion of plasmid pMR207 with restriction endonucleases *Nde*I and *Sph*I. An ~2 kb fragment encoding the AT-C domain was cloned into *Nde*I–*Sph*I-cut plasmid pND707, resulting in the expression plasmid pRJ 002.

pRJ004 (Figure 2B). In order to construct an expression plasmid

Table I. Bacterial strains

E.coli strain	Genotype	Reference
AN1459	F⁻ *lvC leuthr-1B 6 hsdR recA srlA*::Tn*10*	Vasudevan *et al.* (1991)
TG1	K12 Δ(*lac-pro*) *sup*E *thi hsd* D5/F′ *tra*D36 *pro* A+B+*lacI*^{q} *lac*ZΔM15	Gibson (1984)
DH5α	F⁻ *supE44 hsdR17 recA1 endA1*(m80*lacZ*ΔM15)	Raleigh *et al.* (1989)
RB9017	*endA1 thi-1 hsdR17 supE44* Δ*lacU168 hutC*$_{klebs}$ Mu lysogen *glnE*::Tn5	Bueno *et al.* (1985)
RB9040	*endA1 thi-1 hsdR17 supE44* Δ*lacU169 hutC*$_{klebs}$ Mu lysogen *glnD* ::Tn*10*	Bueno *et al.* (1985)

Table II. Plasmids used in this work

Plasmid/vectors	Description	Source/reference
pWVH57	GS, *gln*A	van Heeswijk *et al.* (1996)
pRJ1	PII, *glnB*	Jaggi *et al.* (1996)
pNV101	UTase, *glnD*	Jaggi *et al.* (1996)
pND707	λ P_R–P_L promoter vector, *c*I857, Ap^r	Love *et al.* (1996)
pMR207	ATase, *glnE*	van Heeswijk *et al.* (1993)
pRJ002	AT-C	this work
pRJ004	ATase	this work
pRJ007	AT-N	this work
pRJ009	ATase	this work

containing the complete *glnE*, a 2.0 kb *Eco*RI fragment containing the 5′-untranslated region of the *glnE* DNA sequence was obtained from the plasmid pMR207 and cloned into the same site of the vector pRJ002, resulting in the plasmid pRJ004.

pRJ009. To improve the level of expression of ATase, the 5′-untranslated region of *glnE* was removed by cloning a 2353 bp *Sal*I–*Sph*I fragment from pMR207 containing the coding region for the C-terminal end of AT-N and the entire AT-C into the same sites in pRJ007 to yield pRJ009.

Protein expression and purification

Purification of ATase, AT-N and AT-C. The cells containing the appropriate expression plasmids were grown to an OD_{595} of 0.5 at 30°C in a Braun Biolab CP fermentor (5 l) at a stirrer speed of 500 r.p.m. and an aeration rate of 3 l/min. At a temperature of 30°C, the tandem λ P_R and P_L promoters are repressed by the temperature-sensitive gene product cI857. Overexpression of the proteins was induced by increasing the temperature rapidly to 42°C, which caused the derepression of the tandem λ promoters. The cells were grown at 42°C for 4 h (Figure 1C). The harvested cells were suspended in sodium phosphate buffer (20 mM, pH 7.2) and lysed in a French pressure cell at 14 000 p.s.i. The lysate was centrifuged at 10 000 *g* in a Beckman J2-MC centrifuge. To the supernatant, streptomycin sulfate was added to a final concentration of 1.5% (w/v) and the solution was centrifuged at 10 000 *g*. To the resulting supernatant, ammonium sulfate was added to a final concentration of 144 mg/ml and, after centrifugation, the pellet was discarded. The ammonium sulfate concentration in the supernatant was increased to a final concentration of 277 mg/ml and the protein fraction containing ATase was pelleted by centrifugation. The corresponding ammonium sulfate concentrations for the precipitation of AT-N and AT-C were 144–430 mg/ml and 242–430 mg/ml respectively. The protein pellets were resuspended in sodium phosphate buffer, 20 mM, pH 7.2, and dialysed against the same buffer overnight. ATase and AT-C were purified further by separation in an ISCO HPLC system. The protein solutions were filtered through a 22 μm filter and applied to a self-packed Poros 20HQ anion exchange column (4.6×100 mm, PerSeptive Biosystems) equilibrated with sodium phosphate buffer, 20 mM, pH 7.2, and NaCl, 100 mM at a flow rate of 4 ml/min. The proteins were eluted with a linear gradient in sodium phosphate buffer, 20 mM, pH 7.2, of 100–600 mM NaCl over 4 min at a flow rate of 4 ml/min. ATase and AT-C eluted at ~450 mM NaCl. Glycerol to a final concentration of 10% was added and the samples were stored at –20°C. Both proteins were ~80% pure as judged from an SDS–polyacrylamide gel stained with Coomassie brilliant blue R-250.

Purification of PII, UTase, GS and GS-AMP. The production and purification of PII and UTase was essentially as described previously (Vasudevan *et al.*, 1994; Jaggi *et al.*, 1996). The purification of GS and GS-AMP will be described elsewhere (D.Molenaar, W.C.van Heeswijk and H.V.Westerhoff, in preparation).

Uridylylation of PII

The uridylylation of PII was carried out in a 1 ml reaction solution containing purified PII (2 mg), HEPES pH 7.5 (25 mM), α-ketoglutarate (1.25 mM), $MgCl_2$ (25 mM), dithiothreitol (DTT, 1.25 mM), KCl (125 mM), ATP (40 mM) and UTP (4 mM). After pre-warming the reaction solution at 30°C for 5 min, the uridylylation reaction was started by adding partially purified uridylyl transferase (10 μg as judged by SDS–PAGE) and the uridylylation was allowed to proceed for 20 min at 30°C. The reaction was stopped and the uridylylated PII isolated by applying the reaction mixture to an HPLC anion exchange column (Poros 20HQ) equilibrated with HEPES buffer 20 mM, pH 7.5 containing EDTA (1 mM) and β-mercaptoethanol (1 mM). Purified PII-UMP was eluted on a linear gradient of HEPES buffer 20 mM, pH 7.5 containing EDTA (1 mM) and β-mercaptoethanol (1 mM) to HEPES buffer 20 mM, pH 7.5 containing EDTA (1 mM) and β-mercaptoethanol (1 mM) also containing 1 M NaCl over 12 min at a flow rate of 4 ml/min. PII-UMP eluted at ~750 mM NaCl. The degree of uridylylation of PII-UMP was found to be >90% as estimated by non-denaturing polyacrylamide gel electrophoresis (Forchhammer and Tandeau de Marsac, 1994).

SDS–PAGE and Western blot analysis

The proteins were analysed under denaturing conditions on a 10% SDS–polyacrylamide gel and under non-denaturing conditions on an 8% polyacrylamide gel (Laemmli, 1970; Forchhammer and Tandeau de Marsac, 1994). For quantification, the ATase domains AT-N and AT-C were blotted onto nitrocellulose and probed with ATase polyclonal antibodies (D.Molenaar and H.V.Westerhoff, unpublished). The bands of the primary antibodies were probed with alkaline phosphatase-conjugated secondary antibodies and visualized after exposure to AttoPhos detection reagent (Amersham) by scanning with a Storm PhosphorImager (Molecular Dynamics).

Glutamine synthetase adenylylation and deadenylylation assay

The adenylylation/deadenylylation of GS was monitored as the rate of formation of γ-GH utilizing the γ-glutamyl transferase activity of GS as described by Stadtman *et al.* (1970). The γ-glutamyl transferase activity was measured in microtitre plates at 30°C as described by van Heeswijk *et al.* (1996). Essentially, the assay was started by adding solutions of 100 μl of ATase or either of its domains (AT-N or AT-C) diluted as indicated in bovine serum albumin (BSA, 1 mg/ml) containing PEG 6000 (10%), to 100 μl of 2× ATase reaction mixture containing HEPES–HCl (100 mM, pH 7.6), BSA (2 mg/ml), potassium phosphate (50 mM), $MgCl_2$ (10 mM), ATP (2 mM, pH 7.2), as well as glutamine (2 mM) and GS (100 nM) for the adenylylation reaction or α-ketoglutarate (40 mM) and GS-AMP (100 nM; n = 11) for the deadenylylation reaction and PII or PII-UMP as indicated. At indicated time intervals, an aliquot of 10 μl was withdrawn from the ATase reaction mixture and

added to 100 µl of the γ-glutamyl transferase assay mixture (pH 7.2) containing imidazole–HCl (0.16 M), glutamine (0.16 M), NH_2OH (42 mM), ADP (0.42 mM), Na_2HAsO_4 (21 mM), $MgCl_2$ (62.5 mM) and $MnCl_2$ (0.42 mM). The γ-glutamyl transferase assay was terminated after 30 min by adding 200 µl of the stop mixture containing $FeCl_3$ (206 mM), trichloroacetic acid (123 mM) and HCl (249 mM). The amount of γ-GH produced was determined by measuring the absorbance at 490 nm. The quoted molarities refer to the GS dodecamer, ATase monomer and PII and PII-UMP trimer. Note also that phosphate which is a substrate in the deadenylylation reaction is also present in the adenylylation assay so as to reduce or eliminate the PII-independent adenylylation reaction of ATase when glutamine is present.

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