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Regulation of synthesis of glutamine synthetase by adenylylated glutamine synthetase

(autoregulation/control of nitrogen metabolism/*Klebsiella aerogenes*/enzyme regulation)

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ABSTRACT **We have examined three mutants of *Klebsiella aerogenes* whose genetic lesions (*glnB, glnD,* and *glnE*) are in loci unlinked to the structural gene for glutamine synthetase (*glnA*) and in which the control of both the level and state of adenylylation of glutamine synthetase is altered. Each mutation alters a different component of the adenylylation system of glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2]. Inability of the cell to deadenylylate glutamine synthetase (*glnB* and *glnD*) greatly decreases its production, while inability to adenylylate glutamine synthetase (*glnE*) results in its constitutively high production. These results together with our previous results indicate that adenylylated glutamine synthetase inhibits the transcription of *glnA*.**

The rate of synthesis of glutamine synthetase [GS; L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] in *Klebsiella aerogenes* is controlled by the nitrogen source of the growth medium: it is high in media whose nitrogen source is growth-rate limiting, and low in media containing an excess of ammonia. We have found that mutations in the site of the structural gene for GS, *glnA*, can alter this response: some of the mutants produce enzymatically inactive GS antigen and others active enzyme at a high rate in an ammonia-rich medium (1, 2, 9). The study of merodiploid cells, containing a mutated, as well as a normal *glnA* gene, has shown that the mutant phenotype is recessive (3). These observations led us to the hypothesis that normal GS is converted in the presence of an excess of ammonia to a form that represses the synthesis of GS.

Studies in the laboratories of Stadtman (4) and Holzer (5) have shown that the ability of GS to catalyze the formation of glutamine can be rapidly decreased by the attachment of adenylyl groups to the enzyme, and, conversely, rapidly increased by the removal of these groups (see Fig. 1). Both unadenylylated and adenylylated GS have transferase activity, that is, the ability to transfer the glutamyl group of glutamine to hydroxylamine in the presence of ADP; thus the presence of either form of GS is readily determined.

Adenylylation and deadenylylation are catalyzed by the same enzyme, adenylyltransferase (ATase). The reaction which ATase catalyzes is governed by a regulatory protein (P_{II}). The unmodified form of P_{II}, P_{IIA}, stimulates adenylylation, whereas, a uridylylated form, P_{IID}, is required for deadenylylation. The interconversion of P_{IIA} and P_{IID} is accomplished by a third enzyme, uridylyltransferase (UTase).

UTase is activated by 2-ketoglutarate and inhibited by glutamine. Consequently, a high ratio of 2-ketoglutarate to glutamine, characteristic of cells growing in an ammonia-deficient medium, will stimulate deadenylylation of GS, and conversely, a low ratio of 2-ketoglutarate to glutamine, characteristic of cells growing with an excess of ammonia, will stimulate its adenylylation. And, indeed, in cells growing in the ammonia-deficient medium, GS is largely present in the free form, and in cells growing with an excess of ammonia, GS is largely present in the adenylylated form (5).

In view of these findings we postulate that the adenylylated form of GS is responsible for the repression of GS. Accordingly, mutants deficient in the ability to deadenylylate GS should produce very little GS and require glutamine for growth; mutants deficient in the ability to adenylylate GS should contain a high level of GS, even when grown in a medium containing an excess of ammonia. Furthermore, a mutant requiring glutamine because of its inability to deadenylylate GS should revert to glutamine independence by a second mutation leading to the loss of ability to adenylylate GS.

We have previously reported that a mutation in the *glnB* site results in the almost complete failure to produce GS (1). A similar deficiency results from a mutation in the *glnD* site (6). These sites are not linked to one another or to *glnA*, the structural gene for GS. The glutamine requirement of the *glnB* mutant can be suppressed by a mutation in *glnE*, linked to *glnB*, but separable from it by transduction with phage P1 (7). The results presented in this paper show that the mutations in *glnB* and *glnD* prevent deadenylylation of GS, by altering P_{II} and UTase, respectively. The mutation in *glnE* prevents adenylylation of GS by altering ATase.

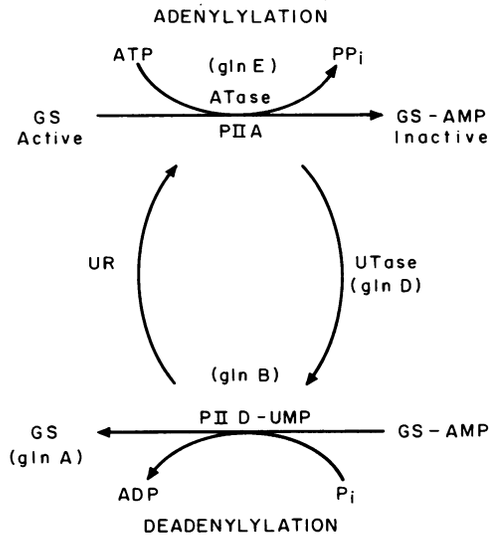

FIG. 1. Scheme for adenylylation and deadenylylation of glutamine synthetase, adapted from Ginsburg and Stadtman (4). UR, uridylyl-removing enzyme.

Abbreviations: GS, glutamine synthetase; ATase, ATP:glutamine synthetase adenylyltransferase; P_{IIA}, unmodified protein component stimulating adenylylation of GS; P_{IID}, uridylylated P_{IIA} stimulating deadenylylation; P_{II}, undefined mixture of P_{IIA} and P_{IID}; UTase, UTP:P_{IIA} uridylyltransferase.

MATERIALS AND METHODS

Bacterial Strains. The presence of GS in extracts interferes with the adenylylation and deadenylylation assays to be described below. Strain MK 9011 (relevant genotype, *glnA6*) was therefore used as a source of wild-type ATase, P_{II}, and UTase. This strain contains a small deletion in the structural gene for GS (2). Strain MK 9401 (*glnB3*) contains such a low level of GS that the *glnA6* mutation need not be introduced (1, 7). The same is true for strain MK 9157 (*glnD17*) (6). The suppressor mutation at *glnE*, when moved by transduction to a $glnB^+$ strain, causes the production of a constitutively high level of unadenylylated GS (7). Strain MK 9195 (*glnE54 glnA6*), which contains the deletion in the GS structural gene, was therefore used in examining the effect of the *glnE* mutation on the adenylylation system.

Preparation of Cell Extracts. Bacteria were grown at 30° with strong aeration on minimal medium containing 10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, and 50 mg of $MgSO_4$ per liter of distilled water, supplemented with 0.4% glucose and 0.2% glutamine. Other amino acids were added when required at a final concentration of 0.01%. Cells were harvested during exponential growth when a concentration of about 10^9 cells per ml was reached, and were stored frozen at −20°. Frozen cell paste (10 g) was thawed and resuspended with 10 ml of buffer, pH 7.15, containing 0.10 M imidazole·HCl, 0.10 M KCl, 0.30 mM $MgCl_2$, 0.25 mM EDTA, 7 mM 2-mercaptoethanol, and 5 mg of DNase I (Sigma catalog no. DN-100). All subsequent steps were carried out at 4°. The suspension was passed through a French pressure cell at 12,000 lb/in^2 (83 MPa), and the cell debris was removed by centrifugation for 1 hr at 35,000 × *g*.

GS Transferase Assay. The state of adenylylation of GS was determined by measuring the reverse γ-glutamyl transferase activity of GS as described by Stadtman *et al.* (8) for *Escherichia coli* W, except that 0.10 M imidazole, pH 7.15, was used as the buffer. At this pH the enzyme from *K. aerogenes* has a transferase activity which is about 2.5-fold higher when the enzyme is fully adenylylated than when it is fully unadenylylated. The two forms have identical transferase activities at pH 7.55; however, this higher pH was not used, since precipitates form when 60 mM $MgCl_2$ is added to inhibit the adenylylated enzyme. Precipitates do not form at pH 7.15. The completely unadenylylated enzyme is stimulated about 1.6-fold by the addition of Mg^{++}, while the fully adenylylated enzyme is inhibited by 93%. For preparations having an intermediate state of adenylylation, the ratio of the activity in the presence of Mg^{++} to the activity in the absence of Mg^{++}, divided by a factor of 1.6, was used as a rough estimate of the fraction of subunits which have adenylyl groups attached. An exact correlation of this number to the actual state of adenylylation as determined by an independent method has as yet not been carried out for the enzyme from *K. aerogenes*.

Deadenylylation Assay. The solution to be assayed was incubated at 37° with about 0.3 unit of adenylylated GS, prepared as previously described (9), in a 0.20 ml assay mixture, pH 7.15, containing 0.10 M imidazole·HCl, 15 mM potassium 2-ketoglutarate, 10 mM potassium phosphate, 15 mM $MgCl_2$, and 1.0 mg/ml of bovine serum albumin. After 4 hr of incubation, portions of 20 μl were removed and added to 0.50 ml of transferase assay mixture, and the state of adenylylation of GS was determined. Deadenylylation activity is expressed as the difference between the percent adenylylation of GS before and after incubation.

Table 1. Ability of the various mutant extracts to catalyze adenylylation and deadenylylation of GS

Extract*	% Adenylylation	% Deadenylylation
Wild type	65	90
glnB	70	3
glnD	69	0
glnB + *glnD*	66	50
glnE	7	67

* Twenty-five microliters of each extract was assayed as described under *Materials and Methods*, except that 1.0 mM UTP was added to the deadenylylation assay mixture.

Adenylylation Assay. The solution to be assayed was incubated at 37° with about 0.3 unit of unadenylylated GS, prepared as previously described (9), in a 0.20 ml assay mixture, pH 7.15, containing 0.10 M imidazole·HCl, 10 mM glutamine, 4 mM ATP, 8 mM $MgCl_2$, and 1.0 mg/ml of bovine serum albumin. After 30 min portions of 20 μl were removed and added to 0.50 ml of transferase assay mixture, and the state of adenylylation of GS was determined. Adenylylation activity is expressed as the difference between the percent adenylylation before and after incubation.

Fractionation of Crude Extracts on Sephadex G-200. Fresh crude extract (8.0 ml) was applied to a column (3.1 × 111 cm) of Sephadex G-200 (40–120 μm particle diameter) that had been equilibrated with buffer at pH 7.15, containing 20 mM imidazole·HCl, 0.10 M KCl, 0.30 mM $MgCl_2$, 0.25 mM EDTA, and 7 mM 2-mercaptoethanol. The column was developed with the same buffer at 4°. Fractions of 8.0 ml were collected at a flow rate of 40 ml/hr.

RESULTS

We tested extracts of the *glnB* and of the *glnD* mutants for their ability to adenylylate and deadenylylate GS. The results presented in Table 1 show that either extract alone can catalyze adenylylation of GS, but that neither extract can catalyze its deadenylylation. A mixture of the extracts is able to catalyze deadenylylation. We conclude, therefore, that the two mutants have defects in different components of the system required for deadenylylation.

We attempted to identify the defective components by complementing the mutant extracts with fractions of extracts of wild-type cells containing individual components of the system. We separated the components of the wild-type extract on Sephadex G-200. Our results, shown in Fig. 2, are similar to those reported by Stadtman and his coworkers (10–12) for the fractionation of extracts of *E. coli* W. None of the column fractions alone was capable of adenylylation or deadenylylation of GS. However, when material from the low-molecular-weight (P_{II}) region was included in the assay mixture, the position of the ATase became evident [panel (a) of Fig. 2]. The complementary assay, in which excess ATase was added to each assay, revealed the exact location of P_{II} [panel (b) of Fig. 2]. These results showed clearly that our fractionation procedure had succeeded in quantitatively separating P_{II} from ATase, and that P_{II} was a mixture of P_{IIA} and P_{IID}.

We next tested the column fractions for their ability to stimulate deadenylylation when added to mutant extracts deficient in this activity. Panel (c) shows the results with the *glnD* extract. Three components were found, the first two of which did not correspond in position to either ATase or P_{II}. The stimulation of deadenylylation activity provided by

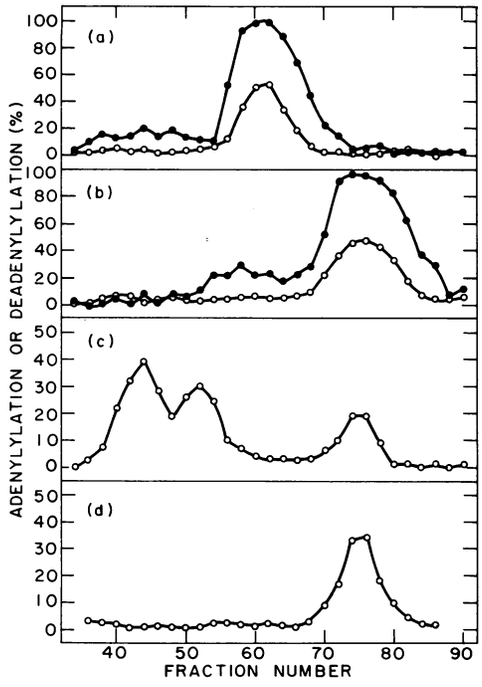

FIG. 2. Fractionation of the wild-type extract on Sephadex G-200. (a) *Assay for ATase activity:* 50 μl of the column fraction was mixed with 50 μl of P_{II} (obtained by pooling 6.0 ml from each of fractions 73–77) and assayed, as described under *Materials and Methods*, for: ●, adenylylation; and ○, deadenylylation. (b) *Assay for P_{IIA} and P_{IID} activity:* 50 μl of the column fraction was mixed with 50 μl of ATase (obtained from fractions 58–62) and assayed for: ●, adenylylation; and ○, deadenylylation. (c) *Complementation of the glnD extract:* 50 μl of the column fraction was mixed with 10 μl of *glnD* extract, and the mixture was tested for deadenylylation activity in the presence of 1.0 mM UTP. (d) *Complementation of the glnB extract:* 50 μl of the column fraction was mixed with 10 μl of *glnB* extract, and the mixture was tested for deadenylylation activity, in the presence of 1.0 mM UTP.

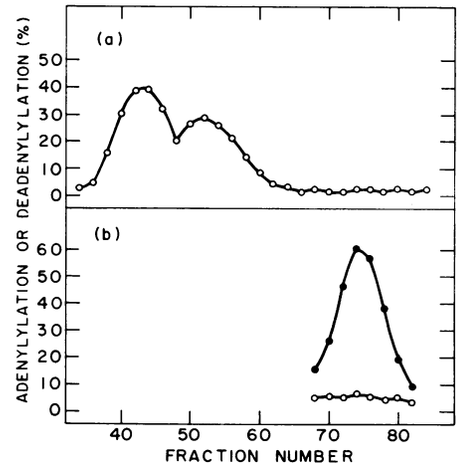

FIG. 3. Fractionation of the *glnB* extract on Sephadex G-200. (a) *Complementation of the glnD extract:* 50 μl of the column fraction was mixed with 10 μl of *glnD* extract, and the mixture was assayed for deadenylylation activity, as described under *Materials and Methods*, except that 1.0 mM UTP was included in the assay mixture. (b) *Assay for P_{IIA} and P_{IID} activity:* 50 μl of the column fraction was mixed with 50 μl of ATase (obtained from the wild-type extract as described in Fig. 2) and assayed for: ●, adenylylation; and ○, deadenylylation.

these high-molecular-weight species was almost completely dependent on the presence of UTP, as well as 2-ketoglutarate (see Table 2). The third component, corresponding to P_{II} in position of elution, required only 2-ketoglutarate. The requirement for UTP confirms that the first two peaks of activity are due to the presence of UTase in these fractions. Since the addition of UTase alone to the mutant extract gives it the ability to deadenylylate GS, we concluded that the *glnD* mutant is deficient in this activity. The presence of two molecular weight species of UTase may be due to partial aggregation of the UTase under our conditions of chromatography.

Table 2. Requirement for 2-ketoglutarate and UTP in the deadenylylation reaction catalyzed by the *glnD* extract complemented with fractions from the wild-type extract

Fraction no.*	2-Keto-glutarate	UTP	% Deadenylylation
43	—	—	1
	+	—	8
	+	+	50
52	—	—	0
	+	—	9
	+	+	87
75	—	—	0
	+	—	17
	+	+	16

* One hundred microliters of the indicated fraction (from the column described in Fig. 2) was incubated with 10 μl of *glnD* extract as described under *Materials and Methods*, except that 15 mM 2-ketoglutarate and 1.0 mM UTP were omitted or added as indicated in the table.

The fractions containing P_{II} also complement the *glnD* extract. It is clear that the *glnD* extract itself contains P_{II}, since it can catalyze deadenylylation upon the addition of UTase. However, since the mutant lacks UTase, its P_{II} remains completely in the unmodified (P_{IIA}) form. The P_{II} fractions from the column, however, contain material from the wild strain, a certain fraction of which is in the uridylylated (P_{IID}) form. Thus, the *glnD* extract is rendered active for deadenylylation by the addition of either the missing enzyme, UTase, or its product, P_{IID}.

Panel (d) of Fig. 2 presents our results when we tested the ability of the fractions from the column to stimulate deadenylylation by the *glnB* extract. In this case only the addition of P_{II} led to deadenylylation. This suggests that the *glnB* extract lacks P_{II}. However, this extract catalyzed adenylylation of GS normally (see Table 1), suggesting that P_{IIA} was present. We fractionated the extract from the *glnB* mutant on the Sephadex column, as shown in Fig. 3. The column fractions were first assayed for components capable of complementing the *glnD* extract (upper panel). The results show that the *glnB* mutant contains a normal amount of UTase but lacks P_{IID} activity, as predicted by our previous result. When the fractions in the P_{II} region were assayed with ATase from the wild strain, P_{IIA} activity was readily apparent (lower panel). Since the *glnB* mutant contains normal UTase, yet was incapable of carrying out the deadenylylation of GS even in the presence of UTP, we must conclude that its P_{II} protein is altered in such a way that it can no longer be converted by the action of UTase into a form capable of activating deadenylylation.

We turned next to the *glnE* mutant. The *glnE* mutation suppresses the effect of the *glnB* mutation by apparently

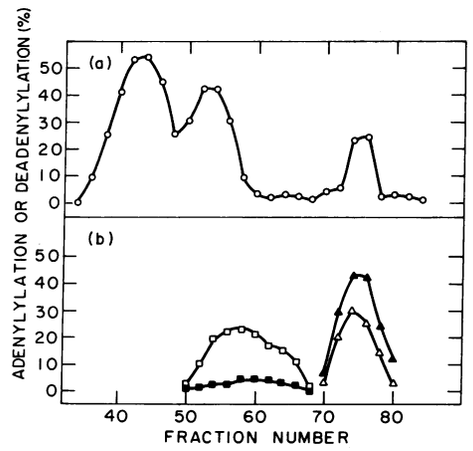

FIG. 4. Fractionation of the *glnE* extract on Sephadex G-200. (a) *Complementation of the glnD extract:* 50 μl of the column fraction was mixed with 10 μl of *glnD* extract, and the mixture was tested for deadenylylation activity, as described under *Materials and Methods*, except that 1.0 mM UTP was added to the assay mixture. (b) *Assay for ATase activity:* 50 μl of the column fraction was mixed with 50 μl of P_{II} (obtained from the wild-type extract as described in Fig. 2) and assayed for: ■, adenylylation; and □, deadenylylation. *Assay for P_{IIA} and P_{IID} activity:* 50 μl of the column fraction was mixed with 50 μl of ATase (obtained from the wild-type extract as described in Fig. 2) and assayed for: ▲, adenylylation; and △, deadenylylation.

preventing full adenylylation of GS, even under conditions normally leading to high adenylylation. When the *glnE* mutation was transduced into another strain, giving a *glnB*$^+$ *glnE54* genotype, this effect was even more pronounced (7). A crude extract of the *glnE* mutant was tested for its ability to adenylylate and deadenylylate purified GS. It was found to have only deadenylylation activity (see bottom of Table 1). We therefore fractionated the mutant extract on the Sephadex column. The results are shown in Fig. 4. Assay for components complementing the *glnD* extract revealed the presence of normal amounts of UTase and P_{IID} (Fig. 4, upper panel). Assay for P_{II} activity, by complementation with wild-type ATase, showed the presence of normal amounts of P_{IIA} and P_{IID} (see the second peak in the lower panel of Fig. 4). When the column was assayed for ATase by complementation with wild-type P_{II}, the ATase from the *glnE* mutant appeared to have normal deadenylylating ability, but was much less active in the adenylylation of GS (see the first peak in the lower panel of Fig. 4). This defect accounts for the failure of the *glnE* mutant to adenylylate GS. We conclude, therefore, that the *glnE54* mutation has altered the ATase so that it is less effective in carrying out the adenylylation of GS.

DISCUSSION

Our results show that *glnB*, *glnD*, and *glnE* are the likely structural genes for P_{II}, UTase, and ATase, respectively. Alterations of P_{II} and UTase leading to the inability of the cell to deadenylylate GS result in an abnormally low level of GS, as measured by antigenicity (9) and transferase activity (6, 7). Conversely, an alteration causing a failure to adenylylate results in high levels of GS, even when the cells are grown under conditions of nitrogen excess (7).

One possible explanation for these results is that intracellular adenylylated GS is less stable than unadenylylated GS. We have evidence that allows us to reject this hypothesis. Normal cells grown on nitrogen-limited media accumulate a large pool of unadenylylated GS. When the cells are shifted from nitrogen-limited to energy-limited growth, GS immediately becomes highly adenylylated. Nevertheless, there is no decrease in the accumulated GS, other than that expected from dilution by further growth (unpublished experiment).

Recent experiments by P. Weglenski and B. Tyler (personal communication) have shown that the level of GS reflects the intracellular concentration of RNA specific for *glnA*, the structural GS gene (9); the level of this *glnA* specific RNA was greatly reduced in the strain having the altered P_{II} (*glnB*). This reduction in transcription of *glnA* can be corrected by a mutation in *glnA* that results in a high level of GS even when the cells are grown in an ammonia-rich medium (1); this *glnA* mutation is recessive to *glnA*$^+$ (3). These facts together with our present results suggest that adenylylated GS is the inhibitor of transcription of *glnA*. Consequently, a defect in the ability to deadenylylate GS (as in *glnB* and *glnD* mutants) leads to permanent repression of GS; conversely, a defect in the ability to adenylylate GS (as in the *glnE* mutant) leads to nonrepressible synthesis of unadenylylated GS. The mutation in *glnB* (no deadenylylation) can be suppressed by a mutation in *glnE* (no adenylylation) or by certain mutations in *glnA* (GS inactive as repressor).

Enzymes required for the production of ammonia or glutamate from other nitrogen-containing compounds are only produced in cells starved for ammonia. The synthesis of these enzymes is stimulated by GS (3). In the case of the *hut* operon, coding for histidine-degrading enzymes, it has been shown that unadenylylated GS activates transcription of *hut* specific DNA (13).

We may now explain how the message that ammonia is required is transmitted through GS to the responsive systems. Deadenylylation of GS is stimulated by a high intracellular ratio of 2-ketoglutarate to glutamine, which results from ammonia deficiency. The conversion of adenylylated to unadenylylated GS results in derepression of GS. The consequent accumulation of nonadenylylated GS activates transcription of genes like *hut* coding for enzymes whose role it is to provide ammonia and glutamate from other nitrogen compounds. Conversely, a low ratio of 2-ketoglutarate to glutamine which results from an excess of ammonia brings about adenylylation of GS, cessation of the activation of transcription of systems like *hut*, and repression of GS.

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