JOURNAL OF BACTERIOLOGY, Sept. 1982, p. 1299–1313
0021-9193/82/091299-15$02.00/0

Vol. 151, No. 3

Asparagine Synthetases of *Klebsiella aerogenes*: Properties and Regulation of Synthesis

LAWRENCE J. REITZER AND BORIS MAGASANIK*

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received 13 November 1981/Accepted 29 April 1982

We isolated pleiotropic mutants of *Klebsiella aerogenes* with the transposon Tn*5* which were unable to utilize a variety of poor sources of nitrogen. The mutation responsible was shown to be in the *asnB* gene, one of two genes coding for an asparagine synthetase. Mutations in both *asnA* and *asnB* were necessary to produce an asparagine requirement. Assays which could distinguish the two asparagine synthetase activities were developed in strains missing a high-affinity asparaginase. The *asnA* and *asnB* genes coded for ammonia-dependent and glutamine-dependent asparagine synthetases, respectively. Asparagine repressed both enzymes. When growth was nitrogen limited, the level of the ammonia-dependent enzyme was low and that of the glutamine-dependent enzyme was high. The reverse was true in a nitrogen-rich (ammonia-containing) medium. Furthermore, mutations in the *glnG* protein, a regulatory component of the nitrogen assimilatory system, increased the level of the ammonia-dependent enzyme. The glutamine-dependent asparagine synthetase was purified to 95%. It was a tetramer with four equal 57,000-dalton subunits and catalyzed the stoichiometric generation of asparagine, AMP, and inorganic pyrophosphate from aspartate, ATP, and glutamine. High levels of ammonium chloride (50 mM) could replace glutamine. The purified enzyme exhibited a substrate-independent glutaminase activity which was probably an artifact of purification. The tetramer could be dissociated; the monomer possessed the high ammonia-dependent activity and the glutaminase activity, but not the glutamine-dependent activity. In contrast, the purified ammonia-dependent asparagine synthetase, about 40% pure, had a molecular weight of 80,000 and is probably a dimer of identical subunits. Asparagine inhibited both enzymes. Kinetic constants and the effect of pH, substrate, and product analogs were determined. The regulation and biochemistry of the asparagine synthetases prove the hypothesis strongly suggested by the genetic and physiological evidence that a glutamine-dependent enzyme is essential for asparagine synthesis when the nitrogen source is growth rate limiting.

In the course of our studies on nitrogen assimilation in *Klebsiella aerogenes* we isolated mutants that required ammonia for normal growth. Unlike previously isolated mutants with a similar phenotype, these mutants were not defective in glutamate synthase or in the product of *glnG*, a regulator of the expression of glutamine synthetase. The phenotype of these mutants results from a mutation in a previously described gene coding for an asparagine synthetase (8, 14). This observation led us to investigate the properties and the regulation of the formation of the two asparagine synthetases of *K. aerogenes*, the products of the *asnA* and *asnB* genes.

MATERIALS AND METHODS

Chemicals. Common chemicals were reagent grade and were used without purification unless mentioned below. For cell growth, Calbiochem (grade A) glutamine was used. The following chemicals were from Sigma Chemical Co.: reactive red-120 cross-linked agarose (product no. R-0503), 6-diazo-5-oxo-norleucine, and amino-oxyacetic acid. Ammonium sulfate (special enzyme grade) was from Schwarz/Mann, azaserine from P-L Biochemicals, Inc., and 5-diazo-4-oxo-norvaline was the gift of R. E. Handschumacher (Yale University) via Andrew Wright (Tufts University). Potassium chloride (Fisher Scientific Co.) had to be filtered before use or the columns got clogged. All enzymes used for analytical purposes were from Boehringer Mannheim Corp., except myokinase (EC 2.7.4.3) which was from Sigma. All chromatographic resins were from Pharmacia Fine Chemicals except for the anion exchange resin for asparagine synthetase assays, AG 1-X8 (100 to 200 mesh, chloride form), which was from Bio-Rad Laboratories. [*U*-^{14}C]aspartate was from New England Nuclear Corp. or ICN Chemicals and Radioisotopes; the labeled aspartate was purified as previously described (28).

TABLE 1. List of *K. aerogenes* strains

Strain	Genotype	Derivation or source
MK9011	*glnA6 ilvA1*	MIT[a]
MK9361	*glnA51 ilvA1*	MIT
MK9520	*gtf-2 metB4 rha-1*	MIT
FF1025	*rha-1 glnA20 rbs-7*	MIT
CG253	*rha-1*	MIT
CG566	*metB4 rha-1 nadB1*	MIT
CG632	*glnG229*::Tn*5*	MIT
CG777	*rha-1 asnB1*::Tn*5*	Tn*5* mutagenesis of CG253
CG784	*glnA263*::Tn*5 ilvA1*	MIT
CG785	*glnG229*::Tn*5 ilvA1*	P1 transductant of CG632 into MK9011
LR83	*gtf-2 metB4 rha-1 asnB1*::Tn*5*	P1 transductant of CG777 into MK9520
LR84	*gtf-2 metB4 rha-1 asnA1 asnB1*::Tn*5*	EMS mutagenesis of LR83
LR87	*gtf-2 metB4 rha-1 asnA1*	P1 transductant of MK9011 into LR84
LR88	*gtf-2 metB4 asnB1*::Tn*5 glnA6*	P1 transductant of MK9011 into LR84
LR89	*metB4 rha-1 nadB1 trp-1*	EMS mutagenesis of CG566
LR90	*gtf-2 metB4 asnA1 asnB1*::Tn*5*	P1 transductant of LR84 into LR88
LR95	*metB4 rha-1 nadB1 nag-3*	EMS mutagenesis of CG566
LR96	*gtf-2 metB4 rha-1 asnB1*::Tn*5 nag-1*	EMS mutagenesis of LR83
LR146	*gtf-2 asnA1 glnG229*::Tn*5* (*rha*?)	P1 transductant of CG785 into LR87, *asnA* verified by assay
LR147	*gtf-2 asnA1 glnA263*::Tn*5* (*rha*?)	P1 transductant of CG784 into LR87; *asnA* verified by assay
LR148	*gtf-2 glnG229*::Tn*5* (*rha*?)	P1 transductant of CG785 into MK9520
LR149	*gtf-2 glnA263*::Tn*5* (*rha*?)	P1 transductant of CG784 into MK9520
LR153	*gtf-2 glnA51* (*rha*?)	P1 transductant of MK9361 into MK9520

[a] Laboratory stock, Massachusetts Institute of Technology.

Media and cell growth. Media and cell growths were those described previously (27). Large-scale cell preparations were made in 1.25- to 1.5-liter batches in 6-liter flasks with vigorous aeration. The cells were grown to late log growth at 30°C, harvested by centrifugation, washed once or twice in 0.85% NaCl, and stored as frozen pellets at −20°C. Purified agar (Oxoid Ltd.) or agarose (Sigma or Calbiochem) was used in place of normal agar when growth on poor nitrogen sources was being tested. This precaution was necessary because normal agar contained residual ammonia.

Cell strains and construction. Table 1 lists the strains used. All of the strains were *K. aerogenes*. Strains were constructed as previously described (10). Sometimes transductions with P1 clr100CM or the P1 *vir* phages present in our lab were not successful with kanamycin-resistant strains. A positive selection for lysogens was possible through the construction of a P1.TC phage. The phage was constructed by introducing a temperature-sensitive F′ episome (9) carrying Tn*5* (kanamycin resistance) and Tn*10* (tetracycline resistance) into EG47 (10), a strain of *Escherichia coli* with no restriction or modification systems. The episome recipient was a P1 clr100CM lysogen of EG47: the phage is thermally induced, and therefore the cells were grown at 30°C. Lysis was induced by growth at 37°C, and temperature-sensitive, tetracycline-resistant lysogens of EG47 were obtained. Lysis and subsequent transductions into *Klebsiella* strains gave a high frequency of transfer of tetracycline resistance. The phage was not characterized further.

Mutagenesis and mutant selection. Ethyl methane sulfonic acid (EMS) mutagenesis has been described previously for *K. aerogenes* (27). A 2-h EMS treatment at 37°C gave 10 to 15% survival.

We isolated the *asnB*::Tn*5* strains from a culture mutagenized with the insertion element Tn*5*, which codes for kanamycin resistance, by a method described previously (9). After generation of a population with random transpositions and two rounds of penicillin treatment for 1.5 generations in glucose-proline medium, the survivors were screened for growth with low levels of ammonia and inability to grow on proline as nitrogen source. The phenotype cotransduced with kanamycin resistance.

The *nag* mutants (*N*-acetyl-D-glucosamine non-utilizers) were obtained by EMS mutagenesis and one round of penicillin selection with *N*-acetyl-D-glucosamine (0.1%) as carbon source.

Enzyme assays and other analytical methods. Cells for the assay of asparagine synthetase were prepared by ultrasonic disruption in 20 mM imidazole–5 mM $MgCl_2$–0.5 mM EDTA–0.5 mM dithiothreitol brought to pH 7.0. Protein concentration was at least 0.5 mg/ml in the crude extract. After sonication, the extract was centrifuged at 12,000 × *g* for 10 to 15 min to remove cell debris. The final assay mixture contained 20 mM imidazole, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 2 mM K-aspartate, and 2 μCi of [*U*-^{14}C]aspartate per ml at pH 7.0; this was called the basic mixture. For the assay of glutamine-dependent asparagine synthetase and ammonia-dependent asparagine synthetase in the same extract, three reactions were run: (i) the basic mixture plus 2.5 mM NH_4Cl

(this was the blank); (ii) same as i, but 3.0 mM ATP was added (this gave ammonia-dependent asparagine synthetase activity); and (iii) same as ii, but 2.0 mM glutamine (freshly prepared) was added (this measured the sum of glutamine-dependent asparagine synthetase and ammonia-dependent asparagine synthetase). The activity of the glutamine-dependent asparagine synthetase was determined by the subtraction of assay ii from assay iii.

For crude extract analysis, the asparagine synthetase assays were conducted in 0.6 ml of basic assay mixture at 30°C. Five 0.1-ml samples were added to 0.4 ml of 95% ethanol at room temperature (about 23°C). If they were not to be processed within 0.5 h, the tubes were kept at −20°C. The ethanol solution was mixed with 1 ml of 10 mM imidazole plus 5 mM asparagine at pH 7.5, applied to a small ion-exchange column as described previously (28), and washed with 1 ml of the above-mentioned imidazole-asparagine solution. The voided liquid, which contained asparagine, was collected directly into scintillation vials. A 5-ml portion of scintillation fluid (Scinti Verse; Fisher) was added, and the contents were vigorously mixed and counted. Counts per minute were converted to nanomoles by taking a sample of the assay mixture, adding 2 ml of wash solution and 0.4 ml of ethanol to a scintillation vial, and counting as above. Assays were linear if less than 20,000 cpm were observed. Preliminary assays, 50 μl of extract plus 50 μl of double-strength assay mixture, were run to determine how much extract to add.

For the purification a rapid, more sensitive assay was derived. Aspartate was reduced to 0.5 mM, and only one time point was taken. The total reaction was done in 100 μl, with 50 μl of sample and 50 μl of double-strength assay mixture. For purification of the glutamine-dependent asparagine synthetase, glutamine addition was optional because all of the buffers contained glutamine.

The small Dowex columns were recycled by the following steps: three washes with 0.03 N HCl, one wash with 0.01 N NaOH, and five washes with 20 mM imidazole at pH 7.5. About 1.5 ml was used for each wash. With this procedure, the background was consistently low.

Glutaminase activity was assayed by two methods, both measuring ammonia generation. The first assay is a two-step assay: the first step contained 20 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM glutamine in 0.2-ml total volume, pH 7.0, at 30°C. The reaction was terminated by adding 0.05 ml of 1 N perchloric acid, and the mixture was centrifuged. A 0.2-ml portion was removed and neutralized with 1 N KOH. The mixture was again centrifuged, and 0.2 ml was removed and assayed for ammonia with glutamate dehydrogenase (EC 1.4.1.3). The final mixture contained 50 mM Tris, 0.1 mM ADP, 10 mM 2-ketoglutarate, 0.2 mM NADH, and 12 U of beef liver glutamate dehydrogenase in 1 ml at pH 7.6. The decrease in NADH was monitored at 340 nm in a spectrophotometer.

The second glutaminase assay was simpler and continuous. The assay was performed in 1 ml at 30°C and pH 7.0. The mixture contained 20 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM ADP, 0.5 mM 2-ketoglutarate, 1 mM glutamine, 0.15 mM NADH, and 24 U of glutamate dehydrogenase per ml. The coupling enzyme was not limiting, and a blank without glutamine was always run in parallel. NADH disappearance was monitored as described above.

The products and substrates of the asparagine synthetase reaction for the reaction stoichiometry experiment were determined by enzymatic methods. AMP, ADP, ATP, aspartate, and glutamate levels were determined by fluorometric techniques in a Farrand filter fluorometer as previously described (18). Asparagine was assayed by adding 0.4 U of *E. coli* L-asparaginase (EC 3.5.1.1) per ml to the aspartate reaction after the aspartate was depleted. Inorganic pyrophosphate (PP_i) was determined by adding 1 mM UDP-glucose and 0.4 U of UDPglucose pyrophosphorylase (EC 2.7.7.9) per ml to the previously described glucose-1-phosphate determination (18). Ammonia was determined as described above for the two-step glutaminase assay. Protein was assayed (19) and gel electrophoresis was carried out (15) as previously described.

Purification of glutamine-dependent asparagine synthetase. Strain LR87 (*asnA asnB*$^+$) was used for the purification. The first large-scale purification used cells grown to stationary phase and the second used exponentially growing cells: no difference in the properties of the enzyme were seen between them. Cells were grown in glucose-glutamine medium for maximal induction of activity. The cells from 15 1.5-liter cultures were used for each preparation.

All operations were performed at 4°C. All buffers contained 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (or dithioerythritol), and 10 mM glutamine. Buffer A also contained 20 mM 2(*N*-morpholino)ethane sulfonic acid at pH 6.0; buffer B contained 20 mM imidazole at pH 7.0; and buffer C contained 20 mM potassium phosphate at pH 7.0. The pH of the buffer was adjusted at room temperature. Two methods of concentration were used: ultrafiltration with Amicon apparatus and PM10 filters or ammonium sulfate precipitation.

Frozen cell pellets were thawed in 100 to 200 ml of buffer A and sonically disrupted. The homogenate was centrifuged at 12,000 × *g* for 20 min, and the pellet was resuspended in buffer A, sonicated again, and centrifuged. Solid streptomycin sulfate was added to the pooled supernatants to 1% (wt/vol), slowly agitated for at least 20 min, and centrifuged. The pellet was discarded.

Solid ammonium sulfate was added to 40% saturation (corrected for 4°C). This step had to be done in pH 6.0 buffer (see below). The solution was slowly agitated for at least 30 min and centrifuged for 30 min at at least 5,000 × *g*, and the pellet was saved. The actual recovery may be lower than those we recorded (see Table 2) because $(NH_4)_2SO_4$ was not removed before assay. Furthermore, if the enzyme had dissociated, it would react with the ammonia present but not with glutamine.

The ammonium sulfate pellet was dissolved in buffer B. In earlier preparations, the next steps were gel filtration and then ion-exchange chromatography. In later purifications these steps were reversed. The latter procedure is recommended.

For ion-exchange chromatography, the enzyme was diluted until the conductivity was less than 75 mM KCl and loaded on a 2.5- by 10-cm column which had 30 ml of DEAE–Sephadex A-50 equilibrated with buffer B

plus 0.1 M KCl. The enzyme was loaded at about 40 ml/h and washed with 0.1 to 0.15 M KCl. This was considered a mistake in retrospect: prolonged washing probably dissociated this enzyme. A gradient between 0.10 or 0.15 and 0.45 M KCl in buffer B (200 ml, each chamber) was run at 80 ml/h, and 5-ml fractions were collected. Activity was eluted in a symmetrical peak centering at 250 mM KCl. All fractions with ≥10% of the peak fraction were pooled and concentrated.

Preparative gel filtration was performed with an ascending 2.6- by 100-cm column packed with Sephadex G-200. The flow rate was 10 to 15 ml/h, and 5-ml fractions were collected. Glutamine-dependent asparagine synthetase came out just after, but cleanly separated from, the peak of void volume protein. The column was equilibrated and run in buffer B plus 0.1 M KCl. All fractions with ≥10% of the peak fractions were pooled and concentrated.

The enzyme was loaded on 5 ml of red agarose after the conductivity had been reduced to 50 mM KCl. The red agarose was equilibrated with buffer B. The glutamine-dependent asparagine synthetase was eluted with 100 ml of 10 mM magnesium ATP after being washed with 100 ml of 3 mM magnesium ATP. Fractions (5 ml) were collected. Washing the enzyme with high salt after the enzyme was loaded led to loss of activity.

The purified enzyme was stored in saturated ammonium sulfate plus 5 mM $MgCl_2$ and 1 mM EDTA at 4°C. Before the experiments, the glutamine-dependent asparagine synthetase was centrifuged and passed through a Sephadex G-25 column (1.5 by 5 cm) to remove residual ammonia.

Purification of ammonia-dependent asparagine synthetase. Cells (4.5 to 6 liters) were grown as described earlier in glucose-glutamine medium to 100 to 150 Klett units. LR83, the starting strain (*asnA*$^+$ *asnB1*::Tn*5*), had the highest level of ammonia-dependent asparagine synthetase. This enzyme was indefinitely stable in a phosphate buffer at pH 7.0 or a Tris buffer at pH 8.0. The same buffers were used in this purification as for the glutamine-dependent enzyme, but glutamine was not included.

The first two steps were done exactly as described for glutamine-dependent asparagine synthetase, but buffer C was used. Enzyme precipitated between 50 and 70% ammonium sulfate saturation (corrected for 4°C) and was dissolved in buffer B (note change) and applied to the DEAE-Sephadex column as described above for the glutamine-dependent enzyme. Buffer C was used for elution, but otherwise all was done as in the purification for the glutamine-dependent asparagine synthetase. Ammonia-dependent asparagine synthetase activity came off in a peak centering at 250 mM KCl. The fractions were pooled and concentrated, and preparative gel filtration was performed as described above for the glutamine-dependent asparagine synthetase except that buffer C was used. The ammonia-dependent asparagine synthetase was stored and processed before experiments in the same way as was the glutamine-dependent enzyme.

Molecular weight determinations. The molecular weight of the glutamine-dependent asparagine synthetase was determined on a 1.5- by 50-cm column packed with Sepharose 6B. The standards used were chromotrypsinogen A, ovalbumin, bovine serum albumin, alcohol dehydrogenase, aldolase, pyruvate kinase, catalase, urease, β-galactosidase, and thyroglobulin. The molecular weights ranged from 25,000 to 669,000. Linear regression analysis was used to determine molecular weight.

The molecular weight of the ammonia-dependent asparagine synthetase was determined on a 1.5- by 50-cm column packed with Sephadex G-200 superfine, with ovalbumin, bovine serum albumin, aldolase, and catalase used as standards. The molecular weight was read off a standard curve.

RESULTS

Isolation of an *asnB* mutant. In the course of our studies on the regulation of nitrogen metabolism, we generated, by the insertion of transposon Tn*5* (Km^r), mutants of *K. aerogenes* that had lost the ability to grow with proline, aspartate, nitrate, or glutamate as the source of nitrogen. They grew slowly with glutamine, but grew as well as did the wild-type strain with ammonia or asparagine as a source of nitrogen. This observation suggested the possibility that the mutants had lost the ability to produce asparagine when supplied with a poor source of nitrogen. Exogenously supplied asparagine is rapidly destroyed by a periplasmic asparaginase; therefore, we transferred the mutation responsible for the inability to grow on poor sources of nitrogen to a mutant lacking this asparaginase by phage P1-mediated transduction and selection for kanamycin resistance (29). The transductant, strain LR83, or one of its derivatives was used in all subsequent experiments. We found that strain LR83 grew as did the wild-type strain on glucose with glutamine or glutamate as source of nitrogen when the medium was supplemented with 0.1 mg of asparagine per ml; supplementation with an equivalent mixture of aspartate and ammonia was not effective.

These results indicated that the slow growth of strain LR83 with glutamine as source of nitrogen reflected starvation for asparagine and not for glutamine or glutamate. This conclusion was supported by the finding that cells of strain LR83 cultivated on glucose-glutamine had low levels of glutamine synthetase and histidase similar to the levels found normally in cells growing with an excess of ammonia; the addition of asparagine to the growth medium increased these enzyme levels to those found in the cells of the wild-type strain grown on glucose-glutamine (data not shown).

It is known that in *E. coli* two genes code for asparagine synthetases and that asparagine auxotrophy requires both genes to be defective (8, 14). To prove that the conditional asparagine auxotrophy of strain LR83 had resulted from the insertion of transposon Tn*5* into one of these genes, we used mutagenesis with EMS to isolate mutants of strain LR83 unable to grow without

TABLE 2. Genetics of *asn*

Cross no.	Relevant genotype		Selected phenotype	Unselected phenotype[a]	Linkage (%)
	Donor	Recipient			
1	MK9011 *glnA6 ilvA1*	LR84 *metB4 asnA1 asnB1*::Tn*5 rha-1*	Asn$^+$ Km^r	Gln (31/156) Rha$^+$ (19/156) Met$^+$ (0/156) Ilv (0/156)	*asnA-glnA* (20) *asnA-rha* (8) *asnA-metB* (0) *asnA-ilvA* (0)
2	FF1025 *rha-1 glnA20 rbs-7*	LR90 *metB4 asnA1 asnB1*::Tn*5*	Asn$^+$ Km^r	Rbs (27/44)[b] Gln (12/44)[c] Rha (2/44)	*asnA-rbs* (62) *asnA-glnA* (27) *asnA-rha* (5)
3	LR84 *asnA1 asnB1*::Tn*5 rha-1*	LR88 *asnB1*::Tn*5 glnA6*	Gln$^+$	Asn (76/104) Rha (17/104)	*glnA-asnA* (76) *glnA-rha* (16)
4	LR96 *asnB1*::Tn*5 nag-1*	MK9520	Km^r	Nag (77/108)	*asnB-nag* (71)
5	LR83 *asnB1*::Tn*5*	LR95 *nag-3*	Nag$^+$	Km^r (88/100)	*nag-asnB* (88)

[a] The number shown in parentheses is the ratio of unselected to selected phenotypes; the denominator is the total number of transductants examined.
[b] Of the 27 Rbs transductants, 12 were Gln$^+$.
[c] All 12 of the Gln transductants were Rbs.

asparagine in an ammonia-containing medium. If the unconditional asparagine auxotrophy indeed resulted from two mutations, then transduction with a phage P1 lysate of the wild-type strain and selection for asparagine independence on the glucose-ammonia medium should yield two classes of transductants distinguishable by their sensitivity or resistance to kanamycin. These two classes were in fact observed, with kanamycin-resistant prototrophs predominating by a ratio of 1.6 over kanamycin-sensitive ones. The reason for this divergence from a 1-to-1 ratio is not known, but transductional anomalies have been observed for other mutations in the region of the chromosome where *asnA* is located (2). The kanamycin-resistant transductants had the phenotype of strain LR83. The kanamycin-sensitive transductants grew with poor sources of nitrogen as well as did the wild-type strain.

It has been shown in *E. coli* that *asnA* is linked to *rbs* and *asnB* is linked to *nag* (8, 14). In *K. aerogenes*, *rbs* is closely linked to *glnA*. Using phage P1-mediated transduction, we obtained the results shown in Table 2. The data show that of the two mutations responsible for the unconditional asparagine requirement in strain LR84, the one resulting from EMS mutagenesis was closely linked to *glnA* and *rbs* (Table 2, crosses 1–3). The linkage of *glnA* to *asnA* depended on the selected phenotype; the reason for this is not understood. Our results indicate the order *asnA rbs glnA rha metB*; there was no linkage between *asnA* and *metB*. The mutation resulting from the insertion of transposon Tn*5* was closely linked to *nag* (Table 2, crosses 4 and 5).

According to these results, the mutants unable to grow on poor sources of nitrogen when not supplied with asparagine are defective in *asnB*, the gene coding for an asparagine synthetase. This conclusion could be confirmed by the demonstration that strain LR83 lacks a glutamine-dependent asparagine synthetase.

Assay of asparagine synthetases. The assay for the enzymes is given in detail above. We found that we could measure each enzyme in the presence of the other by carrying out the assay at pH 7 in a reaction mixture containing 2.5 mM NH_4Cl. Extracts of cells of strain LR87 (*asnA1 asnB*$^+$) catalyzed the synthesis of asparagine only when glutamine was added to the reaction mixture; on the other hand, extracts of cells of strain LR83 (*asnA*$^+$ *asnB1*::Tn*5*) were fully active in the absence of glutamine. In this case, some asparagine was produced in a reaction mixture lacking ammonia as well as glutamine, presumably owing to the fact that the cell extract was not free of ammonia (data not shown). In the control reaction, ATP was omitted.

It was therefore possible to assay both the glutamine- and ammonia-dependent enzymes in the same extract by carrying out the reaction in the presence and absence of 2.0 mM glutamine. The latter value is a measure of the activity of the ammonia-dependent enzyme, and the difference between the values is a measure of the glutamine-dependent enzyme.

Regulation of the synthesis of the asparagine synthetases. Levels of both of the asparagine synthetases depended on the nitrogen source of the medium (Table 3).

In the wild-type strain MK9520, the level of ammonia-dependent asparagine synthetase was high when ammonia was present in the medium and low when the nitrogen source was poor. The reverse was true for the glutamine-dependent asparagine synthetase (Table 3). The addition of

TABLE 3. Regulation of the asparagine synthetases

Expt. no.	Strain	Relevant genotype	Growth medium[a]	NH_4^+-dependent activity (nmol/min per mg of protein)[b]	Gln-dependent activity (nmol/min per mg of protein)[b]
1	MK9520	Wild type	GNgln	11.3 (2)	1.7 (2)
2			GN	16.7 (4)	2.0 (2)
3			Ggln	4.8 (2)	15.8 (2)
4			Gglt	1.8 (2)	6.6 (2)
5			GNasn	0.7 (3)	1.0 (3)
6	LR83	*asnA*$^+$ *asnB1*::Tn*5*	GNgln	12.0 (2)	
7			GN	13.6 (5)	
8			Ggln	189 (3)	
9			GNasn	0.7 (2)	
10	LR87	*asnA1 asnB*$^+$	GNgln		4.5 (2)
11			GN	≤0.44 (1)	8.8 (2)
12			Ggln		19.9 (2)
13			GNasn		1.4 (2)
14	LR84	*asnA1 asnB1*::Tn*5*	GNasn	≤0.15 (3)	≤0.23 (3)
15	LR146	*glnG229*::Tn*5 asnA1 asnB*$^+$	GNgln		5.6 (3)
16			GN		12.9 (3)
17			Ggln		14.8 (4)
18	LR148	*glnG229*::Tn*5 asnA*$^+$ *asnB*$^+$	GNgln	13.0 (2)	2.7 (2)
19			GN	13.5 (2)	3.4 (2)
20			Ggln	57 (2)	14.2 (2)
21	LR147	*glnA263*::Tn*5 asnA1 asnB*$^+$	GNgln		12.3 (3)
22			Ggln		15.9 (3)
23	LR149	*glnA263*::Tn*5 asnA*$^+$ *asnB*$^+$	GNgln	10.2 (2)	2.2 (2)
24			Ggln	14.5 (2)	5.4 (2)
25	LR153	*glnA51 asnA*$^+$ *asnB*$^+$	GNgln	2.4 (1)	9.9 (1)
26			Ggln	2.4 (1)	8.8 (1)

[a] G, 0.4% glucose; N, 0.2% ammonia sulfate; gln, 0.2% glutamine; asn, 0.01% asparagine; glt, 0.2% glutamate.
[b] The number of different extracts assayed is shown in parentheses.

asparagine (0.76 mM) to the growth medium resulted in the repression of both enzymes.

In strain LR83 (*asn*$^+$ *asnB1*::Tn*5*), the level of the ammonia-dependent asparagine synthetase was similar to that in the wild-type strain in ammonia-containing medium (Table 3) and was asparagine repressible, but reached astonishingly high levels in the glucose-glutamine medium where this strain grew poorly.

Strain LR87 (*asnA1 asnB*$^+$) grew normally in all media tested, but the loss of the *asnA* gene product affected the levels of the glutamine-dependent asparagine synthetase. When ammonia was present, the level of glutamine-dependent asparagine synthetase was higher than in the wild-type *asnA*$^+$ strain (Table 3). The glutamine-dependent asparagine synthetase compensated for the loss of the ammonia-dependent asparagine synthetase.

Mutations in *glnG*, a regulatory gene of nitrogen assimilation, had no effect on glutamine-dependent activity (Table 3, compare lines 1–3 to 18–20 and lines 10–12 to 15–17). The regulation of the ammonia-dependent asparagine synthetase in *glnG* mutants was more complex. When nitrogen was growth rate limiting, a *glnG* mutation caused a dramatic increase in the ammonia-dependent asparagine synthetase. However, the *glnG* mutation had little effect on cells grown in nitrogen-rich media.

We examined the effects of mutations in *glnA*, the structural gene for glutamine synthetase, on the levels of the asparagine synthetases. We found that in these mutants, the addition of ammonia to the growth medium did not alter the levels of these enzymes (Table 3, lines 21–26). The loss of glutamine synthetase activity in strain LR149 was due to the insertion of transposon Tn*5* into *glnA*; in this strain, the levels of the asparagine synthetases corresponded to the levels in the wild-type strain MK9520 grown on glucose-ammonia-glutamine. The loss of glutamine synthetase activity in strain LR153 was due to a mutation resulting in a high constitutive level of inactive enzyme; in this strain the levels of both asparagine synthetases corresponded to the levels in the wild-type strain grown on a poor source of nitrogen.

Comments on the purification of the glutamine-dependent asparagine synthetase. A summary of the purification of the glutamine-dependent asparagine synthetase is shown in Table 4. Analy-

TABLE 4. Purification of glutamine-dependent asparagine synthetase

Step no.	Prepn or process	Total units[a]	Total protein (mg)	Sp act	Yield (%)	Purification (×)
1	Crude extract	34,020	1,800	18.9	100	
2	Streptomycin sulfate	32,400	1,660	19.5	95	1.0
3	Ammonium sulfate (0–40%, pH 6.0)	19,420	217	89.4	57	5
4	Sephadex G-200	16,340	68	240	48	13
5	Ion exchange (DEAE)	10,640	38	280	31	15
6	Red agarose	4,740	11	430	14	23

[a] The assay used 0.5 mM aspartate, which is the K_m value. The units have been multiplied by two so that maximal velocities are recorded.

sis by polyacrylamide gel electrophoresis revealed only one major band in the 23-fold-purified enzyme preparation (Fig. 1, lanes c, d, and e). Fully induced glutamine-dependent asparagine synthetase represented 4 to 5% of soluble protein in a crude extract and could be seen after denaturation in SDS gels of crude extract.

The major problem in purifying any asparagine synthetase has been stability. Glutamine-dependent asparagine synthetase was stable in crude extracts when the protein concentration exceeded about 1 mg/ml. The initial steps of the purification gave no trouble until we attempted gel filtration, when all activity was lost. Reasoning by analogy to other amidotransferases, which when dissociated into subunits still retain partial activities, asparagine synthetase activity with 50 mM NH_4Cl used in lieu of glutamine was sought and found. Dilution of the glutamine-dependent asparagine synthetase also caused loss of glutamine-dependent activity but retention of activity with high ammonia. With dilution used as a model system, increasing EDTA and the sulfhydryl reagent and adding glutamine stabilized the activity. With these additions in low-ionic-strength buffers the enzyme was indefinitely stable. A gel filtration column equilibrated with glutamine retained all of the glutamine-dependent activity. If the glutamine in the buffers had been completely degraded during one of the purification steps, the generated ammonia would have been insufficient for asparagine synthesis.

The second major technical problem was the ammonium sulfate precipitation. With a pH 7 buffer (buffer B), recovery of activity varied from 0 to 70%. Changing to a pH 6 buffer (buffer A) allowed reproducible recovery and a better purification, since the activity was precipitated at a lower salt concentration. The isoelectric point of this protein was 6.4 to 6.5 (data not shown); therefore, the charge of the protein was reversed by the change in buffer. Subsequent steps were done at pH 7 since changing the pH did not alter the behavior of glutamine-dependent asparagine synthetase. For example, glutamine-dependent asparagine synthetase did not bind to cation-exchange columns at either pH.

The purest preparation had no visible spectrum but a UV peak at 260 nm, a result of the ATP elution and binding to the enzyme at the last step. A second preparation made with omission of the last step had an absorption peak at 280 nm. Almost all of the properties were identical in both enzyme preparations; the nucleotide binding had no effect.

The best purification gave a single major band on a denaturing gel (Fig. 1) with a subunit molecular weight of 57,000. The enzyme was estimated to be about 95% pure. The second

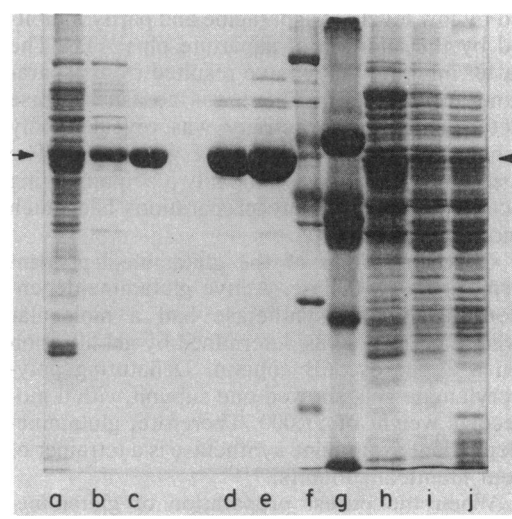

FIG. 1. Polyacrylamide gel analysis of the glutamine-dependent asparagine synthetase. Lanes: (a) 30 μg of protein after Sephadex G-200 (Table 4, step 4); (b) 15 μg of protein after ion exchange (Table 4, step 5); (c, d, and e) 8, 16, and 32 μg of protein, respectively, after red agarose (Table 4, step 6); (f and g) overloaded standards; (h) 48 μg of protein after ammonium sulfate (Table 4, step 3); (i) 40 μg of protein after streptomycin sulfate (Table 4, step 2); (j) 40 μg of crude extract protein (Table 4, step 1).

TABLE 5. Glutamine-dependent asparagine synthetase: reaction stoichiometry and product identification

Time elapsed (min)	Substrate consumed[a]		Product generated[a]					
	Aspartate	ATP	Asparagine	AMP	ADP	PP_i	Glutamate	NH_4^+
10	153	128	130	122	<16	129	274	149
20	170	149	164	169	<16	169	372	234

[a] Total nanomoles generated or consumed for a 1-ml reaction.

preparation (omitting the last step) had the same specific activity but was only about 85% pure.

Reaction stoichiometry and side reactions. Purified enzyme catalyzed the stoichiometric production of asparagine, AMP, and PP_i and the depletion of aspartate and ATP (Table 5): ADP formation was not detectable. An excess of glutamate was generated which corresponded with ammonia generation, indicating the presence of glutaminase activity. Since enzymatic assays were used for all analyses, all products were of the correct biological configuration. For example, the product was indeed L-asparagine since asparaginase treatment yielded L-aspartate. ATPase and pyrophosphatase were not major contaminating or side reactions because ATP and PP_i were depleted and generated stoichiometrically with respect to asparagine formation.

The potential ammonia-generating reactions were partially characterized (data not shown). The glutaminase activity was completely inhibited by low levels of asparagine and partly inhibited by the addition of aspartate plus ATP. The latter inhibition may have resulted from asparagine accumulation. There was no asparaginase activity; aspartase activity was revealed only when high levels of aspartate were used. The aspartase activity was probably a contaminating activity since less pure preparations had much more of this activity.

Characterization of the glutamine-dependent asparagine synthetase. Active glutamine-dependent asparagine synthetase had a molecular weight of 230,000 as determined by gel filtration on a Sepharose 6B column. Denaturing polyacrylamide gels showed one subunit with a molecular weight of 57,000. Therefore, glutamine-dependent asparagine synthetase is a tetramer of four identical subunits.

When the purest preparation of glutamine-dependent asparagine synthetase was analyzed on a two-dimensional gel, there was only one molecular weight species but four evenly-spaced charge species (two major peaks, two minor ones). This could represent a covalent modification or some form of damage such as protein deamidation. The only evidence against covalent modification was negative. Strain LR87 (*asnA1 asnB*$^+$) was grown under fully derepressing conditions and shocked by the addition of asparagine or ammonia to the culture. Synthesis of new enzyme ceased but activity did not disappear from the culture (data not shown).

Activity with glutamine had a broad pH optimum from 6.5 to 8.0 which was independent of the buffer system; with 50 mM ammonium chloride as substrate, the activity increased steadily

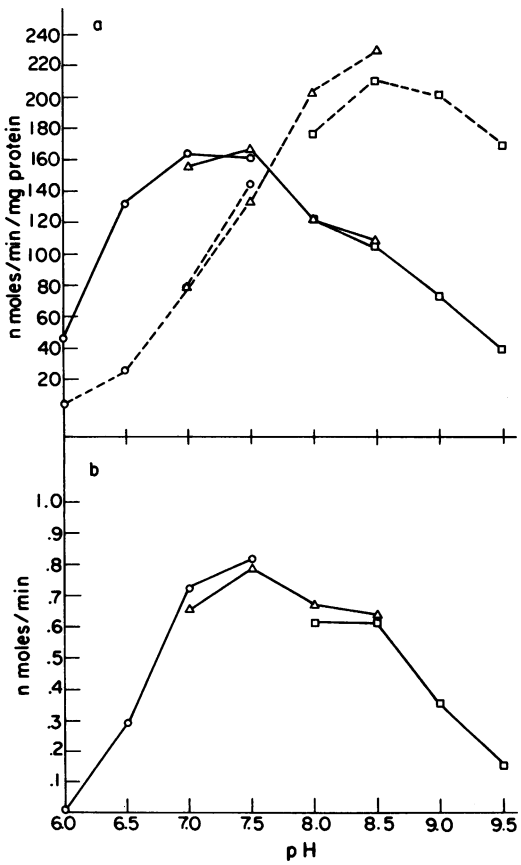

FIG. 2. pH profile of the asparagine synthetases. (a) Glutamine-dependent asparagine synthetase (*asnB* gene product). Substrate: ———, 2 mM glutamine; - - - -, 50 mM NH_4Cl. (b) Ammonia-dependent asparagine synthetase (*asnA* gene product). A constant but arbitrary amount of enzyme was used. The different symbols refer to the buffer system used. Each buffer was present at 20 mM; otherwise the assay system was as described in the text. Symbols: ○, 2(*N*-morpholino)ethane sulfonic acid plus imidazole; △, imidazole plus Tris; □, Tris plus histidine.

TABLE 6. Kinetics of the asparagine synthetases

Enzyme	Variable substrate[a]	pH	K_m (mM)
Glutamine-dependent	Glutamine	7.0	0.55
	ATP	7.0	0.24
	Aspartate	7.0	Biphasic, linear portion near 0.5
	NH_4Cl	7.0	22
Ammonia-dependent	Aspartate	7.0	0.91
	ATP	7.0	0.27
	NH_4Cl	7.0	0.33
	NH_4Cl	8.0	~0.06[b]

[a] The other substrates were 2 mM aspartate or 3 mM ATP for both enzymes, 10 mM glutamine for the glutamine-dependent enzyme, and 2 mM NH_4Cl for the ammonia-dependent enzyme.

[b] Since ammonia is a common contaminant of glassware and many solutions, this value is considered only approximate.

from pH 6.0 to 8.5, with a slight decrease in activity after pH 8.5 (Fig. 2a). This is suggestive of NH_3 instead of NH_4^+ as the reactive species. All previous asparagine synthetase assays have been done at or near pH 8.0 (1, 6, 7, 11–14, 16, 20, 22, 23, 26, 30–33). At this pH the substrate specificity is completely obscured. At physiological pH values, the bacterial glutamine-dependent asparagine synthetase should function almost exclusively with glutamine as the nitrogen donor.

The K_m values for glutamine and ATP (Table 6) when the other two substrates were fixed were 0.55 and 0.24 mM, respectively. The K_m for aspartate was 0.5 mM when aspartate was less than 0.5 mM. Higher levels of aspartate inhibited the enzyme. This was not a property of the glutamine-dependent asparagine synthetase in crude extracts. The apparent K_m at pH 7.0 for NH_4Cl was 22 mM. If NH_3 were the actual substrate, then the K_m for NH_3 would be 0.11 mM. If the K_m value for NH_3 is the same at pH 7 and 8, then the K_m at pH 8 for total ammonia (NH_4^+ plus NH_3) is 2.2 mM: the pH value of the assay is critical for determining substrate specificity.

The glutamine-dependent asparagine synthetase was highly specific for ATP; GTP, CTP, and UTP did not synthesize asparagine significantly over a slight background reaction (data not shown).

The effect of various inhibitors is shown in Table 7. The transaminase inhibitor amino-oxyacetic acid was not inhibitory. Both enzymes

TABLE 7. Effect of inhibitors on asparagine synthetases

Enzyme[a]	Effector	Concn (mM)	% Inhibition		
			Glutamine-dependent activity	NH_4-dependent activity	Glutaminase
Glutamine-dependent asparagine synthetase	Azaserine	1	3	—[c]	—
		10	36	—	—
	6-Diazo-5-oxo-norleucine	0.001	—	—	46
		0.01	—	—	90
		0.1	100	44	94
		1.0	100	41	—
	Asparagine	0.1	48	36	75
		1.0	100	58	100
	5-Diazo-4-oxo-norvaline	2.5	23	28	60
		10	—	—	84
	PP_i	1	60	—	—
		10	95	—	4
	AMP	2.5	23	49	—
	Amino-oxy-acetic acid	10	0	—	—
Ammonia-dependent asparagine synthetase[b]	Asparagine	0.1	—	24	—
		1.0	—	79	—
	5-Diazo-4-oxo-norvaline	1.0	—	0	—
	PP_i	1.0	—	34	—
		10	—	100	—
	5-Diazo-4-oxo-norleucine	2.5	—	0	—
	Amino-oxy-acetic acid	10	—	0	—

[a] The glutamine-dependent activity was measured with 1 mM glutamine, 1 mM ATP, and 0.5 mM aspartate; for the ammonia-dependent activity the glutamine was replaced with 50 mM NH_4Cl. The glutaminase activity was measured with 1 mM glutamine and the continuous assay method. Otherwise assay conditions were as described in the text.

[b] The assay was as described in the text, but 2 mM NH_4Cl was used.

[c] —, Not determined.

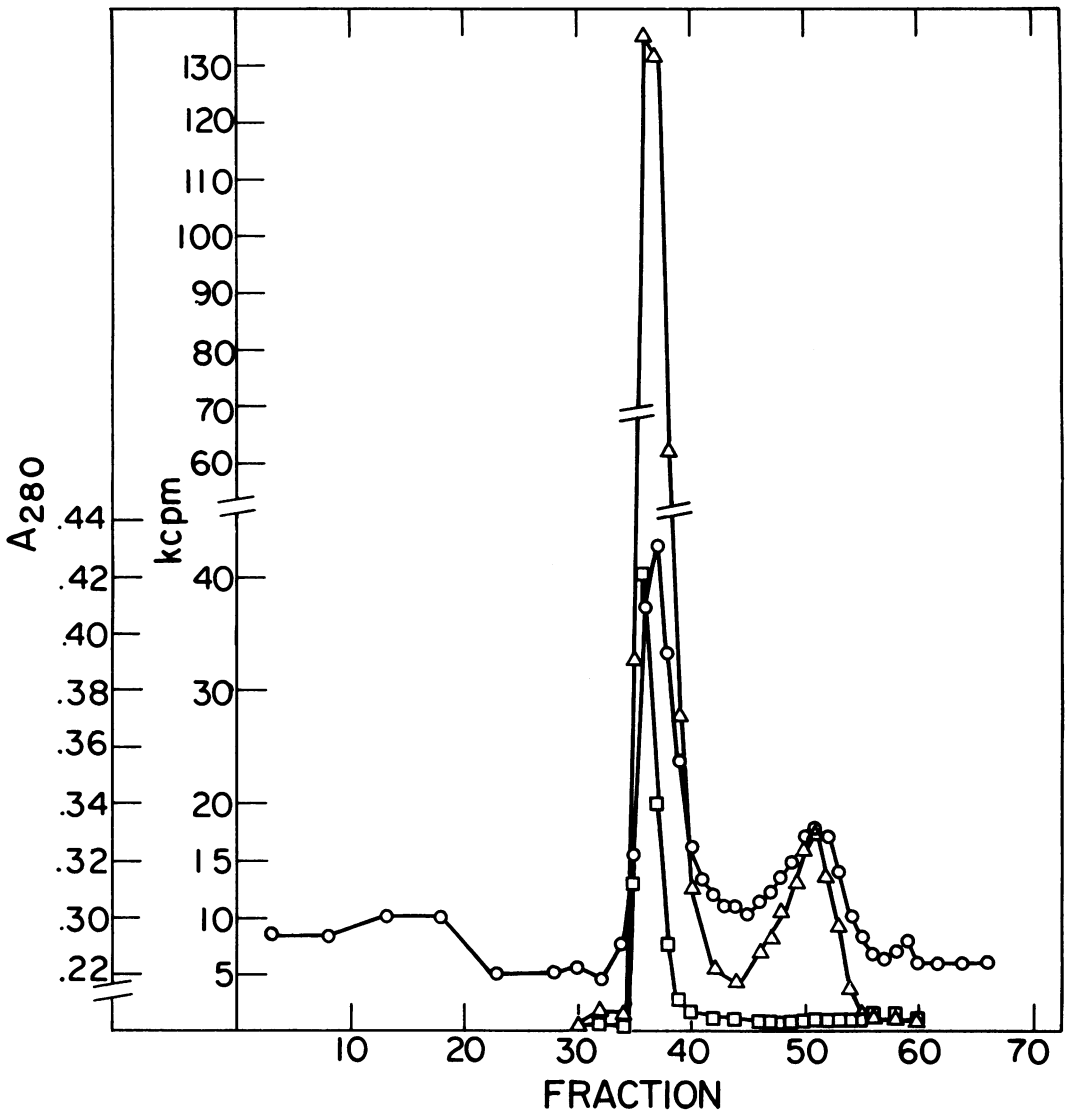

FIG. 3. Dissociation of the glutamine-dependent asparagine synthetase into subunits at pH 8.0. Enzyme was applied to a Sephadex G-200 superfine column and assayed for activity with (△) 50 mM NH_4Cl or (□) 2 mM glutamine as substrate. ○, Protein as determined by absorbance at 280 nm.

were subject to inhibition by asparagine and its analog 5-diazo-4-oxo-norvaline; only the glutamine-dependent enzyme was inhibited by the glutamine analogs 6-diazo-5-oxo-norleucine and azaserine. The fact that the glutaminase and glutamine-dependent enzyme activities were more sensitive to inhibition by asparagine than was the ammonia-dependent enzyme activity suggests that asparagine acts as an analog of glutamine and interferes with the binding of glutamine.

Dissociation of glutamine-dependent asparagine synthetase. Figure 3 shows the elution pattern from gel filtration without glutamine in the buffer for the purified glutamine-dependent asparagine synthetase. There were two peaks of activity. The first peak was due to a tetramer and contained glutamine and high ammonia-dependent activity. The second peak, corresponding to a molecular weight of the monomer (57,000), had only high ammonia-dependent activity. Both peaks had glutaminase activity. The monomer had lower specific activities for both reactions than did the tetramer. The experiment was performed at pH 8.0, but identical results were obtained at pH 7.0. A denaturing poly-

TABLE 8. Partial purification of ammonia-dependent asparagine synthetase

Step no.	Prepn or process	Total units[a]	Total protein (mg)	Sp act	Yield (%)	Purification (×)
1	Crude extract	59,360	352	169	100	1
2	Streptomycin sulfate	58,720	—[b]	—	99	—
3	Ammonium sulfate (50–70%)	41,270	86.4	478	70	3
4	Ion exchange (DEAE)	25,450	30.7	829	43	5
5	Sephadex G-200	24,080	10.1	2,380	40	14

[a] The purification assay did not use optimal assay conditions (see the text). The total units have been multiplied by 2.8 to correct for the suboptimal aspartate levels used.
[b] —, Not determined.

acrylamide gel of the second peak showed only one polypeptide. Reassociation was not attempted. The tetramer did not dissociate completely (pH 7.0 or 8.0), unlike crude extract activity which did not survive gel filtration without glutamine.

Purification of the ammonia-dependent asparagine synthetase. We partially purified the ammonia-dependent asparagine synthetase (Table 8). We started with a crude extract activity 140 times greater than that used by Cedar and Schwartz (6). Figure 4 shows the gel filtration elution pattern. A denaturing gel of the gel filtration fractions revealed only one band whose stain intensity varied with enzyme activity: it had a molecular weight of 38,000 (Fig. 5). There was a major band with a molecular weight of 42,000 throughout all fractions. The total molecular weight of ammonia-dependent asparagine synthetase as determined by gel filtration was about 80,000, which agrees well with previous results (6). It is tempting to postulate a dimer of two 38,000-dalton subunits. A mixed dimer of 38,000- and 42,000-dalton subunits was not rigorously excluded because the 42,000-dalton band was generally more intense than the 38,000-dalton band. The ammonia-dependent asparagine synthetase was estimated to be 40% pure by visual examination.

Characterization of the ammonia-dependent asparagine synthetase. No problems of stability were encountered with the ammonia-dependent

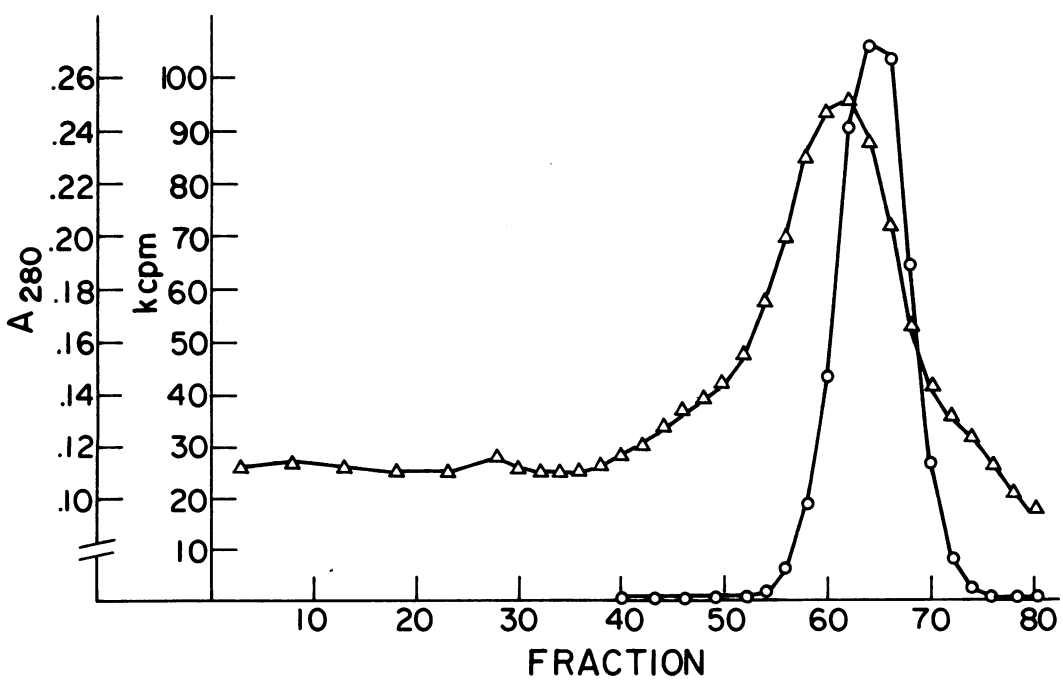

FIG. 4. Gel filtration of the ammonia-dependent asparagine synthetase. Enzyme was applied to a Sephadex G-200 column. Symbols: △, absorbance at 280 nm; ○, asparagine synthetase activity.

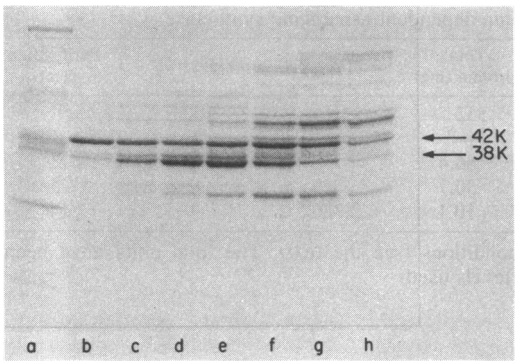

FIG. 5. Polyacrylamide gel analysis of the fraction from gel filtration of the ammonia-dependent asparagine synthetase (see Fig. 4). Lanes: (a) standards; (b–h) fractions 58, 60, 62, 64, 66, 68, and 70 (shown in Fig. 4).

asparagine synthetase. The pH profile (Fig. 2b) was remarkably similar to that of the glutamine-dependent activity of the glutamine-dependent asparagine synthetase.

The kinetic constants with saturating amounts of the other two substrates are shown in Table 6. The values for aspartate and ATP agree well with the limiting constants derived by Cedar and Schwartz (7). Cedar and Schwartz (6) estimated a K_m of 0.1 mM for NH_4Cl at pH 7.8. We found a K_m of 0.33 and 0.06 for NH_4Cl at pH 7.0 and 8.0, respectively.

Low levels of asparagine and high levels of PP_i were inhibitory (Table 7). 6-Diazo-5-oxo-norleucine, the glutamine analog, amino-oxyacetic acid, the transaminase inhibitor, and 1 mM 5-diazo-4-oxo-norvaline, the asparagine analog, were not inhibitory.

DISCUSSION

We found that a mutation in the *asnB* gene, resulting in the loss of glutamine-dependent asparagine synthetase, caused *K. aerogenes* to be unable to grow on poor sources of nitrogen. This unexpected observation led us to study the properties and regulation of the synthesis of this enzyme and of the ammonia-dependent asparagine synthetase, the product of the *asnA* gene.

The two genes are located on the chromosome of *K. aerogenes* in positions corresponding to those previously determined for *asnA* and *asnB* on the *E. coli* chromosome. We found that *asnA* is closely linked to *rbs* as is the corresponding gene in *E. coli* (8, 14). It has previously been shown that *rbs* is closely linked to *glnA* in *K. aerogenes* but not in *E. coli*; accordingly, we found close linkage of *asnA* and *glnA*.

Our study of the levels of the enzymes in cells of the wild type and in mutants grown with ammonia or glutamine as source of nitrogen (Table 3) enabled us to use cell extracts with the greatest abundance of either enzyme as starting materials for the respective purifications.

The purified glutamine-dependent asparagine synthetase had a specific activity of about 430 nmol/min per mg at 30°C. The specific activities of other glutamine-dependent asparagine synthetases, based on estimates of purity, are similar (16, 23, 30). Glutamine-dependent asparagine synthetase cleaves ATP to AMP and PP_i. This is a property of all of the known asparagine synthetases. The estimated specific activity of pure ammonia-dependent asparagine synthetase is at least 6,000 nmol/min per mg. Cedar and Schwartz did not estimate the purity of their preparation of ammonia-dependent enzyme (6, 7); however, it was probably only about 5% pure after a 370-fold purification.

The glutamine-dependent asparagine synthetase found in *K. aerogenes* shared two side reactions with other amidotransferases: it used ammonia at high concentrations in place of glutamine and possessed glutaminase activity (5, 13, 17, 24, 34, 35, 37). The glutaminase activity was about as high as the asparagine biosynthetic activity and was inhibited completely by asparagine and analogs of asparagine and glutamine. The degree of the glutaminase activity relative to total synthetase is unusual for amidotransferases, but not for asparagine synthetases. The asparagine synthetase of mouse leukemia cells also had glutaminase activity as a major side reaction (13).

Wellner and Meister (37) convincingly argued that structural changes which affected subunit interactions in carbamyl phosphate synthetase were responsible for glutaminase activity. A circumstantial case for alterations in the glutamine-dependent asparagine synthetase causing glutaminase activity can be made. The purified enzyme had properties which differed from those in crude extracts. Furthermore, two-dimensional gel analysis of purified glutamine-dependent asparagine synthetase showed four charge species. These charge species were not the product of an assayable physiological modification. When fully derepressed, the addition of repressor, asparagine, stopped new synthesis but did not lead to the disappearance of activity from the culture. The possible cause of this damage may be deamidation. We believe that the glutaminase activity is not expressed in the cell.

The subunit structure of an asparagine synthetase was revealed for the first time. The *K. aerogenes* glutamine-dependent asparagine synthetase has subunits of 57,000 daltons and a total molecular weight of about 230,000, indicating that the enzyme is a tetramer. This is similar to

PRPP amidotransferase and CTP synthetase (cited in reference 5); both are tetramers with 55,000-dalton subunits. The glutamine-dependent asparagine synthetase dissociated to a monomer. Reassociation was not attempted. The monomer did not catalyze the complete reaction, but had ammonia-dependent activity and glutaminase activity. This situation is similar to the carbamyl phosphate synthetase of *E. coli*, in which only the associated subunits can synthesize carbamyl phosphate with glutamine (34).

The assays of asparagine synthetase activities in crude extracts are a good reflection of which synthetase is active in vivo. When ammonia was provided in the medium to the wild-type strain MK9520, the ammonia-dependent activity was high compared with the glutamine-dependent activity. The reverse was true for nitrogen-limited growth, when the intracellular level of ammonia was presumably low.

The glutamine-dependent asparagine synthetase is controlled only by asparagine through repression and inhibition. The glutamine-dependent enzyme varied from 1.0 to about 20 nmol/min per mg. When fully derepressed, this enzyme was about 5% of the soluble protein of the cell. On poor nitrogen sources, when the ammonia-dependent enzyme was repressed, the glutamine-dependent enzyme was high (Table 3). Furthermore, the levels of the glutamine-dependent asparagine synthetase invariably compensated for the loss of ammonia-dependent enzyme. These results can be explained solely on the basis of intracellular asparagine pools. When asparagine is low, the glutamine-dependent asparagine synthetase becomes derepressed. Consistent with this hypothesis, a *glnG* mutation did not affect regulation of the glutamine-dependent asparagine synthetase (Table 3).

The regulation of the ammonia-dependent asparagine synthetase is more complex. Asparagine repressed (Table 3) and inhibited the activity (Table 7). The normal physiological range of activity was from 0.7 to 16.7 nmol/min per mg. Only in mutant strains and under special growth conditions did the ammonia-dependent enzyme reach fully derepressed levels (Table 3). When fully derepressed this enzyme, like the glutamine-dependent enzyme, represented up to a few percent of the soluble protein of the cell.

The existence of a control mechanism independent of intracellular asparagine pools must be postulated for the ammonia-dependent enzyme. Asparagine pools cannot explain why ammonia-dependent asparagine synthetase is sometimes more active than glutamine-dependent activity when at other times the reverse is true (Table 3). The data suggest that the *glnG* protein is needed to repress the ammonia-dependent enzyme (Table 3, line 20). The *glnG* product, a regulatory protein of nitrogen assimilation, is high whenever glutamine synthetase (the *glnA* gene product) is high (e.g., during growth with glutamine or glutamate as a nitrogen source). The *glnG* product is low whenever glutamine synthetase is low (e.g., growth with ammonia as nitrogen source or in strains with a polar mutation in *glnA* [25]). When the *glnG* product was known to be high, ammonia-dependent asparagine synthetase varied from 1.8 to 4.8 nmol/min per mg; when the *glnG* product was low or missing, ammonia-dependent asparagine synthetase varied from 10.2 to 57 nmol/min per mg (Table 3). When the ammonia-dependent asparagine synthetase was fully derepressed, low levels of *glnG* product could be surmised since under this growth condition the synthesis of histidase is not activated (data not shown). In summation, the control mechanism that operates independent of asparagine availability appears to be repression in response to the *glnG* product.

This repression of the ammonia-dependent asparagine synthetase explains the inability of mutants lacking the glutamine-dependent enzyme to grow on poor sources of nitrogen. In such media the utilization of the nitrogen source requires a high level of the *glnG* product, which can only be achieved by the initiation of transcription of the *glnALG* operon at the promoter for *glnA*, the structural gene for glutamine synthetase (25). The glutamine synthetase present at a high level in these cells will compete with the ammonia-dependent asparagine synthetase, kept at a low level by the *glnG* product, for the limited supply of ammonia. Therefore, although the reaction catalyzed by the asparagine synthetase has a favorable equilibrium and its affinity for ammonia is approximately the same as that of glutamine synthetase (21), the enzyme cannot produce asparagine in an amount sufficient for growth. This interpretation is supported by the observation that mutants of strain LR83 (*asnB1*::Tn*5*) selected for their ability to grow with glutamate as source of nitrogen generally had mutations that resulted in a lower level of glutamine synthetase or in a less active enzyme (unpublished data).

Mutants of *Salmonella typhimurium* unable to grow on poor nitrogen sources that regained this ability by mutations which reduced the activity of glutamine synthetase have previously been described (4). We found that such a mutant, kindly provided by S. Kustu, University of California, Davis, was not deficient in the glutamine-dependent asparagine synthetase. It is possible that this mutant has a defect in an enzyme such as anthranilate synthase that utilizes glutamine but also functions with ammonia. The loss

of affinity for glutamine would result in a requirement for ammonia or for the final product of the affected biosynthetic sequence. We have isolated such a mutant of *K. aerogenes* (unpublished data). In this case, glutamine synthetase, present at a high level, may lower the intracellular concentration of ammonia below the level required for the function of the defective enzyme.

The control of the ammonia-dependent asparagine synthetase by the *glnG* product has the obvious physiological advantage that, when growth is nitrogen limited, asparagine and glutamine synthetase do not compete for the available ammonia. Two enzymes are also responsible for the synthesis of glutamate: the glutamine-dependent enzyme glutamate synthase catalyzes glutamate formation when growth is nitrogen limited, a condition that results in the repression of the ammonia-dependent glutamate dehydrogenase. Furthermore, glutamate dehydrogenase is repressed in an ammonia-containing medium if glutamine synthetase and presumably the *glnG* product levels are high (3). Therefore, when growth is nitrogen limited and glutamine synthetase and *glnG* product levels are high, the only ammonia-harvesting reaction is glutamine synthetase, because glutamate dehydrogenase and the ammonia-dependent asparagine synthetase are repressed.

Enzymes exist that can synthesize glutamate and asparagine with ammonia instead of glutamine when the concentration of ammonia in the medium is high. Possibly all amidotransferases can use ammonia, if sufficiently high, in place of glutamine as the nitrogenous precursor. However, the only essential nitrogenous precursor is glutamine. On a poor nitrogen source, when intracellular ammonia is low, glutamine becomes the nitrogen currency of the cell. The amide nitrogen of glutamine is necessary for the synthesis of glutamate, asparagine, purine, and pyrimidine nucleotides and certain amino acids and coenzymes. Loss of the ammonia-dependent asparagine synthetase (*asnA*) or of glutamate dehydrogenase (36) does not result in an observable growth phenotype. On the other hand, loss by mutation of the glutamine-dependent asparagine synthetase or glutamate synthase results in the inability to grow on poor nitrogen sources since the presumably low intracellular ammonia cannot replace glutamine.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grants GM-07446 and AM-13894 from the National Institute of General Medical Sciences and the National Institute of Arthritis, Metabolism and Digestive Diseases, respectively, and by grant PCM78-08576 from the National Science Foundation.

We thank June Bossinger for performing the two-dimensional gel analysis on purified glutamine-dependent asparagine synthetase.

LITERATURE CITED

1. **Arfin, S. M.** 1967. Asparagine synthesis in the chick embryo liver. Biochim. Biophys. Acta **136**:233–244.
2. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116–167.
3. **Brenchley, J. E., M. J. Prival, and B. Magasanik.** 1973. Regulation of the synthesis of the enzyme responsible for glutamate formation in *Klebsiella aerogenes*. J. Biol. Chem. **248**:6122–6128.
4. **Broach, J., C. Neumann, and S. Kustu.** 1976. Mutant strains (*nit*) of *Salmonella typhimurium* with a pleiotropic defect in nitrogen metabolism. J. Bacteriol. **128**:86–98.
5. **Buchanan, J. M.** 1973. The amidotransferases. Adv. Enzymol. **39**:91–183.
6. **Cedar, H., and J. H. Schwartz.** 1969. The asparagine synthetase of *Escherichia coli*. I. Biosynthetic role of the enzyme, purification, and characterization of the reaction products. J. Biol. Chem. **244**:4112–4121.
7. **Cedar, H., and J. H. Schwartz.** 1969. The asparagine synthetase of *Escherichia coli*. II. Studies on mechanism. J. Biol. Chem. **244**:4122–4127.
8. **Felton, J., S. Michaelis, and A. Wright.** 1980. Mutations in two unlinked genes are required to produce asparagine auxotrophy in *Escherichia coli*. J. Bacteriol. **142**:221–228.
9. **Foor, F., Z. Reuveny, and B. Magasanik.** 1980. Regulation of the synthesis of glutamine synthetase by the P_{II} protein in *Klebsiella aerogenes*. Proc. Natl. Acad. Sci. U.S.A. **77**:2636–2640.
10. **Goldberg, R. B., R. A. Bender, and S. L. Streicher.** 1974. Direct selection for P1-sensitive mutants of enteric bacteria. J. Bacteriol. **118**:810–814.
11. **Hongo, S., T. Matsumoto, and T. Sato.** 1978. Purification and properties of asparagine synthetase from rat liver. Biochim. Biophys. Acta **522**:258–266.
12. **Horowitz, B., B. K. Madros, A. Meister, L. J. Old, E. A. Boyse, and E. Stockert.** 1968. Asparagine synthetase activities of mouse leukemias. Science **160**:533–535.
13. **Horowitz, B., and A. Meister.** 1972. Glutamine-dependent asparagine synthetase from leukemia cells. Chloride dependence, mechanism of action, and inhibition. J. Biol. Chem. **247**:6708–6719.
14. **Humbert, R., and R. D. Simoni.** 1980. Genetic and biochemical studies demonstrating a second gene coding for asparagine synthetase in *Escherichia coli*. J. Bacteriol. **142**:212–220.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Lea, P. J., and L. Fowden.** 1975. The purification and properties of glutamine-dependent asparagine synthetase isolated from *Lupinus albus*. Proc. R. Soc. London Ser. B **192**:13–26.
17. **Li. H.-C., and J. M. Buchanan.** 1971. Biosynthesis of the purines XXIII: catalytic properties of the glutamine site of formylglycinamide ribonucleotide amidotransferase from chicken liver. J. Biol. Chem. **246**:4713–4719.
18. **Lowry, O. H., and J. V. Passonneau.** 1972. A flexible system of enzymatic analysis, p. 172–174. Academic Press, Inc., New York.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **Meister, A.** 1974. Asparagine synthetase, p. 561–580. *In* P. D. Boyer (ed.), The enzymes, vol. X. Academic Press, Inc., New York.
21. **Miller, E. S., and J. E. Brenchley.** 1981. L-Methionine *SR*-sulfoximine-resistant glutamine synthetase from mutants of *Salmonella typhimurium*. J. Biol. Chem. **256**:11307–11312.

22. **Milman, H. A., and D. A. Cooney.** 1974. The distribution of L-asparagine synthetase in the principal organs of several mammalian and avian species. Biochem. J. **142**:27–35.
23. **Milman, H. A., and D. A. Cooney.** 1979. Partial purification and properties of L-asparagine synthetase from mouse pancreas. Biochem. J. **181**:51–59.
24. **Nagano, H., H. Zalkin, and E. J. Henderson.** 1970. The anthranilate synthetase-anthranilate-5-phospho-ribosyl-pyrophosphate phosphoribosyl transferase aggregate. J. Biol. Chem. **245**:3810–3820.
25. **Pahel, G., D. M. Rothstein, and B. Magasanik.** 1982. The complex *glnA-glnL-glnG* operon of *Escherichia coli*. J. Bacteriol. **150**:202–213.
26. **Patterson, M. K., Jr., and G. R. Orr.** 1968. Asparagine biosynthesis by the Novikoff hepatoma. J. Biol. Chem. **243**:376–380.
27. **Prival, M. J., and B. Magasanik.** 1971. Resistance to catabolite repression of histidase and proline oxidase during nitrogen limited growth of *Klebsiella aerogenes*. J. Biol. Chem. **246**:6288–6296.
28. **Prusiner, S., and L. Milner.** 1970. A rapid radioactive assay for glutamine synthetase, glutaminase, asparagine synthetase, and asparaginase. Anal. Biochem. **37**:429–438.
29. **Resnick, A. D., and B. Magasanik.** 1976. L-Asparaginase of *Klebsiella aerogenes*. J. Biol. Chem. **251**:2722–2728.
30. **Rognes, S. E.** 1975. Glutamine-dependent asparagine synthetase from *Lupinus leuteus*. Phytochemistry **14**:1975–1982.
31. **Streeter, J. G.** 1973. *In vivo* and *in vitro* studies on asparagine biosynthesis in soybean seedlings. Arch. Biochem. Biophys. **157**:613–624.
32. **Stulen, I., G. F. Israelstam, and A. Oaks.** 1979. Enzymes of asparagine synthesis in maize roots. Planta **146**:237–241.
33. **Stulen, I., and A. Oaks.** 1977. Asparagine synthetase in corn roots. Plant Physiol. **60**:680–683.
34. **Trotta, P. P., M. E. Burt, R. H. Haschemeyer, and A. Meister.** 1971. Reversible dissociation of carbamyl phosphate synthetase in a regulated synthesis subunit and a subunit required for glutamine utilization. Proc. Natl. Acad. Sci. U.S.A. **68**:2599–2603.
35. **Trotta, P. P., K. E. B. Platzer, R. H. Haschemeyer, and A. Meister.** 1974. Glutamine-binding subunit of glutamate synthase and partial reactions catalyzed by this glutamine amidotransferase. Proc. Natl. Acad. Sci. U.S.A. **71**:4607–4611.
36. **Tyler, B.** 1978. Regulation of the assimilation of nitrogen compounds. Annu. Rev. Biochem. **47**:1127–1162.
37. **Wellner, V. P., and A. Meister.** 1975. Enhancement of the glutaminase activity of carbamyl phosphate synthetase by alterations in the interaction between the heavy and light subunits. J. Biol. Chem. **250**:3261–3266.