Biochemical Parameters of Glutamine Synthetase from *Klebsiella aerogenes*

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The glutamine synthetase (GS) from *Klebsiella aerogenes* is similar to that from *Escherichia coli* in several respects: (i) it is repressed by high levels of ammonia in the growth medium; (ii) its biosynthetic activity is greatly reduced by adenylylation; and (iii) adenylylation lowers the pH optimum and alters the response of the enzymes to various inhibitors in the γ-glutamyl transferase (γGT) assay. There are, however, several important differences: (i) the isoactivity point for the adenylylated and non-adenylylated forms in the γGT assay occurs at pH 7.55 in *K. aerogenes* and at pH 7.15 in *E. coli*; (ii) the non-adenylylated form of the GS from *K. aerogenes* is stimulated by 60 mM $MgCl_2$ in the γGT assay at pH 7.15. A biosynthetic reaction assay that correlates well with the number of non-adenylylated enzyme subunits, as determined by the method of Mg^{2+} inhibition of the γGT assay, is described. Finally, we have found that it is necessary to use special methods to harvest growing cells to prevent changes in the adenylylation state of GS from occurring during harvesting.

The enzyme glutamine synthetase {GS; EC 6.3.1.2; L-glutamate:ammonia ligase (adenosine 5'-diphosphate (ADP)-forming)} is at the center of nitrogen metabolism in enteric bacteria (15). As such, it is subject to a complex set of regulatory mechanisms (8, 25). Stadtman and his colleagues showed that GS from *Escherichia coli* strain W is regulated by repression (26) and by rapid, reversible inactivation via the covalent attachment of adenosine 5'-monophosphate moieties to the GS molecule when ammonia is present at high levels (11). Much interest has focused on the two forms of GS, adenylylated and non-adenylylated, and on their interconvertibility (1, 16).

Recently, several other groups began to study GS in other enteric bacteria (2, 3, 14). In particular, work on *Klebsiella aerogenes* showed that the GS of this organism is responsible for regulating the synthesis of many of the enzymes of nitrogen metabolism. Tyler et al. (24) showed that the GS molecule activates the transcription of the *hut* operons in vitro, and Magasanik et al. (15) implicated GS in the control of glutamate dehydrogenase, L-asparaginase, a tryptophan transaminase system, proline oxidase, and possibly GS itself.

Since the basic similarity of the GS from several enteric bacteria was already shown by Holzer and co-workers (25), the procedures developed for studying GS from *E. coli* were generally adopted for use in these other organisms. In so doing, the properties of GS from the various strains were assumed to be identical. In many respects this assumption has proved justified, but several discrepancies have appeared, i.e., stimulation of the γ-glutamyl transferase (γGT) activity of non-adenylylated GS by Mg^{2+} (2, 6, 14). We present here a characterization of the GS from *K. aerogenes*, showing that the GS from this organism is indeed similar to that from *E. coli* but that the two enzymes are not identical in their properties. Therefore, certain assay methods must be modified for use with *K. aerogenes*. We also show that special precautions must be taken when harvesting growing cells if the adenylylation state of the GS measured by various assays is to truly reflect the adenylylation state of GS in the growing cells.

MATERIALS AND METHODS

Chemicals. All chemicals of reagent grade were used without further purification. L-Histidine hydrochloride, L-proline, L-glutamate (monosodium salt), ADP, and adenosine 5'-triphosphate (ATP) were obtained from Sigma. L-Glutamine (A grade) was obtained from Calbiochem. Glutamine solutions were prepared immediately before use, sterilized by filtration, and used at 30°C to avoid breakdown and release of ammonia into the growth medium.

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Bacterial growth conditions. *K. aerogenes* strains MK9000 (19) and MK9011 (9) were grown in W medium (22) containing 10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, 0.1 g of $MgSO_4$, and water to 1 liter. This minimal medium was supplemented with glucose (G) at 0.4% as a carbon source and with ammonium sulfate (N), L-glutamine (Gln), potassium nitrate (NO_3), L-histidine (H), or L-proline (Pro) as nitrogen source at 0.2%. G-NO_3, G-H, and G-Pro are all media in which GS levels are derepressed; G-N-Gln, G-N-H, G-N-Pro, and G-N are media in which GS levels are repressed. G-Gln medium is a derepressing medium if care is taken to prepare the glutamine immediately before the experiment, sterilize it by filtration, and incubate the cells aerobically at 32°C or lower. Strains of *K. aerogenes* are maintained in medium LB (3) (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 1 ml of 1 N NaOH, and water to 1 liter) supplemented with glutamine at 0.2%.

For purification of GS, cells were grown in large carboys sparged with compressed air, harvested by continuous flow centrifugation late in exponential growth, and kept frozen as a cell paste at −20°C until needed. For preparation of crude extracts, cells were grown in 250 ml of medium in Fernbach flasks (1 liter) on a reciprocating shaker at 30°C. These cells were harvested when the turbidity of the culture reached 100 Klett units (about 5×10^8 cells/ml). The cells were collected by centrifugation, washed once with 1% KCl, and resuspended in 2.5 ml of buffer at pH 7.15 containing 10 mM imidazole and 2.5 mM $MgCl_2$. The cells were disrupted by sonic oscillation, and the cell debris was removed by centrifugation at 30,000 × g for 30 min.

Harvesting. For whole cell assays, 10-ml cultures were grown in Erlenmeyer flasks (125 ml) with vigorous shaking in a gyratory shaker bath at 30°C. When the turbidity of the culture reached 100 Klett units, growth was stopped by the additon of 1 ml of hexadexyltrimethylammonium bromide (CTAB; 1 mg/ml), and the shaking was continued for 1 to 3 min. The cells were than harvested by centrifugation in the cold, washed once with 1% KCl, resuspended in 1.0 ml of 1% KCl, and stored at 4°C.

GS assay. The γGT assay is used to measure the total amount of GS present, since both the adenylylated and deadenylylated forms of the enzyme are active in this assay (23). The assay mixture is adapted from Shapiro and Stadtman (21). A fresh concentrated assay mixture is prepared daily by mixing the stock solution in the order and proportions shown in Table 1. The concentration of each reagent in the final assay mixture is also given. Use of this procedure avoids the formation of precipitates and leads to higher reproducibility of the assay. A blank can be prepared in the same manner by replacing the arsenate and ADP solutions with water. CTAB, which renders the cell permeable to the reactants, may be replaced with water when purified enzyme or crude extracts rather than whole cells are to be assayed. For the standard assay in *K. aerogenes*, the concentrated assay mixture is adjusted to pH 7.55 at room temperature with 2 M KOH. For the pH curves shown in Fig. 1, 2, and 3,

TABLE 1. *Composition of GS reaction mixtures*

| Prepn	Volume (ml)	Stock solution	Final concn (mM)
γGT assay mixture	7.53	Water	
	2.25	1.0 M imidazole-hydrochloride, pH 7.15	135
	0.37	0.80 M hydroxylamine-hydrochloride	18
	0.045	0.10 M $MnCl_2$	0.27
	1.5	0.28 M potassium arsenate, pH 7.15	25
	0.15	40 mM sodium ADP, pH 7	0.36
	1.5	CTAB (1 mg/ml)	(90 μg/ml)
Forward reaction assay mixture	7.2	Water	
	2.0	1.0 M imidazole-hydrochloride, pH 7.15	94
	1.25	0.80 M hydroxylamine-hydrochloride	47
	0.40	3.0 M $MgCl_2$	56
	4.2	0.85 M monosodium L-glutamate	168
	2.0	CTAB (1 mg/ml)	(94 μg/ml)
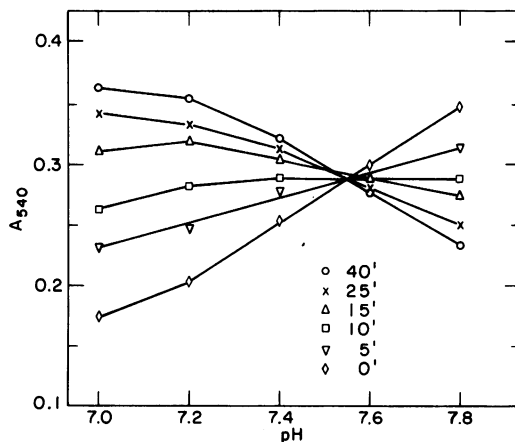
FIG. 1. *Determination of the isoactivity point in the γGT assay of purified GS from K. aerogenes. GS, purified from G-NO_3-grown cells, was incubated with an extract of strain MK9011 in a 0.20-ml assay mixture (pH 7.15) containing 0.50 M imidazole-hydrochloride, 10 mM glutamine, 5 mM ATP, and 25 mM $MgCl_2$. At 0, 5, 10, 15, 25, and 40 min, 20-μl samples were diluted 1:10 into ice-cold buffer (2.5 mM $MgCl_2$, 5 mM imidazole). Each of these time samples was assayed by the γGT method (without Mg^{2+}) at pH 7.0, 7.2, 7.4, 7.6, and 7.8.*

the concentrated assay mixture was adjusted with either 1 M HCl or 2 M KOH to the pH indicated. After adjusting the pH, it is best to cool the mixture to 4°C, if it is not to be used immediately. Sample and water are added to 0.40 ml of the concentrated assay mixture to give a volume of 0.45 ml. The solu-
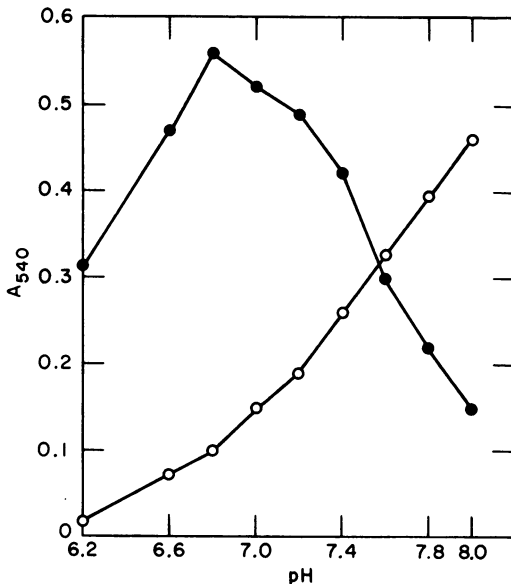
FIG. 2. *Determination of the isoactivity point in crude extracts. A culture of MK9000 was grown on G-Gln medium to mid-exponential growth phase and split. One half of this culture was directly harvested, the other half was "shocked" by the addition of 15 mM* $(NH_4)_2SO_4$ *and harvested 7 min later. Crude extracts were prepared by passage through a French pressure cell at 12,000 lb/in^2 with subsequent centrifugation (see Materials and Methods). These extracts were assayed by the γGT method (without Mg^{2+}) at a variety of pH's between pH 6.2 and 8.0. Open circles represent the results for the extract derived from the G-Gln culture; closed circles represent the results for the extract of the ammonia-shocked cells.*

tion is equilibrated for 5 min at 37°C, and the reaction is initiated by the addition of 0.050 ml of 0.20 M L-glutamine (final concentration, 20 mM). The reaction is terminated by the addition of 1.0 ml of "stop mix" containing 55 g of $FeCl_3 \cdot 6H_2O$, 20 g of trichloroacetic acid, and 21 ml of concentrated HCl per liter (21). The samples are centrifuged to remove any precipitate before absorbance at 540 nm is measured. Under these conditions, 1 μmol of glutamyl hydroxamate gives 0.532 units of absorbancy at 540 nm. One unit of GS activity is defined as the amount of enzyme producing 1 μmol of glutamyl hydroxamate per min.

The forward reaction assay, adapted from Holzer et al. (5, 12), is a measure of the ability of the GS to form glutamine. The concentrated assay mixture is prepared fresh daily by mixing the stock solutions in the order and proportions shown in Table 1. It is then adjusted with 10 M KOH to pH 7.7 at room temperature. Sample and water are added to 0.40 ml of the concentrated assay mixture to give a volume of 0.44 ml. After 5 min of equilibration at 37°C, the reaction is initiated by adding 0.06 ml of 0.20 M ATP (adjusted to pH 7.7 with KOH). The reaction is terminated by the addition of 1.0 ml of stop mix.

This mixture is immediately mixed in a Vortex mixer to dissolve the precipitate that forms transiently upon the addition of the stop mix. The samples are then centrifuged to remove any remaining precipitate, and the absorbancy at 540 nm is measured as for the γGT assay. Blanks without ATP or with cells lacking GS because of mutation give values of less than 0.01 unit per mg of protein and are ignored in this assay (see text).

Immunological methods. The procedure described by DeLeo and Magasanik (4) was used with some modifications. Increasing amounts of extracts (0.015 to 0.105 ml) were incubated with fixed amounts of 10-fold diluted antiserum, equivalent to 50 and 75 mU of glutamine synthetase. The reaction volume of 0.2 ml was incubated at 37°C for 30 min and then in ice water for another 30 min. After centrifugation at 7,700 × *g* for 20 min, 0.075 ml of the supernatant fluid was carefully removed for the assay of GS activity.

RESULTS

Harvesting procedure. In enteric bacteria, the adenylylation level of GS is usually considered to be a reflection of the level of the ammonia supply available to the culture (25). During nitrogen starvation, GS is largely non-adenylylated, and if ammonia is added to such a starved culture, GS very quickly becomes adenylylated (10, 20). However, it has been reported that cells grown in a medium rich in ammonia (G-N-Gln) contain mostly non-adenylylated GS. This suggests that the adenylylation state may change during the harvesting procedure.
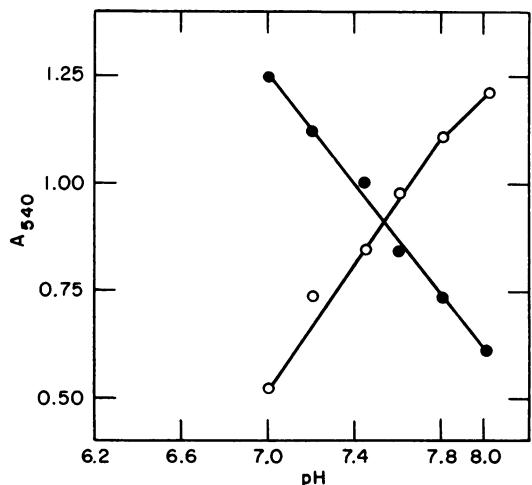

FIG. 3. *Determination of the isoactivity point in whole cells. A culture of MK9000 was grown and ammonia shocked as described in the legend to Fig. 2. Whole cells were harvested as described in Materials and Methods and assayed by the γGT method at pH's between 7.0 and 8.0. The activities of ammonia-starved cells are represented by open circles and those of ammonia-shocked cells by closed circles.*

TABLE 2. *Effect of harvesting procedure on GS activities and adenylylation state*

Growth medium	Addition of CTAB[a] during harvesting	GSA (A_{540}/min per ml)[b]				
		Apparent			Calculated	
		$-Mg^{2+}$	$+Mg^{2+}$	%Mg/Mn	$-Mg^{2+}$	%Mg/Mn
G-N$_3$	−	0.4	0.31	77	0.50	62
	+	0.2	0.25	125	0.45	55
G-N-Gln	−	0.07	0.08	110	0.11	73
	+	0.15	0.02	15	0.10	22
G-N$_{30}$	+	0.20	0.08	40	0.18	43
G-Gln→G-N-Gln	−	0.40	0.07	18	0.29	24
	+	0.40	0.06	15	0.28	21

[a] One hundred micrograms of CTAB per ml was added to the growth medium where indicated. Shaking of the culture was continued at 30°C for 1 min, and then the culture was removed onto ice.

[b] GS was assayed for γGT activity in the presence and absence of 60 mM MgCl$_2$ at pH 7.15 (apparent activities) and subsequently converted to (calculated activities) at pH 7.55 (see text). Activity is expressed as units of absorbancy measured at 540 nm per minute per milliliter.

[c] Subscript of N denotes the concentration (mM) of NH$_4^+$ in growth medium. Growth media are defined in Materials and Methods.

[d] Cells were harvested 5 min after the addition of 30 mM NH$_4^+$ to the culture growing on G-Gln (see text).

Table 2 shows the results comparing two ways of harvesting *K. aerogenes* cells. The GS levels were assayed by the γGT assay at pH 7.15 as described by Shapiro and Stadtman for *E. coli* W (21). In this assay, the extent of inhibition of γGT activity in the presence 60 mM MgCl$_2$ is used to approximate the adenylylation state of GS, with the fully adenylylated enzyme being inactive in the presence of Mg^{2+} and the unadenylylated enzyme unchanged in its activity (23). Two conclusions can be drawn from this table: (i) unless CTAB is used, the adenylylation state of GS may change during harvesting procedure (compare GN$_3$ and G-N-Gln); (ii) the change in adenylylation due to different harvesting procedures is most pronounced when the level of enzyme is relatively low. As a result, previous data reporting adenylylation values should be interpreted cautiously, especially when the levels of GS are quite low. Chilling of the harvested cells in the absence of CTAB somewhat slows this change in adenylylation state. Allowing CTAB to act on the cells at 30°C while they are being agitated seems to stop it most quickly. It should be noted that the treated cells retain GS activity if stored at 4°C overnight but lose all activity if frozen. The addition of sodium deoxycholate, L-glutamine, or potassium arsenate does not significantly change the measured adenylylation values as long as CTAB is used in the harvesting procedure.

From Table 2 it is also immediately apparent that whenever the two harvesting procedures gave different adenylylation levels, they also gave substantially different GS levels. In performing this assay, we assumed that the adeny-lylated and non-adenylylated forms were isoactive in the γGT assay at pH 7.15, as had been shown to be true for *E. coli* W (23). In fact, this assumption has proved false. Figures 1, 2, and 3 show the results of experiments designed to determine the isoactivity point for the adenylylated and non-adenylylated forms of *K. aerogenes* in the assay mixture described in Materials and Methods.

Isoactivity point of the two forms of GS from *K. aerogenes*. Although we have not yet rigorously shown that the two forms of GS from *K. aerogenes* differ by the attachment of an adenosine 5'-monophosphate residue, we will present the evidence for this assumption later in this paper and will refer to the forms here as adenylylated and non-adenylylated for the sake of simplicity.

For the experiment shown in Fig. 1, highly non-adenylylated (not inhibited by 60 mM Mg^{2+}) GS was purified from *K. aerogenes* by the Zn^{2+} precipitation method of Miller et al. (17). This enzyme was then incubated with an extract of strain MK9011 (which lacks GS because of the *glnA6* mutation) in the presence of glutamine and ATP (conditions that lead to adenylylation) for varying lengths of time. Adenylylation was monitored by measuring the increased inhibition of γGT activity in the presence of 60 mM MgCl$_2$. Each time sample was assayed for γGT activity (without Mg^{2+}) at several pH values. The data in Fig. 1 clearly show that as the enzyme becomes more and more adenylylated, the pH profile changes radically; however, there is an isoactivity point for all levels of adenylylation at pH 7.55. Thus, we can assume that at pH 7.55 the assay conditions

described in Materials and Methods will yield equal activities for equal amounts of GS, no matter what the state of adenylylation. We frequently perform assays with crude extracts or whole cells grown under various conditions. For this reason, we determined that the isoactivity point of pH 7.55 does not vary with the level of purity of GS. For the data shown in Fig. 2, crude extracts were prepared from cells grown under different conditions, and the GS activity of each extract was measured at a variety of pH values. The values obtained were normalized to the amount of GS antigen present in each extract, as measured by antibody precipitation of GS activity (see Materials and Methods). The isoactivity point of the two GS activities (one adenylylated and one non-adenylylated) was again found to be pH 7.55. Finally, the value was determined for whole cells. A single culture was grown under nitrogen-limiting conditions and, when the cell density reached about 5×10^8 cells/ml, half of the culture was harvested (with CTAB); ammonium sulfate (15 mM) was added to the other half to cause rapid adenylylation of the GS (10). After 5 min, this half of the culture was also harvested (with CTAB), and both portions of the culture were assayed for γGT activity at a variety of pH values. During this time, the cell mass increased less than 2%, so the amounts of GS in each portion were very nearly equal. Once again, the isoactivity point occurred at approximately pH 7.55.

Since the GS activities of whole cells, extracts, and purified enzyme gave the same isoactivity point, we can assume that this is not a property dependent upon the level of purity of the enzyme. In addition, the Zn^{2+} purification procedure does not change this property. One may then also conclude from these data that the antiserum raised against purified, non-adenylylated GS does not distinguish between adenylylated and non-adenylylated GS. Using the pH profiles for γGT activity (Fig. 1, 2, and 3), we can calculate isoactivity values from the apparent values (activities at pH 7.15) of Table 2. Thus, we can correct for the absolute amount of GS present. In addition, the ratio of Mg^{2+} activity at pH 7.15 to the calculated activity in the absence of Mg^{2+} at pH 7.55 is given. We suggest that this ratio is an accurate estimate of the fraction of non-adenylylated subunits in the enzyme. This is based on results presented in the following sections.

It is important to emphasize that the isoactivity point is valid only for this organism and only under these exact conditions of the assay. The isoactivity point for GS from *E. coli* W is

considerably lower under these conditions (data not shown), and the isoactivity points for GS from both sources (*E. coli* and *K. aerogenes*) are somewhat lower when 130 mM "complex imidazole" (23) replaces the 130 mM imidazole used here. Varying other conditions of the assay can have a significant influence on the total activity measured, and the adenylylation state is important in these changes. For example, we find that higher concentrations of imidazole and the presence of sodium ions both selectively inhibit the adenylylated form of the enzyme. Higher concentrations of potassium ion stimulate the non-adenylylated form. Increasing the glutamine concentration in the assay mixture selectively increases the activity of the adenylylated form.

Under the conditions of the γGT assay, we find that the non-adenylylated form of GS from *K. aerogenes* has a much greater affinity for glutamine, arsenate, ADP, and Mn^{2+}. The K_m of the substantially non-adenylylated GS for glutamine is 2.7 mM, whereas the fully adenylylated enzyme has a K_m for glutamine of 30 mM.

Interconvertability of the adenylylated and non-adenylylated forms of GS from *K. aerogenes*. Several lines of evidence support the assumption that the modification responsible for the inactivation of GS by ammonia shock (Fig. 3) is, in fact, adenylylation. (i) Adenylylated GS can be de-adenylylated by treatment with snake venom phosphodiesterase (Fig. 4), producing a change from Mg^{2+} inhibition to Mg^{2+} stimulation and a reduction in γGT activity at pH 7.15. (ii) Incubation of a partially adenylylated preparation of GS and a crude extract of strain MK9011 (which lacks GS) with 10 mM glutamine, 5 mM ATP, and 25 mM $MgCl_2$, a treatment that leads to adenylylation of the *E. coli* enzyme (16), leads to increased Mg^{2+} inhibition and an increase in the γGT activity at pH 7.15 (see Fig. 1 and 5). This change does not occur if ATP, glutamine, or the extract of strain MK9011 is omitted from the adenylylation reaction mixture. (iii) The ratio of units of absorbancy measured at 260 nm to units of absorbancy measured at 280 nm was 0.55 for a purified preparation of our non-adenylylated GS, whereas the ratio for the adenylylated form was 0.80. These ratios would correspond to the adenylylation states of $E_{\bar{1}}$ and $E_{\overline{10}}$, respectively, if the spectrophotometric data from *E. coli* W (11) can be applied to the enzyme from *K. aerogenes*. (iv) The response of the two forms to various inhibitors is similar to that reported for *E. coli* (7). As already mentioned, the Mg^{2+} inhibition is observed for only one
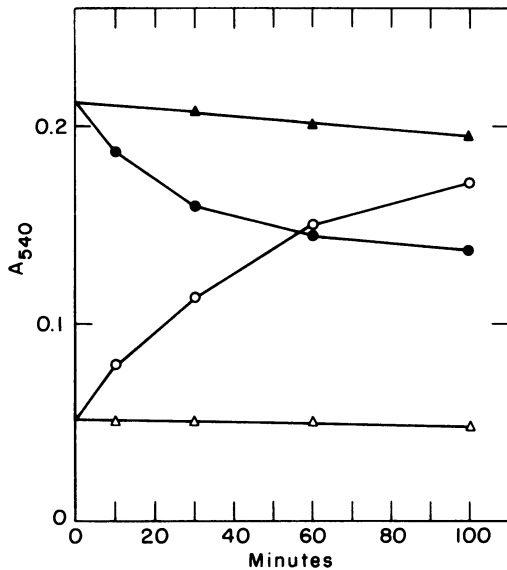
FIG. 4. Treatment of purified, adenylylated GS with snake venom phosphodiesterase. Purified, adenylylated GS was incubated with snake venom phosphodiesterase in a ratio of 10 parts of GS to 1 part of snake venom phosphodiesterase by weight at pH 7.15. Samples were removed every 10 min during 100 min of incubation and assayed for γGT activity in the presence and absence of Mg^{2+}. The activity of GS is plotted as a function of incubation time. ▲ represents the activity of the control (untreated GS without Mg^{2+}); △, the activity of the control with Mg^{2+}. The activity of snake venom phosphodiesterase-treated GS without Mg^{2+} is represented by ● and that with Mg^{2+} by ○.
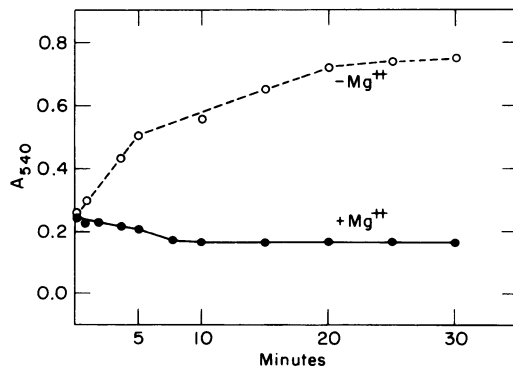

FIG. 5. Adenylylation of purified GS. GS was purified from $G-NO_3$-grown cells and incubated with an extract of MK9011 under conditions which promote adenylylation (see legend to Fig. 1). Every 10 min after the start of the incubation, samples were removed and assayed for γGT activity at pH 7.15. Closed circles show the results of assays in the presence of 60 mM $MgCl_2$. Open circles represent the results of the assay without Mg^{2+}.

form (Fig. 1 and 3). In addition, there are differences in the patterns of inhibition of the two forms observed for a variety of other compounds (Table 3). These are quite similar to the differences seen for the two forms of the E. coli enzyme (7).

Forward reaction assay for the GS of K. aerogenes. Most of the studies of GS from enteric bacteria have employed the inhibition of the γGT activity by 60 mM Mg^{2+} as the measurement of the adenylylation state (2, 6). Stadtman et al. (23) have shown that the non-adenylylated enzyme of E. coli W has comparable γGT activities in the presence and absence of 60 mM $MgCl_2$ at pH 7.15. However, the γGT activity of the non-adenylylated GS of Salmonella typhimurium (2, 14) and of K. aerogenes (6) is stimulated by Mg^{2+} at pH 7.15. Therefore, we examined a reaction that was more directly related to the biosynthetic activity and which could be routinely used to assay large numbers of samples easily. The assay is a modification of the one developed by Holzer and co-workers (12), which measures the γ-glutamyl hydroxamate produced from glutamate and ATP rather than from glutamine. Thus, with no new reagents except ATP and glutamate, the standard "reverse" (γGT) assay (measuring the total amount of GS present) can be run "forward" to measure the biosynthetically active GS that is present.

Under the conditions described in Materials and Methods, the reaction has a moderately broad pH profile, with an optimum at about pH 7.7 (Fig. 6). The reaction is linear for at least 30 min over a range of 1 to 30 mU of enzyme activity per ml in the reaction mixture for purified enzyme, crude extracts, and for whole cells. At times greater than 30 min, ATP may become limiting in both extract and whole cell assays, especially when the biosynthetic activity is also high. A blank to which no ATP is added gives a

TABLE 3. Effect of inhibitors on the adenylylated and non-adenylylated forms of GS

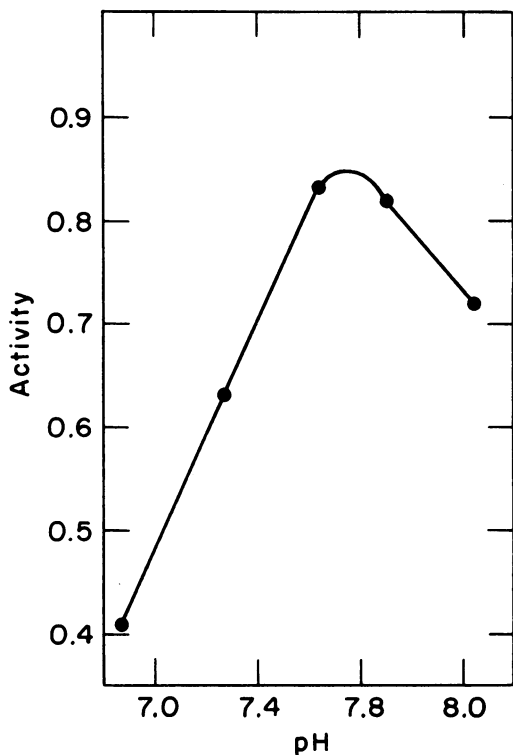| Inhibitor	Final concn (mM)	% γGT activity ($-Mg^{2+}$) remaining in:	
		GS	GS-AMP[a]
Alanine	5	22	48
	40		14
Histidine	5	86	95
	40		63
Adenosine 5' monophosphate	33		19
Tryptophan	5	95	85

[a] GS-AMP, adenylylated GS.

FIG. 6. *Determination of the pH optimum for the forward reaction. A culture of MK9000, grown in G-Gln medium, was harvested with CTAB (see Materials and Methods). The whole cells were assayed by the forward reaction for biosynthetic activity at pH points between 7.0 and 8.0. Activity is expressed as units of absorbancy measured at 540 nm per minute per milliliter.*

negligible value of about 0.01 units per mg of protein, even when whole cells are used. A similar value is obtained when cells lacking GS (due to mutation) are assayed in the complete assay mixture.

Correlation of forward assay with the Mg^{2+} inhibition assay. We now routinely assay GS from *K. aerogenes* at pH 7.55, the isoactivity point. Unfortunately, a precipitate is formed upon the addition of 60 mM $MgCl_2$ to the reaction mixture. Therefore, we cannot use the Mg^{2+} inhibition assay at this higher pH. However, we do measure the γGT activity in the presence of 60 mM $MgCl_2$ at pH 7.15 as an estimate of the amount of biosynthetically active GS in our preparations. We observed that the γGT activity of our most adenylylated preparation of GS in the presence of Mg^{2+} at pH 7.15 is less than 10% of the γGT activity (without Mg^{2+}) at pH 7.55 (data not shown). Furthermore, the γGT activity of our most non-adeny-

lylated GS, measured in the presence of Mg^{2+} at pH 7.15, has never been higher than 100 to 105% of the γGT activity (without Mg^{2+}) at pH 7.55. We, therefore, define the value R2 as the ratio of the γGT activity with Mg^{2+} (pH 7.15) to the γGT activity without Mg^{2+} (pH 7.55). This ratio seems to be a reasonable estimate of the percentage of total GS that is biosynthetically active (unadenylylated).

This ratio (R2) correlates well with the activity measured by the forward assay (Fig. 7). The value of R1 is obtained by dividing the activity of a preparation of GS as measured by the forward assay by the activity as measured by the γGT assay at pH 7.55. We observe a linear correlation between R1 and R2 regardless of
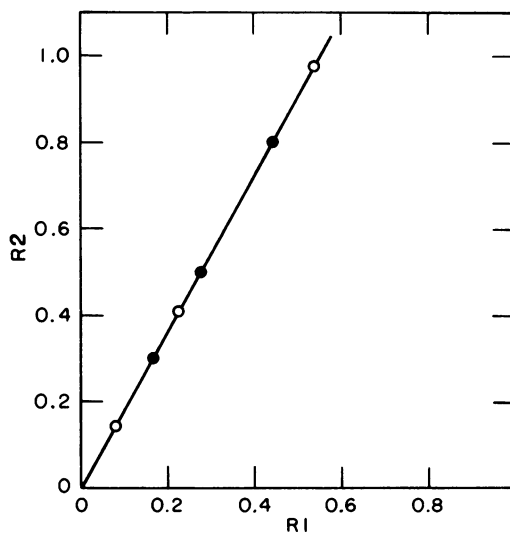

FIG. 7. *Correlation between Mg^{2+} inhibition (of the γGT assay) and biosynthetic activity (forward reaction). A culture of strain MK9000 was prepared as described in the legend to Fig. 3, except that after removal of one third of the culture, excess ammonia (30 mM) was added to the remaining culture. Half of the ammonia-shocked cells were harvested 2 min after the addition of ammonia, and the remaining culture was harvested after 6 min. Each of these batches was prepared for whole cell assays. Whole cells were assayed for γGT activity in the presence (pH 7.15) and absence (pH 7.55) of 60 mM $MgCl_2$ and for biosynthetic activity by the forward reaction. R1 and R2 are calculated as described in the text and plotted against one another. The results of the assays using whole cells (R1 versus R2) are shown by the closed circles. Three preparations of purified glutamine synthetase of different adenylylation states were assayed as described for biosynthetic activity and GT activity. R1 and R2 values, determined from the activities of the purified enzymes, are represented by the open circles.*

whether the GS measured is the Zn^{2+}-purified enzyme or is present in a whole cell preparation (see Fig. 7).

DISCUSSION

The data presented here provide a partial characterization of the biochemical parameters of GS from K. $aerogenes$. As expected, there are many similarities to the GS of E. $coli$, in particular, inactivation by adenylylation, repression by high levels of ammonia, patterns of feedback inhibition, inhibition of the adenylylated form in the γGT assay by Mg^{2+} and different pH profiles for adenylylated and non-adenylylated forms of GS. In addition, the GS from these two organisms are antigenically similar (4), and both can be highly purified by the Zn^{2+} precipitation procedure (4, 17). There are, however, several differences. The most important difference from a practical standpoint is that under our assay conditions, the isoactivity point of the two forms of K. $aerogenes$ GS is pH 7.55; if the K. $aerogenes$ enzyme is assayed at pH 7.15, variations of up to twofold can be seen in the values for total enzyme measured, depending upon the adenylylation state. As the results of Fig. 1 and 5 suggest, the γGT activity without Mg^{2+} at pH 7.15 increases with the increasing adenylylation of GS.

The isoactivity point is independent of the level of purity of the GS preparation, since purified enzyme, crude extracts, and whole cells yield the same value. It is, however, dependent upon the exact conditions of the assay system; for example, the isoactivity point is slightly reduced when 130 mM complex imidazole (23) replaces the 130 mM imidazole used here; any changes in the concentrations of glutamine, arsenate, ADP, imidazole, or salt will also alter the isoactivity point.

The fraction of GS that is non-adenylylated can be inferred from R2. This conclusion stems from the observation that only non-adenylylated GS is active in the transferase assay in the presence of 60 mM $MgCl_2$. No correction factor need be applied to R2 because of the fortuitous result that the transferase activity of non-adenylylated GS is the same in the presence of Mg^{2+} at pH 7.15 as it is in its absence at pH 7.55.

The characterization of the forward assay described here gives us an independent way of estimating the amount of biosynthetically active GS in the cell. Since some studies in this laboratory focus on mutations in the structural gene for GS that affect the regulation of GS synthesis, we are concerned that the reliability of an inhibition assay might be questionable. In at least two other systems, the $hisG$ product of $Salmonella$ (13) and the $ilvA$ product of E. $coli$ K-12 (18), a confusing coincidence of regulatory defects and changes in feedback inhibition makes one cautious of relying on inhibitors for assaying enzymatic activities in regulatory mutants. Thus, the forward assay should allow us to measure the levels of biosynthetically active GS with more confidence, even in such mutants.

Finally, the CTAB harvesting procedure allows a resolution of some apparent contradictions regarding the correlation of the adenylylation state of GS with the presence or absence of an excess supply of ammonia. We should now be able to measure the adenylylation state of GS reliably, even when the total GS levels are low.

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