REGULATION OF GLUTAMINE SYNTHETASE, VII. ADENYLYL GLUTAMINE SYNTHETASE: A NEW FORM OF THE ENZYME WITH ALTERED REGULATORY AND KINETIC PROPERTIES

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During the investigation of the kinetics of regulation of *Escherichia coli* glutamine synthetase, it became obvious that conditions of growth of the organism played a decisive role in the catalytic properties of the enzyme purified from it. It was found that enzyme purified from *E. coli* grown on NH_4Cl and glucose (preparation I) was in general less sensitive to inhibition by feedback effectors than was enzyme purified from *E. coli* grown on glutamate and glycerol (preparation II). Moreover, in the biosynthetic reaction catalyzed by the enzyme, the two preparations show markedly different responses to divalent cations. Examination for structural differences in the two forms of the enzyme has shown that the dramatic kinetic alterations are not due to differences in primary structure, but rather to adenylylation of glutamine synthetase I which converts it to glutamine synthetase II. This report presents experiments which demonstrate that the two forms of the enzyme have identical amino acid composition but differ in the amount of adenosine 5′-phosphate (5′-AMP) bound in phosphodiester linkage to the protein.

Materials and Methods.—E. coli strain W was grown aerobically in a 300-liter fermenter as previously described.[1, 2] Preparation I was the enzyme purified from cells grown on 4 mM NH_4Cl and 11 mM glucose. Preparation II was the enzyme purified from cells grown on a medium in which 19 mM glutamate replaced the NH_4Cl and 21 mM glycerol replaced the glucose. Cells were harvested and frozen in liquid nitrogen until ready for the purification procedure, which was as previously described,[1] and the resultant enzyme preparations were homogeneous on disk gel electrophoresis and in the ultracentrifuge. Samples of enzyme were dialyzed exhaustively against 0.01 *M* imidazole buffer, pH 7.0, before chemical analyses were performed. All chemicals were of the highest quality commercially available. Cellulose and polyethylenimine-cellulose thin-layer chromatography plates were purchased from Brinkmann Instruments, Inc. Snake venom phosphodiesterase (*Crotalus ademanteus*) and *E. coli* alkaline phosphatase were purchased from Worthington Biochemical Co. 5′-Adenylic acid deaminase (muscle) was obtained from Sigma Chemical Co. Intestinal adenosine deaminase was a gift of Dr. Leon Heppel.

Amino acid analyses: Amino acid analyses were performed according to Spackman *et al.*[3] on a Spinco automatic amino acid analyzer, equipped for high-sensitivity analyses. Protein samples were hydrolyzed for 23 hr in constant boiling HCl at 108° in a sealed, evacuated tube. Cysteine and tryptophan were not determined.

Enzymatic assays: Glutamine synthetase activity was determined by the release of phosphate from adenosine 5′-triphosphate (ATP) in the presence of glutamate and ammonium ion[1] at 37°. The assays contained either 50 mM $MgCl_2$ and were run at pH 7.8, or 5 mM $MnCl_2$ in place of $MgCl_2$ and run at pH 6.9.

Enzyme concentration: Protein was determined by using $A_{280}^{0.1\%} = 0.77$, obtained from previous dry weight measurement on preparation II.[1] The value obtained for preparation I by this method should be about 3% too low, but this is within the limits of experimental error of the initial dry weight measurement. For the difference spectrum in Figure 1, protein concentrations were normalized by the biuret reaction.[4] Enzyme concentration is expressed either with respect to subunit weight (50,000 gm/mole) or dodecamer weight (native enzyme, 600,000 gm/mole).[1, 5]

Spectroscopy: All spectra were measured in a Cary 15 recording spectrophotometer using matched cuvettes; protein spectra were corrected for light scattering by appropriate extrapolation from the absorption at 340 mμ.

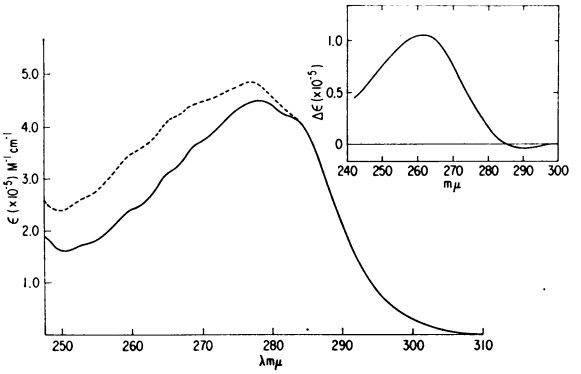

FIG. 1.—Spectra of glutamine synthetase, preparation 1 (*solid line*) and preparation II (*dashed line*), determined as in *Materials and Methods*. The inset is the difference spectrum between the two forms. Extinction coefficients are expressed per mole of enzyme of mol. wt. 600,000, as described in *Methods*.

Phosphate analyses: Phosphate was determined by the method of Ames and Dubin[6] with color development for 30 min at 45°. Total phosphate was determined by measuring the phosphate content of ashed samples. Inorganic phosphate measurements on protein samples were performed after precipitating the protein with 10% perchloric acid. Organic phosphate was calculated as the difference between total and inorganic phosphate values for the same preparation.

Pentose analyses: Quantitative total pentose was determined by the Dische modification of the orcinol reaction,[7] using authentic 5′-AMP as the standard, and having preparation I in some standard tubes to assess the degree of color quenching by protein. There was diminution in the color yield caused by the protein, despite boiling for as long as 40 min to achieve maximal color development. Deoxypentose was estimated by the diphenylamine reaction.[8]

Sulfhydryl determinations: Free SH groups were measured on enzyme preparations which were treated with 0.01 *M* ethylenediaminetetraacetate (EDTA) and then subjected to denaturation with 0.5% sodium dodecyl sulfate in the presence of dithionitrobenzoic acid (Ellman's reagent[9]) as previously described.[10]

Perchloric acid digestion: Glutamine synthetase preparations, after being carefully dried, were digested with 12 *N* perchloric acid (PCA) for 1 hr at 100°. The resultant brownish solution was diluted tenfold and neutralized with saturated KOH. The precipitate was centrifuged off and the supernatant collected and concentrated for thin-layer chromatography on cellulose, in distilled water. The one ultraviolet quenching spot present in preparation II which was fainter in preparation I was eluted for subsequent spectral analysis and chromatographic identification.

Snake venom phosphodiesterase digestion: Analytic venom phosphodiesterase digestion was performed in 0.1 *M* Tris buffer, pH 9.0, 5 mM $MgCl_2$, in a total volume of 0.2 ml at 37°. It was found that maximal release of AMP from glutamine synthetase occurred within 3 hr using 0.1 mg of diesterase and 1.0 mg of glutamine synthetase. The reaction was stopped at 3 hr by adding 16 mM EDTA and diluting to 0.3 ml, at which point the mixture was either analyzed for inorganic phosphate by precipitating the protein with 0.06 ml 60% PCA and taking 0.3-ml aliquots for further analysis, or *E. coli* alkaline phosphatase was added and the reaction was permitted to continue for 2 hr more at 37° before addition of PCA. In order to assess the nature of the material removed by snake venom phosphodiesterase from glutamine synthetase, a large-scale digestion was performed in 1.0 ml, using 26.8 mg of preparation II and 0.25 mg phosphodiesterase, in 0.1 *M* Tris, pH 9.0, 5 mM $MgCl_2$, at 37°C for 5 hr. The digestion mixture was put over Sephadex G-25 to separate the protein from low-molecular-weight compounds, and the latter were collected. The fractions were pooled and lyophilized, then diluted into 0.01 *M* acetic acid and adsorbed on charcoal. The charcoal was separated and eluted with 1% NH_3 in 50% ethanol, and this eluate was concentrated for spectral, chromatographic, and enzymic characterization.

E. coli alkaline phosphatase digestion: All *E. coli* alkaline phosphatase digestions were done with 0.02 mg enzyme at pH 9.0 in 0.1 *M* Tris, 0.3 ml, for 2 hr at 37°C, conditions which release quantitatively the phosphate from authentic 5′-AMP when present at the levels under study in these experiments (45 μM).

Adenylic acid and adenosine assays: The material obtained from charcoal adsorption of phosphodiesterase digestion products was characterized by sequential digestion with 5′-adenylic acid deaminase and adenosine deaminase by following the decrease in absorbancy at 265 mμ upon the

addition of these enzymes to a cuvette containing the unknown material. The reaction was followed in a total volume of 0.835 ml at 25°C, containing 0.045 *M* citrate buffer, pH 6.5, and the unknown compound (0.02 ml), with 50 units of adenylate deaminase in 0.05 ml 1 *M* KCl. When the 5′-adenylate deaminase reaction was completed, as evidenced by a constant optical density, 0.1 ml of 1.0 *M* phosphate buffer, pH 7.2, was added, followed by 0.025 ml of an intestinal adenosine deaminase preparation, and the further total decrement in optical density was measured as an index of the amount of adenosine present. The conditions of adenosine deamination (high phosphate concentration) were selected to inhibit any phosphatase activity present in the intestinal enzyme preparation, making the reaction specific for adenosine.[11] Calculations were based upon the change in optical density undergone by a known concentration of 5′-AMP upon deamination by adenylate deaminase.

Thin-layer chromatography: Cellulose or polyethylenimine-cellulose anion exchange plates were used, and the techniques of spotting and developing were as described by Randerath.[12] The chromatographic systems were as follows: system 1—thin-layer cellulose plates developed in the ascending direction in distilled water; system 2—cellulose plates, ascending; solvent was chloroform:methanol:water, 60:70:26; system 3—polyethylenimine cellulose plates, ascending, plates first washed with distilled water, then spotted, then developed in 0.8 *M* NaCl for 10 min followed by 1.0 *M* NaCl for 50 min, without intermediate drying; system 4—cellulose plates, ascending; solvent was t-amyl alcohol:formic acid:water, 3:2:1. Systems 1, 2, and 4 were allowed to proceed until the solvent front had moved 15 cm from the origin. Spots were visualized under short-wave ultraviolet light.

Results.—Table 1 summarizes data illustrating the differences in response to effectors of glutamine synthetase preparations I and II in the γ-glutamyl transfer assay. With high concentrations of effectors, preparation I is more strongly inhibited than is preparation II by glycine and alanine, whereas preparation II is more strongly inhibited by tryptophan, histidine, AMP, and CTP (preparation I is actually stimulated by the latter three). In addition, Table 1 shows that the two preparations have markedly different responses to divalent cations in the biosynthetic assay. Preparation I is five times more active than preparation II in the assay containing $MgCl_2$, but preparation II is five times more active in the assay containing $MnCl_2$. To state it differently, preparation I is 30 times as active with Mg^{++} as it is with Mn^{++}, whereas preparation II has equivalent activities with the two cations. More complete data on the differences in divalent cation specificities are being reported elsewhere.[13]

It is unlikely that the differences in kinetic behavior of the two preparations are due to differences in primary structure since, as shown in Table 2, they have essentially identical amino acid composition. The results reported in Table 2 agree quite well with the amino acid analysis published for yet another glutamine synthetase preparation[1] but differ significantly from that preparation in threonine and histidine content, for reasons which are unclear at the present time, but may re-

TABLE 1

KINETIC DIFFERENCES BETWEEN GLUTAMINE SYNTHETASE PREPARATIONS I AND II

Glutamine synthetase	Specific Activity in Biosynthetic Assay		Relative Specific Activity in Presence of Effectors in γ-Glutamyl Transfer Assay*					
	Mg^{++} assay	Mn^{++} assay	40 mM AMP	40 mM Histidine	20 mM Tryptophan	8 mM CTP	40 mM Glycine	40 mM L-Alanine
Prep. I	60.1	2.0	110	107	98	104	29	4
Prep. II	12.8	8.9	26	50	55	61	61	9

Specific activities in the biosynthetic assays are expressed as μmoles phosphate produced at 37° per minute per mg of enzyme. Relative specific activities in the transfer assay are calculated by comparison to duplicate controls without effector, arbitrarily set at 100; values above 100 represent stimulation by effector, and values less than 100, inhibition.

* Summarized from a previous communication.[2]

flect experimental errors. Sulfhydryl titrations were performed on preparations I and II as described in *Materials and Methods*, and showed that there were 4.1 and 4.3 SH groups, respectively, per subunit, and this accounts for all of the cysteine in the enzyme.[10] Hence the interconversion of enzyme I to enzyme II during growth[13] does not involve disulfide bond formation.

Ultraviolet absorption spectra: The utraviolet spectra of the two enzyme forms are markedly different. Figure 1 shows that preparation II has much more absorbancy below 280 mμ than does preparation I, and the difference between the two spectra has a peak at 261 mμ. This suggested that preparation II might contain a bound nucleotide that is absent in preparation I. This was further indicated by direct chemical analyses showing that preparation II contained 9.0 moles of organic phosphate and 6 moles of pentose per mole of enzyme, whereas organic phosphate and pentose in preparation I were only 1.2 and 1.0 moles/mole, respectively (Table 3). The lower yield of pentose compared to phosphate may be due to an effect of protein in quenching of color yield of the orcinol reaction. The spectrum of the color produced with orcinol was identical to that obtained with common pentoses. The absence of color production with diphenylamine (Table 3) establishes that the pentose present in preparation II is not deoxyribose. Assuming that the difference in absorbancy between preparation I and II at 260 mμ is due to an adenine nucleotide ($\epsilon_{260} = 15.0 \times 10^3$ cm^{-1} M^{-1}, pH 7.0), it was calculated that preparation II contains 7.4 equivalents of adenine nucleotide per mole more than preparation I. This is in good agreement with the value of 7.8 moles per mole of enzyme, which is the difference in the organic phosphate content of these preparations.

TABLE 2

AMINO ACID COMPOSITION OF HOMOGENEOUS GLUTAMINE SYNTHETASE PREPARATIONS*

Amino acid	Prep. I (moles/mole subunit)	Prep. II (moles/mole subunit)
Aspartic acid	46.0	46.0
Threonine	18.0	18.3
Serine	25.7	24.0
Glutamic acid	42.0	41.0
Proline	24.7	24.0
Glycine	35.7	33.3
Alanine	40.9	41.3
Valine	26.0	26.3
Methionine	14.3	13.7
Isoleucine	22.0	22.0
Leucine	29.2	29.1
Tyrosine	15.1	15.5
Phenylalanine	20.2	22.0
Lysine	26.0	26.0
Histidine	16.1	16.5
Arginine	24.3	24.2

* Values shown are the average of two separate hydrolyses and analyses, and are expressed per minimum mol. wt. of 49,000, the weight of the enzyme subunit.

Covalent nature of the nucleotide binding: Various kinds of evidence indicated that the nucleotide in preparation II is covalently bound to the protein. Thus, the ratio of absorbancy at 280 mμ to 260 mμ was not altered by Sephadex gel filtration, by exhaustive dialysis, or by treatment with charcoal (12.5 mg per mg protein). No

TABLE 3

CHEMICAL ANALYSIS OF GLUTAMINE SYNTHETASE I AND II

Analysis	Prep. I	Prep. II (moles/mole enzyme)	Δ (II−I)
Organic phosphate*	1.2	9.0	7.8
Pentose*	1.0	6.0	5.0
Deoxyribose*	—	<0.6	—
Nucleotide†	—	—	7.4

* Analyses were performed on duplicate samples as described in *Materials and Methods*.
† Nucleotide was determined from the difference spectrum between glutamine synthetases I and II, using the extinction coefficient for AMP at pH 7 for the calculation (15.0×10^3 per mole per cm).

TABLE 4

REMOVAL OF PHOSPHATE FROM GLUTAMINE SYNTHETASE II

Treatment	*E. coli* alkaline phosphatase	Pi liberated (moles/mole enzyme)
None (37°, 3 hr, pH 7.0)	−	0
"	+	0
HCl 0.3 *M*, 37°, 3 hr	−	0
"	+	0
NaOH 0.3 *M*, 37°, 3 hr	−	0
"	+	0
Snake venom phosphodiesterase 37°, 3 hr	−	0.3
"	+	9.2

HCl and NaOH digestions were carried out as indicated in 0.2-ml volume, and at the end of 3 hr the mixtures were neutralized with 0.06 ml of 1 *M* NaOH and HCl, respectively, then had 0.02 ml 1 *M* Tris buffer added and were diluted to a volume of 0.3 ml for subsequent alkaline phosphatase digestion and/or phosphate analysis. *E. coli* alkaline phosphatase digestion and snake venom phosphodiesterase digestion (analytic digestion procedure) were as described in *Materials and Methods*. Before inorganic phosphate analysis, the protein was precipitated by 0.06 ml 60% PCA, centrifuged, and 0.3 ml of the supernatant was used for the assay, as in *Materials and Methods*.

260-mμ absorbing material was released into solution by treatment with 50 per cent saturated $(NH_4)_2SO_4$ at pH 3.2 or by precipitation of the protein with 15 per cent PCA. Moreover, as shown in Table 4, no release of protein-bound phosphate, either as free inorganic orthophosphate (Pi) or in a form susceptible to hydrolysis by *E. coli* alkaline phosphatase, was obtained by treatment for three hours at 37° with 0.3 *M* HCl or 0.3 *M* NaOH. Similar treatments for five hours followed by precipitation of the protein with 15 per cent PCA failed to release significantly more 260-mμ absorbing material from preparation II than from preparation I.

On the other hand, as shown in Table 4, treatment of preparation II with snake venom phosphodiesterase does release essentially all of the protein-bound phosphate as an organic derivative which upon further treatment with *E. coli* alkaline phosphatase yields 9.2 equivalents of Pi per mole of glutamine synthetase. When preparation II was digested with snake venom phosphodiesterase and was then precipitated with PCA, the supernatant solution contained material having a maximum absorbancy at 257 mμ. Assuming this to be free AMP ($\epsilon_{260} = 14.2 \times 10^3$ cm^{-1} M^{-1}, pH 2.0), 9.0 moles of nucleotide per mole of preparation II were released by the phosphodiesterase treatment. Similar enzymic digestion of preparation I yielded a supernatant solution with only 0.6 equivalent of AMP-like absorbing material per mole of protein. Thus, the difference in 260-mμ absorbing material released from preparation I and II was 8.4 equivalents per mole calculated as AMP. This is in good agreement with the value of 7.4 equivalents per mole that was calculated from the difference spectra of the two untreated enzyme preparations (see Fig. 1). Since the purified snake venom phosphodiesterase acts on polynucleotides to produce 5′-mononucleotides, it is likely that the nucleotide released from glutamine synthetase is bound to the protein in a 5′-phosphodiester linkage. The snake venom diesterase preparation is contaminated with slight 5′-nucleotidase activity as is indicated by its ability to catalyze the slow release of Pi from authentic 5′-AMP. The secondary action of this nucleotidase on the nucleotide released from preparation II during diesterase digestion probably accounts for the traces (0.3 equivalent per mole) of Pi that are also formed (see Table 3).

Identification of the bound nucleotide as AMP: The nature of the bound nucleotide was further disclosed by digesting preparation II with 12 *N* PCA under conditions (see *Materials and Methods*) shown previously[14] to give quantitative recovery of bases from nucleic acid. It is evident from the data in Table 5 that the spectral

characteristics and chromatographic behavior of the base released by this PCA digestion are identical with those of adenine.

Finally, large-scale digestion of preparation II with snake venom diesterase (see *Materials and Methods*) was undertaken to obtain a sufficient quantity of the intact nucleotide for its positive identification. The data in Table 5 show that the material thus obtained had the spectral characteristics of AMP or adenosine, and that chromatography in two solvent systems indicated that both of these adenine derivatives were present. To substantiate their identification, the mixture was subjected to sequential digestion with the highly specific 5′-adenylic acid deaminase from muscle and the similarly specific intestinal adenosine deaminase as described in *Materials and Methods*. The results summarized in Table 5 show that 5′-AMP accounts for 17 per cent of the material, and adenosine the remainder.

It is concluded from these results that AMP is the protein-bound nucleotide in preparation II. The relatively high yield of adenosine produced during the large-scale digestion with snake venom diesterase is attributed to the action of the 5′-nucleotidase that contaminates the diesterase preparation. The production of

TABLE 5

IDENTIFICATION OF DIGESTION PRODUCTS OF GLUTAMINE SYNTHETASE II

	λ max	
	pH <1.0	pH 11.5
(1) Perchloric acid (12 *N*) digestion		
(*A*) Spectral		
Unknown	262.5	269
Adenine	262.5	269
	R_f	
	System 1	System 2
(*B*) Chromatographic		
Unknown	0.31	0.65
Adenine	0.30	0.65
Cytosine	0.61	0.65
Guanine	0.27	0.45
Uracil	0.69	0.75
	λ max	
	pH <1.0	pH 7.5
(2) Snake venom phosphodiesterase		
(*A*) Spectral		
Unknown	257	259
AMP (or adenosine)	257	259
	R_f	
	System 3	System 4
(*B*) Chromatographic		
Unknown	0.32, 0.55	0.38, 0.59
5′-AMP	0.32	0.38
Adenosine	0.56	0.59
GMP	0.19	0.29
UMP	0.58	0.41
CMP	0.47	0.37
	mMoles found	Per cent
(*C*) Enzyme assay		
5′ adenylic acid	4.8×10^{-6}	17
Adenosine	24.0×10^{-6}	83
Total	28.8×10^{-6}	

Perchloric acid digestion and isolation of base was as described in *Materials and Methods*. The spectrum was compared with that for authentic adenine under identical conditions. Snake venom phosphodiesterase digestion was described in *Materials and Methods*—the large-scale preparative procedure was used. Enzymic assays for 5′-AMP and adenosine are described in *Materials and Methods*, as are the conditions for thin-layer chromatography. Adenosine and 5′-AMP accounted for 85% of the total material released by phosphodiesterase digestion, as determined by UV absorption.

more adenosine in the large-scale experiment than would have been predicted from the small-scale experiment, described in Table 4, is probably due to the fact that a longer digestion time was employed to ensure maximal digestion of the relatively large amount of glutamine synthetase involved.

Discussion.—It is evident from the data reported in this paper and from previous data,[2] summarized in Table 1, that covalent attachment of adenylyl groups to glutamine synthetase causes marked alterations in the divalent ion specificity, effector response, and in the intrinsic glutamine biosynthetic capacity of the enzyme. Thus, adenylylation and deadenylylation of glutamine synthetase may supplement repression of enzyme synthesis and cumulative feedback inhibition[1, 15, 16] as mechanisms for the regulation of glutamine metabolism. The physiological significance of the adenylylation mechanism is apparent from the fact that the relative intracellular concentrations of the adenylylated and unadenylylated forms of glutamine synthetase vary widely in response to different states of nitrogen nutrition and to the age of the culture.[13] In other experiments,[17] to be reported later, it has been established that cell-free extracts of *E. coli* contain a specific enzyme that catalyzes the *in vitro*, ATP-dependent adenylylation of glutamine synthetase. This enzyme is activated by glutamine and is inhibited by glutamate; it is therefore analogous if not identical with the so-called "glutamine synthetase inactivating enzyme" previously described by Mecke *et al.*[18] In any case it is evident that adenylylation does not lead to complete inactivation of the enzyme but only converts it to a form with altered divalent ion specificity (see Table 1).

Other studies have indicated that the conversion of adenylylated to unadenylylated enzyme probably occurs *in vivo* when *E. coli* grown on glycerol and glutamate is transferred to a growth medium containing glucose and NH_4^+.[13] It has also been found that deadenylylation of the enzyme II and its simultaneous conversion to a preparation with properties analogous to enzyme I is achieved by treatment with snake venom phosphodiesterase. It therefore seems probable that a comparable phosphodiesterase, perhaps specific for adenylyl glutamine synthetase, is normally involved in the conversion of enzyme II to enzyme I.

The regulation of enzyme activity by covalent alterations in structure of preexisting enzyme is not unique for glutamine synthetase. Similar mechanisms are involved in the interconversion of phosphorylase *b* to phosphorylase *a*,[19] in the activation of phosphorylase *b* kinase,[20] and the interconversion of glucose-6-P dependent glycogen synthetase to the independent form.[21] Similar effects may also be involved in the activities of phosphofructokinase.[22] However, these enzyme-catalyzed modifications are all catalyzed by protein-specific kinases and involve phosphorylation rather than adenylylation of the enzyme. The modification of enzymes by adenylylation has been reported for two other enzyme systems, namely, the "DNA-joining enzyme" in which the adenylyl group of DPN serves as the source of adenylyl group,[23] and the adenylylation of polynucleotide ligase.[24, 25]

The release of AMP from glutamine synthetase by treatment with venom phosphodiesterase suggests that the nucleotide is bound to the protein in phosphodiesterase linkage. Although such attachment might logically involve the esterification of hydroxyl groups of serine or threonine residues, the resistance of the adenylyl linkage to alkaline hydrolysis is not typical of phosphoryl derivatives of internal serine residues.[26, 27] Among other possibilities, the increased stability might in-

dicate that a terminal serine or threonine residue, or still a different kind of amino acid residue, is involved in the AMP attachment.

Summary.—*E. coli* elaborates two forms of glutamine synthetase that differ in their responses to end product effectors and in their specificity for divalent cations. These differences in behavior are attributable to the presence or absence of covalently bound 5′-adenylyl groups.

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