GLUTAMINE SYNTHETASE DEADENYLYLATING ENZYME

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The glutamine synthetase of Escherichia coli exists in various forms differing from each other by their content of covalently bound adenylyl groups (Shapiro et al., 1967; Wulff et al., 1967). The biosynthetic activity of unadenylylated glutamine synthetase is very much greater with Mg^{2+} as the activating cation than with Mn^{2+}, and its γ-glutamyltransferase activity is insensitive to inhibition by many end product effectors (Kingdon et al., 1967). The biosynthetic activity of the adenylylated enzyme is relatively specific for Mn^{2+}, and its transferase activity is much more susceptible to feedback inhibition. An enzyme (ATP:glutamine synthetase adenylyltransferase) derived from E. coli catalyzes the covalent attachment of the AMP moiety of ATP to glutamine synthetase in a Mg^{2+} dependent reaction that is stimulated by glutamine and inhibited by glutamate (Kingdon et al., 1967; Wulff et al., 1967). The conditions of growth of E. coli determine the extent of adenylylation of the enzyme (Kingdon and Stadtman, 1967). Kingdon and Stadtman (1967) and Heilmeyer et al. (1967) found evidence for in vivo deadenylylation of glutamine synthetase. The present communication describes some properties of an enzyme in soluble extracts of E. coli that catalyses the release of covalently bound ^{14}C-AMP from glutamine synthetase; it is markedly stimulated by α-ketoglutarate and inhibited by glutamine. The significance of this activity in the control of glutamine synthetase activity and nitrogen metabolism in E. coli is discussed.

METHODS

^{14}C-AMP glutamine synthetase was prepared by incubating 8-^{14}C-ATP (Nuclear Chicago) and purified glutamine synthetase containing 1.2 moles of AMP per mole, with a partially purified preparation of adenylyltransferase (gift of Dr. M.D.Denton), under the conditions outlined by Kingdon et al. (1967). The resultant labeled glutamine synthetase was purified using acetone and acid ammonium sulfate fractionation procedures (Woolfolk et al., 1967). After exhaustive dialysis against 10 mM imidazole·HCl (pH 7.0) and 1 mM $MnCl_2$, the enzyme contained 5.5 moles of AMP per mole (130,000 dpm/mg enzyme). About 4% of the radioactivity of this preparation was released by perchloric acid (PCA)

alone, and this background value was subtracted from all of the determinations below. Non-labeled enzymes containing 1.2 and 9.0 moles AMP per mole, respectively, were purified from E. coli and have been described (Shapiro et al., 1967).

The deadenylylating enzyme was prepared from E. coli cells grown on ammonium chloride and glucose, as previously described (Kingdon and Stadtman, 1967). The cells were harvested, washed with 50 mM imidazole chloride (pH 7) and suspended in the same buffer. The cells were disrupted by sonication (2 min.,30 second bursts, 0°, (Branson S-75, 9 amps). The resulting particulate suspension was centrifuged at 27,000 x g for 20 min. and the supernatant solution was used as the deadenylylating enzyme.

The conditions for the deadenylylation reaction are described in the legends to the Tables. Reactions were stopped by the addition of 50µl of 12% perchloric acid (PCA) to 0.21 ml of reaction mixture and the precipitated protein was removed by centrifugation at 1500 x g for 10 min. A 150 µl aliquot of the supernatant solution was transferred to a counting vial, neutralized with 30 µl of 2.0 N KOH, and 10 ml of Bray's solution was added for counting in a Packard Tri-Carb scintillation counter, at an efficiency of 55%, determined with ^{14}C-toluene. The rate of reaction was linear, under the conditions described in Table I, for 1 hour.

RESULTS

Table I shows that extracts of E. coli catalyze removal of the labeled moiety of ^{14}C-AMP from adenylylated glutamine synthetase. This reaction is markedly stimulated by α-ketoglutarate, which can not be replaced by glutamate, glutamine, or other α-keto acids, such as pyruvate or glyoxylate. Although 65% maximal stimulation is effected by 12 mM α-ketoglutarate, 72 mM was routinely used. The stimulation by α-ketoglutarate varied from 6 to 15-fold, depending upon the extract used. The deadenylylating enzyme was quite labile, and its specific activity varied from extract to extract. Conditions for stabilizing the enzyme have not yet been perfected, but freezing in liquid nitrogen at high extract concentrations preserves activity. Table I also illustrates a requirement for either Mg^{2+} or Mn^{2+}, which cannot be satisfied by Co^{2+} or Ca^{2+}. Fluoride stimulates the deadenylylation reaction by insuring a linear rate to 60% completion; its mechanism of action is unknown. Bovine serum albumin stimulated the deadenylylating activity of various extracts to different degrees. This effect varied with the enzyme concentration and may reflect increased stability of the enzyme in the presence of albumin.

Table I

Requirements for Glutamine Synthetase Deadenylylation

Experiment I		Experiment II	
Conditions of Incubation	^{14}C-AMP released	Conditions of Incubation	^{14}C-AMP released
	μμmoles		μμmoles
Complete system	103	Complete system minus α-ketoglutarate	8
minus α-Ketoglutarate	16	plus 12mM α-ketoglutarate	70
plus glutamate (72 mM)	15	plus 72mM α-ketoglutarate	106
plus glutamate (60 mM)	12	plus 72mM pyruvate	7
minus $MnCl_2$	38	plus 72mM glyoxylate	4
plus EDTA (4.8 mM)	2		
plus $MgCl_2$ (6.6 mM)	70		
plus $MnCl_2$ (6.6 mM)	67		
plus $CoCl_2$ (6.6 mM)	9		
plus $CaCl_2$ (6.6 mM)	1		
minus NaF	66		
plus NaCl (14 mM)	62		
minus bovine serum albumin	91		
minus deadenylylating enzyme	1		
plus adenylyltransferase (790 μg)	4		

The complete system contains: 95 mM imidazole-chloride, pH 7.0; 72 mM α-ketoglutarate; 2.9 mM $MnCl_2$; 14 mM NaF; 2.0 mg bovine serum albumin (BSA); 62 μg adenylylated glutamine synthetase (^{14}C-AMP); and 2.0 mg deadenylylating enzyme extract; final volume, 0.21 ml; pH 7.0. Incubations were at 37° for 25 min (Expt. 1) or 40 min. (Expt. 2). Assay for ^{14}-C-AMP released is described in Methods.

Table II shows that the α-ketoglutarate stimulated deadenylylating activity is strongly inhibited by glutamine. Neither glutamate nor ammonia affects the reaction.

Table II

Inhibition of Deadenylylation by Glutamine

Conditions of Incubation	^{14}C-AMP released	Relative enzyme activity
	μμmoles	
Complete system	156	1.00
plus NH_4Cl (10 mM)	150	0.96
plus glutamate (10 mM)	167	1.07
plus glutamine (10 mM)	32	0.21

The complete system was as in Table I. Incubations were for 40 min. at 37°. Assay for ^{14}C-AMP released is described in Methods.

Table III shows that the enzyme catalyzed removal of adenylyl groups from glutamine synthetase converts it to a form having heightened catalytic activity with Mg^{2+}, less catalytic activity with Mn^{2+}, and which is less susceptible to inhibition by AMP and histidine. These are the expected effects attendent upon deadenylylation (Kingdon et al., 1967).

Table III

Catalytic Changes in Glutamine Synthetase Effected by the Deadenylylating Enzyme

Parameter Observed	Time in Deadenylylation Mixture (hours)		
	0	4	8
Moles of AMP per mole of Enzyme*	8.6	5.1	0.3
Specific activity (μmoles/min/mg enzyme)			
Mg^{2+} biosynthetic assay (pH 6.8)	15.3	26.1	33.2
Mn^{2+} biosynthetic assay (pH 6.9)	20.6	12.7	7.9
Mg^{2+}/Mn^{2+} ratio	0.74	2.05	4.2
Relative Specific Activity (Transferase assay)			
With AMP (40 mM)	0.49	0.82	1.12
With histidine (20 mM)	0.73	0.88	0.97

The deadenylylation mixture contained 80 mM imidazole chloride, pH 7.0; 60 mM α-ketoglutarate; 2.5 mM MnCl; 12 mM NaF; 1.7 mg bovine serum albumin; 0.19 mg ^{14}C-AMP glutamine synthetase (5.5 moles AMP/mole enzyme); 1.34 mg glutamine synthetase (9.0 moles AMP/mole enzyme); and 6.6 mg deadenylylating enzyme preparation, in 0.5 ml. Incubations were at 37°. At the times indicated aliquots were assayed for ^{14}C-AMP released (i.e., acid soluble radioactivity) and glutamine synthetase activity. For acid soluble radioactivity, 0.1 ml of reaction mixture was diluted into 150 μl of 5% PCA, then centrifuged, and 150 μl of the supernatant solution was transferred to a counting vial, neutralized with 40 μl 2.0 M KOH, and counted as in Methods. At the same time, 40 μl of the reaction mixture was diluted into 0.36 ml of imidazole chloride, 0.01 M pH 7.0, containing 5 mM EDTA, at 0°. Aliquots of this solution were taken for biosynthetic and transfer assays at the pH indicated, as described by Kingdon et al.(1967). To obtain a more reliable measure of γ-glutamylhydroxamate produced in the transfer assay in the presence of high AMP levels, the acid ferric chloride reagent was modified to contain 3.3% $FeCl_3$, 2% TCA and 0.25 N HCl.

* The extent of adenylylation was calculated from the amount of acid soluble radioactivity produced and the known initial state of adenylylation of the mixture of glutamine synthetase preparations used. This calculation assumes that the preparations containing 5.5 and 9.0 moles of AMP bound per mole of enzyme, respectively, are deadenylylated at the same rate.

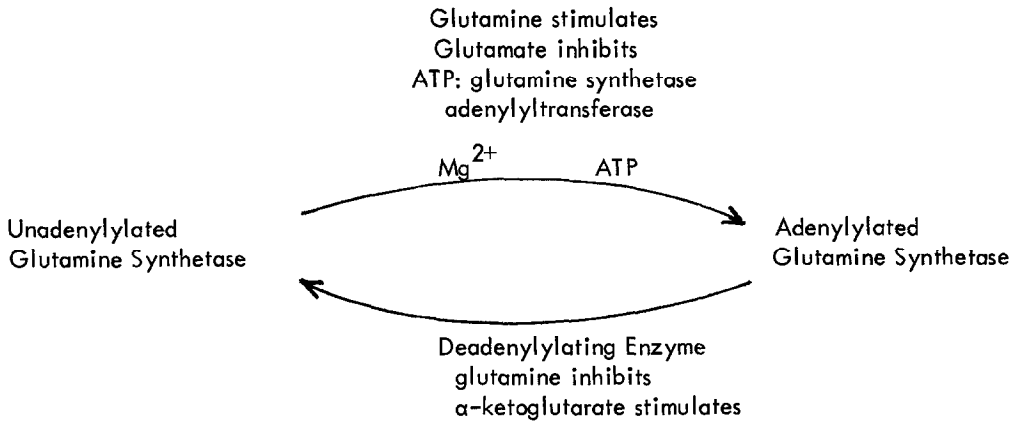
DISCUSSION

The present results clearly demonstrate that E. coli contains an enzyme that catalyzes the removal of a labeled moiety from adenine labeled adenylylglutamine synthetase. Although not positively identified, the cleavage product is probably ^{14}C-AMP since the glutamine synthetase so formed behaves like unadenylylated enzyme;

no attempts to isolate AMP from the crude reaction mixture have yet been made. Since a similar deadenylylation of glutamine synthetase is catalyzed by snake venom phospho diesterase, it seems likely that the deadenylylation enzyme from E. coli is a phospho-diesterase. However, since the latter is specifically activated by α-ketoglutarate and is inhibited by glutamine, its activity appears to be specifically concerned with the deadenylylation of glutamine synthetase.

From the standpoint of cellular regulation it appears significant that adenylylation converts glutamine synthetase from an intrinsically more active form, which is dependent upon Mg^{2+} for activity and which is relatively insensitive to feedback inhibition, to a Mn^{2+} dependent form, which is intrinsically less active and is strongly inhibited by each of the many feedback effectors. Thus, the net effect of adenylylation is to decrease the glutamine synthetase activity, whereas deadenylylation favors glutamine synthesis. The presence of separate adenylylating and deadenylylating enzymes that are themselves subject to regulation by metabolites involved in glutamine metabolism, provides the basis of a highly sophisticated mechanism for the regulation of glutamine synthetase activity. The effectiveness of this control system is easily appreciated by considering the demands of the organism under extreme states of nitrogen nutrition. With nitrogen starvation, the intracellular level of glutamine should be low, and the demand for it great. These conditions should also favor α-ketoglutarate and possibly glutamate accumulation for lack of ammonia needed to convert them to glutamate and glutamine, respectively. The increased demand for glutamine will then be accommodated by the conversion of glutamine synthetase to its most active, deadenylylated form. This will be facilitated, on the one hand, by activation of the deadenylylating enzyme in response to the low concentration of its inhibitor, glutamine, and to the high concentration of its specific activator, α-keto-glutarate and, on the other hand, by a decrease in the activity of the adenylyltransferase caused by low concentrations of its specific activator, glutamine, and/or, the higher concentration of its inhibitor, glutamate. Conversely, with nitrogen saturation, glutamine should accumulate, leading to the production of adenylylated glutamine synthetase, both by activating the adenylyltransferase, and by inhibiting the deadenylylating enzyme. The resultant glutamine synthetase has a low intrinsic activity and is subject to inhibition by many feedback effectors.

Thus, E. coli appears to regulate its nitrogen metabolism by altering the structure of glutamine synthetase, a principal enzyme in nitrogen fixation, according to the following scheme:



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