CUMULATIVE FEEDBACK INHIBITION IN THE MULTIPLE END PRODUCT REGULATION OF GLUTAMINE SYNTHETASE ACTIVITY IN ESCHERICHIA COLI

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The elaboration of multiple enzymes catalyzing a reaction preceeding branch points in metabolism, and the differential control of these isoenzymes by different end-products offers one mechanism by which common enzymatic steps can be regulated through feedback inhibition (1). Whereas this mechanism provides reasonable control of steps concerned with the biosynthesis of only a few different compounds, it seems cumbersome and inefficient when applied to situations in which the common enzymatic step is essential for the biosynthesis of a large number of end-products. To investigate the latter situation we have studied the regulation of glutamine synthetase which occupies a very central role in nitrogen metabolism. The amide nitrogen of glutamine may be used for the synthesis of histidine (2,3), purines (4,5), CTP (6), glucosamine (7), tryptophan and p-aminobenzoic acid (8,9,10), pyridine nucleotides (11), and carbamyl phosphate (12). Transamination of the amino group of glutamine with keto acids (13) suggests a possible role of glutamine in the synthesis of various amino acids.

Our results indicate the existance of a single glutamine synthetase in Escherichia coli strain W that is subject to partial inhibition by a number of possible products of glutamine metabolism. These inhibitors are generally independent in their action; thus the activity of the enzyme is progressively decreased by increasing the number of inhibitors. We refer to this type of multiple inhibition as cumulative feedback inhibition. It should be noted that cumulative feedback inhibition is not exactly

analagous to multivalent repression (14) since in the latter case repression of enzyme synthesis requires the simultaneous presence of all metabolites; individual corepressors have no effect by themselves. The term multivalent feedback inhibition should, therefore, be reserved for the situation where a combination of end-metabolites is required for appreciable inhibition of an enzyme.

METHODS

Derepression of glutamine synthetase. Escherichia coli strain, W, produces glutamine synthetase in low activity when grown on a rich medium containing yeast extract or on a simple medium containing 0.07 M ammonium salts, but is derepressed some 20 fold when grown on glutamate or on a growth limiting concentration of ammonia. Cells used in this study were grown by continuous culture in the Biogen on a medium as previously described (15) but containing only 0.8×10^{-4} M ammonium salt, and harvested at a flow rate sufficiently slow to maintain a reading of 100 as measured with the Klett colorimeter with a No. 660 filter.

Purification of glutamine synthetase. Cell-free extracts were obtained by subjecting cells to sonic oscillation followed by centrifugation. Glutamine synthetase was purified some 200 fold with good yield from these extracts by a combination of treatment with streptomycin, precipitation with acid (pH 5.0-4.4), re-extraction in water buffered at pH 5.7, treatment at this pH with heat at 60°C for 10 minutes in the presence of 10^{-2}M Mn^{++}, and precipitation by addition of solid ammonium sulfate (35% to 50% saturation). The latter precipitate, dissolved in imidazole buffer and dialyzed against distilled water, was used as a source of the enzyme in the experiments described below.

Assay of glutamine synthetase. Glutamine synthetase activity was determined either by the measurement of the formation of γ-glutamylhydroxamate from glutamine and hydroxylamine when the enzyme was incubated in the presence of ADP, arsenate, and manganous ion (16) or by the production of inorganic phosphate from ATP when the enzyme was incubated with gluta-

mate, ammonia, and either manganous or magnesium ions (17). The former method of assay was used for the measurement of the enzyme in crude extracts and in following the purification of the enzyme. The second method of assay was used to obtain the data recorded in the Tables of this report. This assay was adjusted for use in a total volume of 0.20 ml. and was diluted 10 fold just before the addition of the final reagents. Reaction mixtures contained 0.0076 M ATP (apparent K_m=0.00068), 0.1 M l-glutamate (apparent K_m=0.0024), 0.05 M ammonia (apparent K_m=0.0025), 0.05 M Mg^{++} (apparent K_m=0.0176), and sufficient enzyme to give a final concentration of inorganic phosphate of 0.00125 M or less. Of the inhibitors reported here, only alanine (ALA), AMP, and Carbamyl phosphate (CP) effect the maximum velocity of the enzyme. The inhibitions due to glycine (GLY), CTP, and tryptophan (TRY) are competitive with respect to glutamate, and the glucosamine-6-phosphate (GAP) and histidine (HIS) inhibitions are competitive with respect to ammonia. Furthermore, inhibition by HIS is not observed unless Mn^{++} is also present although this ion tends to reduce the amount of inhibition that can be observed with some of the other inhibitors. Because of these complications a variety of reaction conditions were used with the various inhibitors and combinations of inhibitors (Table I). The following concentrations of inhibitors were used

TABLE I

Assay Conditions for the Measurement of Glutamine Synthetase

Assay Condition	Changes in Routine Assay	Used With
A	none	ALA, AMP, CP
B	0.0075 M glutamate	CTP, GLY, TRY and combinations of these with, ALA, AMP, CP
C	0.001 M NH_3	GAP alone and combined with ALA, AMP, CP
D	No Mg^{++}, 0.005 M Mn^{++}	HIS
E	0.0075 M glutamate and 0.001 M NH_3	GAP alone and combined with, ALA, AMP, CP
F	0.015 M Mg^{++}, 0.0035 M Mn^{++}, 0.001 M NH_3	HIS in combination with ALA, AMP, CP, and GAP
G	as in F but with 0.0075 M glutamate	HIS in combination with CTP, TRY, and GLY

in the experiments below as "saturating" concentrations of inhibitor: 0.005 M for ALA, GLY, TRY, AMP, and GAP; 0.0025 M for CTP and CP; 0.01 M for HIS (condition F and G); 0.005 M for HIS (condition D).

RESULTS

The glutamine synthetase when measured in crude extracts by the formation of γ-glutamylhydroxamate can be inhibited to a limited extent by the following compounds: AMP, GAP, CTP, ALA, GLY, TRY, The same compounds inhibit the purified enzyme when measured by either method of assay. In addition, an inhibition due to HIS is observed with the forward assay. The amount of inhibition observed with each inhibitor remained relatively constant over the 4,000 fold increase in specific activity observed from the crude extracts of repressed cells to that of the purified enzyme. These results strongly suggest that only a single glutamine synthetase is present in Escherichia coli W. The following compounds are not inhibitory or show only negligible inhibition when added at the same concentration used with the above compounds: glutamine, leucine, isoleucine, valine, lysine, phenylalanine, serine, aspartate, threonine, cysteine, proline, arginine, methionine, ATP, IMP, UMP, ITP, UTP, CDP, CMP, GMP, GTP, glucosamine, nicotinic acid, TPN, TPNH, DPN, DPNH.

Nearly maximal inhibition by any one of the 8 inhibitors is obtained at concentrations of 0.002 to 0.005 M, (Table II). In no case can complete inhibition be observed with any one of the inhibitors.

TABLE II

The Effect of Concentrations of Inhibitors on the Per Cent Inhibition Observed with Glutamine Synthetase

Inhibitor	Assay Condition	Per Cent Inhibition				
		.001 M	.002 M	.003 M	.005 M	.01 M
AMP	A	16	26	30	35	-
CP	A	6	14	18	18	-
ALA	A	11	19	23	31	-
CTP	B	14	19	23	--	-
GLY	B	17	31	33	34	-
TRY	B	6	12	12	11	-
GAP	C	12	14	16	--	-
HIS	D	8	14	18	22	22

To determine the extent to which these inhibitors are independent in their action various combinations of inhibitors were investigated. The amount of inhibition obtained with all 28 possible pairs of these 8 inhibitors is shown in Table III, along with the amount of inhibition observed with the individual inhibitors in the same experiment.

TABLE III

The Per Cent Inhibition of Glutamine Synthetase by Pairs of Inhibitors

	HIS	GAP	TRY	GLY	CTP	ALA	CP
	12 12	18 10	29 8	51 38	50 28	37 43	45 14
AMP	22 22	25 26	39 35	64 69	63 64	58 64	53 53
	15 4	15 9	9 8	22 38	11 14	28 46	
CP	18 18	22 22	14 16	63 54	21 23	58 61	
	18 5	28 10	39 8	49 38	40 14		
ALA	23 22	38 35	42 44	67 68	50 52		
	11 26	12 9	31 26	31 41			
CTP	38 34	20 20	39 48	55 59			
	28 16	30 11	41 26				
GLY	40 40	42 38	51 55-				
	9 16	7 7					
TRY	20 22	13 14					
	8 11						
GAP	20 18						

With each block of figures the number on the upper left represents the % inhibition observed with saturating concentration (refer to section on methods) of the inhibitor on the left. Similarly, the upper right figure corresponds to the amount of inhibition observed with saturating concentration of the inhibitor designated at the top. The lower left figure is the % inhibition observed when both inhibitors are added simultaneously under the same conditions of assay as that used with the individual inhibitors. The lower right figure is the value calculated (as described in the text) on the assumption that each inhibitor acts independently on the same enzyme. Different conditions of assay were used for the various combinations as described in Table I.

If the partial inhibitions observed with each inhibitor are due to the presence of isoenzymes in the purified enzyme preparation, each enzyme being inhibited by a single inhibitor, the total inhibition for each pair should be equal to the sum of their individual inhibitions. Thus, a mixture of GLY and AMP should give a total inhibition of 51% + 38% = 89%. On the other hand, if these inhibitors act concurrently but independently on a single enzyme the cumulative inhibition would be 51% + 38% (100-51) = 69%, which compares favorably with the observed inhibition of 64%. If the two inhibitors interfered with each other, less total inhibition would be expected. As can be seen in Table III, most of the pairs of inhibitors give a cumulative inhibition close to that predicted on the assumption that they act independently on the same enzyme. As expected from the data of Table III, greater total inhibitions are observed (Table IV) when larger combinations of inhibitors are added, and all 8 inhibitors

give a cumulative inhibition of over 90%.

TABLE IV

The Effect of a Combination of Inhibitors on Glutamine Synthetase Activity

Inhibitor	Assay Condition	Per Cent Inhibition
AMP	A	50
CP	A	23
ALA	A	40
AMP + CP+ALA	A	86 (82)
CTP	B	31
GLY	B	42
TRY	B	26
CTP+GLY+TRY	B	59 (70)
GAP	G	8
HIS	G	10
GAP+HIS	G	20 (18)
AMP+CP+ALA+ CTP+GLY+TRY+ GAP+HIS	G	93

Each vessel contained a saturating concentration of the inhibitor or inhibitors indicated. Figures in parenthesis are calculated as described in the text on the assumption that the inhibitors act independently on a single enzyme.

Other experiments demonstrating the presence of glutamine-pyruvate and glutamine glyoxylate transaminase activities in extracts of Escherichia coli strain W suggest that glutamine may contribute to the synthesis of alanine and glycine. Thus, the possibility that all 8 inhibitors are products of glutamine metabolism is consistent with a regulatory function for the multiple inhibition reported here. The mechanism of action of this cumulative feedback inhibition is under further investigation. The mechanism of action of the 8 inhibitors on the activity of glutamine synthetase from Escherichia coli W is under further investigation.

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