PREFERENTIAL UTILIZATION OF GLUTAMINE FOR AMINATION OF XANTHOSINE 5'-PHOSPHATE TO GUANOSINE 5'-PHOSPHATE BY PURIFIED ENZYMES FROM ESCHERICHIA COLI[1]

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GMP synthetase was purified 180-fold from E. coli B and 18-fold from the derepressed purine auxotroph, E. coli B-96. The enzymes from both sources show the same preference for glutamine over ammonia as amino donor. Each is dimeric, consisting of subunits of molecular weight about 60,000. Thus the two are apparently identical. The similarities between GMP synthetase and xanthosine 5'-phosphate aminase of E. coli B-96 (N. Sakamoto, G.W. Hatfield, and H.S. Moyed, J. Biol. Chem. (1972) 247, 5880-5887) in respect to structure, state of derepression, and behavior during purification, lead us to the conclusion that the synthetase and the aminase are a single entity. We observe no loss or separation of glutamine-dependent activity upon purification of GMP synthetase and we suggest that such loss, reported by other workers, results artifactually by inactivation of an intrinsic glutamine-binding site. GMP synthetase appears not to contain a glutamine-binding subunit which is separable from the xanthosine 5'-phosphate-aminating component.

The amination of XMP[2] to GMP is catalyzed by XMP:L-glutamine amidoligase (AMP), EC 6.3.5.2 (GMP synthetase) and by XMP:ammonia ligase (AMP) EC 6.3.4.1 (XMP aminase) according to Reactions 1 and 2.

$$XMP + ATP + \text{glutamine} + H_2O \xrightarrow{Mg^{++}} GMP + AMP + PP + \text{glutamate} \quad (1)$$

$$XMP + ATP + NH_3 \xrightarrow{Mg^{++}} GMP + AMP + PP \quad (2)$$

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2. Abbreviations used are: XMP, xanthosine 5'-phosphate; DON, 6-diazo-5-oxonorleucine.

Previous studies have shown that, in calf thymus (1) and pigeon liver (2), GMP synthetase preferentially utilizes glutamine as its substrate. However, the amino donor in bacterial systems has been a matter of controversy. Mardashev and Iarovaia reported that extracts from E. coli NCTC 7020 exclusively utilize glutamine (3). Certain purine- or guanine-requiring auxotrophs of Aerobacter aerogenes (4) and Escherichia coli B (5,6), grown under conditions of derepression, have greatly elevated capacity for aminating XMP. Extracts of the Aerobacter mutant use both glutamine and ammonia as substrate, but the ability to use glutamine decreases and eventually disappears during purification of the enzyme. Glutamine was not tested as a substrate for these E. coli B enzymes. Brevet, et al., examined the XMP aminase of E. coli B-24-1 (a mutant lacking IMP dehydrogenase) and observed that ammonium sulfate fractionation of the extract yields components with differential substrate preferences and that upon chromatographic purification an aminase is obtained showing no activity with glutamine (7). They concluded that there are two different forms of the enzyme in E. coli B-24-1, one utilizing glutamine, the other ammonia. However, genetic analysis of E. coli K-12 seems to point to the existence of only one gene for an enzyme which catalyzes amination of XMP, that referred to as gua A (8).

An explanation superficially consistent with the behavior of the derepressed bacterial mutants is based on the conjecture that (wild type) GMP synthetase contains a glutamine-binding subunit distinct from the XMP aminating component and that biosynthesis of the two components is not coordinately controlled. If derepression of XMP aminase in the purine-deficient mutants is not accompanied by a corresponding increase in the glutamine-binding subunit, an enzyme capable of using only ammonia would be expected to predominate. A precedent for this situation is the occurrence of a genetically and physically separable glutamine-binding subunit as a component of anthranilate and p-aminobenzoate synthetases (9). We therefore undertook to examine the GMP synthetase from wild type E. coli B in order to determine whether it contains a dissociable glutamine-utilizing subunit.

GMP synthetase was isolated from frozen cells of E. coli B harvested in the late log phase or early stationary phase by a series of steps adapted from

Sakamoto, et al., (6). The results are summarized in Table I (column 1). It is evident that the specific activities with glutamine and with ammonia as substrates remain in essentially the same ratio throughout the purification of over 180-fold. In those fractions discarded the enzyme showed no significant difference in substrate preference, nor was the presence of other active con-

TABLE I

Purification of GMP Synthetase and XMP Aminase

Stage	Substrate	E. coli B	E. coli B-96	
			(this work)	(Sakamoto, et al.)
Extract	Glutamine	0.0042 (153)	0.372 (461)	-
	NH_3	.0025 (91)	.238 (295)	0.442 (13400)
	Ratio, $\frac{Gln}{NH_3}$	1.68	1.56	
Streptomycin treatment	Glutamine	0.0075 (167)	0.452 (421)	-
	NH_3	.0048 (107)	.322 (300)	0.479 (11700)
	Ratio, $\frac{Gln}{NH_3}$	1.56	1.40	
Ammonium sulfate	Glutamine	0.029 (152)	2.17 (206)	-
	NH_3	.020 (105)	1.53 (145)	1.72 (7150)
	Ratio, $\frac{Gln}{NH_3}$	1.45	1.42	
DEAE chromatography	Glutamine	0.77 (81)	6.75 (109)	-
	NH_3	.51 (54)	4.32 (70)	6.67 (3650)
	Ratio, $\frac{Gln}{NH_3}$	1.51	1.56	
	Net purification	180	18	15

Values given are specific activity, μmoles/min-mg protein; total units recovered are in parentheses. The procedure of Sakamoto, et al., (6) was followed, with the following modifications: for the E. coli B synthetase, the ammonium sulfate factionation was between 45 and 55% saturation, and for both synthetase preparations, DEAE cellulose was employed instead of DEAE Sephadex. The quantities of cells used (wet weight) were 450 g for the E. coli B synthetase, 17 g for the B-96 synthetase, and 340 g for the B-96 XMP aminase (6). Glutamine-dependent activity was determined in the presence of 150 mM Tris Cl, pH 7.4, 15 mM L-glutamine, 6.4 mM $MgCl_2$, 1.3 mM ATP, and 0.23 mM XMP, while the ammonia-dependent reaction was determined in 75 mM Tris Cl, pH 8.5, 55 mM $(NH_4)_2SO_4$, 7.5 mM $MgCl_2$, 1.3 mM ATP, and 0.23 mM XMP. The decrease in XMP concentration was followed by continuous spectrophotometric assay at 30° (4).

stituents indicated during the ammonium sulfate and chromatographic separations. The molecular weight of the partially purified GMP synthetase was determined to be 125,000 ± 10,000 on a calibrated Sephadex G-150 column according to Andrews (10) (Fig. 1).

As observed with other amidotransferases the glutamine analogue, DON, is a powerful inhibitor of GMP synthetase (11), presumably acting as an active site directed, irreversible reagent. We have confirmed these results and find that irreversible binding of 6-[^{14}C]DON is dependent upon the presence of the substrates, XMP, ATP, and Mg^{++}. With covalently bound [^{14}C]DON (12) as a marker, GMP synthetase was subjected to electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate and mercaptoethanol (13) in order to estimate the molecular weight of the DON-binding component. The radioactive protein was

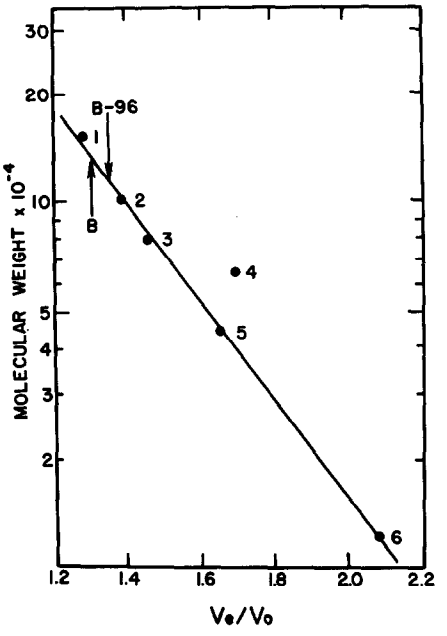

Fig. 1. Gel Filtration of GMP Synthetase. The sample (4 ml containing 20 mg protein) was applied to a column of Sephadex G-150 (5 x 104 cm) and eluted with 0.05 M potassium phosphate, pH 7.4, containing 0.15 M KCl. Fractions of 5.4 ml were collected at a flow rate of 0.45 ml per min. The void volume was determined with Blue Dextran 2000 (Pharmacia) and the calibration proteins (10) were: (1) yeast alcohol dehydrogenase (mol. wt. = 150,000); (2) yeast hexokinase (102,000); (3) E. coli alkaline phosphatase (80,000); (4) hemoglobin (64,500); (5) ovalbumin (45,000); and (6) horse cytochrome C (12,400). GMP synthetase was located as shown by the arrows.

located after accurately sectioning the gels into 1 mm thick slices and extracting each slice with buffer. A single peak of radioactivity was detected in the region corresponding to a molecular weight of 60,000 ± 6,000 (Fig. 2).

These results indicate that GMP synthetase from E. coli B is a dimer consisting of subunits of molecular weight about 60,000. Since Sakamoto, et al. (6) have shown that the XMP aminase from derepressed E. coli B-96 consists of two subunits of M = 63,000, the physical properties of the enzymes from the two strains suggest that they may be the same protein. To examine this possibility further, we grew the B-96 strain on a purine limited medium as described by Zyk, et al. (14). The procedure of Sakamoto, et al., for isolation of XMP aminase was applied to these cells. The results of the purification procedure are compared with those of Sakamoto, et al. in Table I, columns 2 and 3. GMP synthetase from B-96 was chromatographed on a DEAE cellulose column (Fig. 3) rather

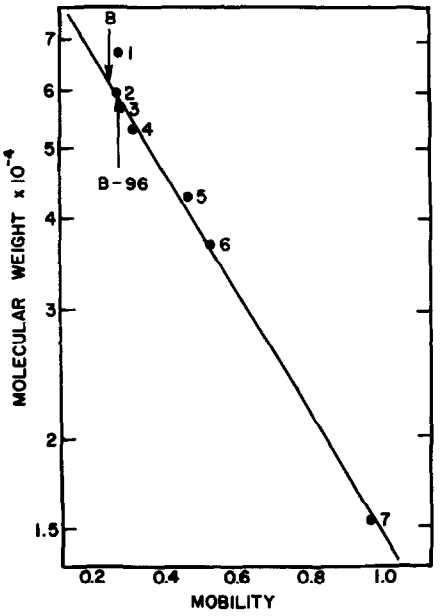

Fig. 2. Polyacrylamide Gel Electrophoresis of GMP Synthetase in the Presence of Sodium Dodecyl Sulfate. The procedure of Weber and Osborn was followed (13). Calibration proteins were: (1) bovine serum albumin (mol. wt. = 68,000); (2) catalase, (60,000); (3) pyruvate kinase (57,000); (4) glutamate dehydrogenase (53,000); (5) ovalbumin (43,000); (6) yeast alcohol dehydrogenase (37,000); and (7) hemoglobin (15,500). GMP synthetase from E. coli B and B-96 were located as indicated by the arrows. Mobilities are relative to bromphenol blue tracking dye.

than on DEAE Sephadex as used by the previous workers. As shown in Fig. 3, the relative activities with glutamine and ammonia remain constant across the single peak observed. The B-96 synthetase at this stage was used for measurement of molecular weight of the native enzyme and its DON-binding subunit as described above. These values were found to be 118,000 and 59,000, respectively (Figs. 1 and 2).

From the structural similarities between the synthetase and the aminase, and from their co-purification, we conclude that E. coli B, whether the wild type or the derepressed mutant, possesses a single enzyme responsible for conversion of XMP to GMP. This conclusion is in agreement with the genetic analysis of the K-12 strain. The enzyme preferentially uses glutamine as amino donor under our conditions of isolation and assay. The nearly constant ratio of activity with glutamine and ammonia during purification of the synthetase does not confirm the report of Brevet, et al., that two forms of the enzyme, with different substrate specificities, can be separated. The purity of the B-96 enzyme

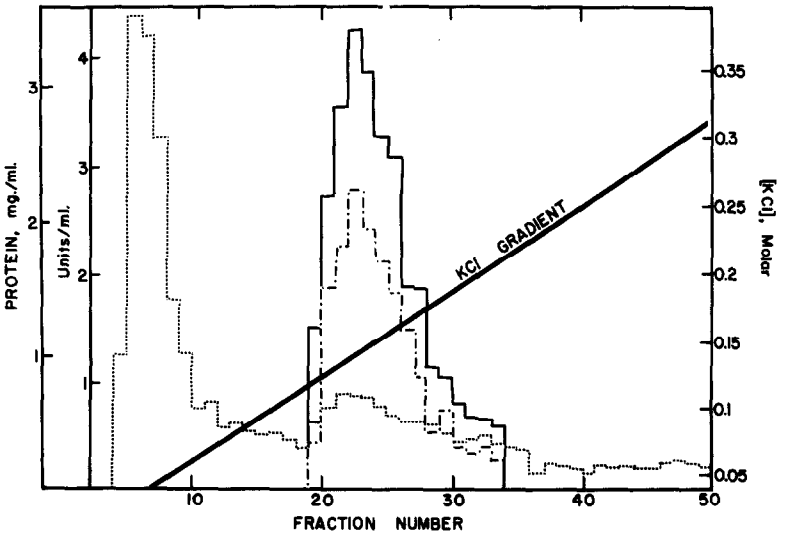

Fig. 3. Chromatography of GMP Synthetase on DEAE Cellulose. The column (0.9 x 16.5 cm) was equilibrated in 0.02 M potassium phosphate, pH 7.4, containing 0.1 mM EDTA. GMP synthetase was eluted with a gradient of potassium chloride from 0.05 to 0.4 M in the above buffer. The total volume of the gradient was 150 ml. Fractions of 2.6 ml were collected. ····· protein; ——— glutamine-dependent activity; —·—·— ammonia-dependent activity.

after chromatography is estimated to be about 50%, based on comparison of its specific activity with that of the homogeneous enzyme (6). Even this material shows no essential change in substrate preference.

The result of Sakamoto, et al., that the aminase consists of identical subunits of molecular weight 63,000 and our observation that DON is covalently bound to a component of similar size lead us to the conclusion that the native enzyme does not contain a separable glutamine-specific subunit but rather that a glutamine (DON) site is part of the same protein constituent which catalyzes the ATP-dependent amination of XMP.

We are unable to account for the fact that other workers observe loss of glutamine-dependent activity in the course of isolation of XMP aminase, other than to suggest that some small differences in their procedures cause selective inactivation of the capacity to bind this substrate. We have noted differential decay of the activity toward glutamine during prolonged storage, and also after exposure to 20 mM hydrogen peroxide at 37° for 30 min. Trotta, et al., have reported that thiols catalyze peroxidative inactivation of the glutamine site in carbamyl phosphate synthetase (15).

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