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AN ENDOGENOUS INHIBITOR OF FOLYLPOLYGLUTAMATE SYNTHETASE IN *NEUROSPORA CRASSA*

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Key Word Index—*Neurospora crassa* (FGSC 853) wild type, *met*-6, *met*-9 and *mac* mutants, folylpolyglutamate synthetase inhibition; endogenous protein inhibitor

Abstract—The *met*-9 mutant (FGSC 552) of *Neurospora crassa* was examined for ability to synthesize folylpolyglutamates *in vivo* and *in vitro* After culture in defined media, the concentration of mycelial folates was *ca* 75% of that found in the Lindegren A wild type (FGSC 853). The derivatives were principally short-chained folates that supported the growth of *Lactobacillus casei* without conjugase treatment. The levels of polyglutamyl folates in the mutant were only 6–20% of those present in the wild type under comparable growth conditions. Crude mycelial extracts of *met*-9 had little folylpolyglutamate synthetase (FPGS) activity but ammonium sulphate fractionation resulted in recoveries in the 150–200% range. A protein fraction precipitating at 30–45% of saturation with ammonium sulphate contained a FPGS inhibitor that was more potent on preincubation. The inhibitor was present in other polyglutamate-deficient mutants and in the wild type. This factor was non-dialysable, thermolabile and inactivated by urea and trypsin treatment. The inhibitor fraction lacked significant protease and conjugase activities. Although several enzymes were not affected by preincubation with this protein fraction, the FPGS activities of *E. coli*, bovine liver and pea cotyledons were strongly inhibited.

INTRODUCTION

The folate-dependent reactions of one-carbon metabolism are preferentially mediated by gamma-glutamyl conjugates of H_4PteGlu [1]. These polyglutamates function as coenzymes in a number of one-carbon transfer reactions vital to cell growth and replication. As a result, most folate-dependent enzymes have greater affinities for polyglutamates than for the corresponding monoglutamyl derivatives [2, 3]. In some cases [2], binding with the polyglutamate substrate increases V_{max} or enhances the affinity for binding the non-folate substrate. The physiological importance of these conjugated folates is also apparent from studies of folylpolyglutamate deficiency Thus mammalian [4, 5] and fungal [6] mutants that lack folylpolyglutamate synthetase (FPGS) require products of one-carbon metabolism for growth in defined media On the other hand, transfectants, expressing the human FPGS gene, are not auxotrophic for these products [8]

There is now strong evidence that folylpolyglutamates have regulatory roles. For example, enzymes of one-carbon metabolism are often inhibited by polyglutamates that are not substrates for the reaction catalysed [2, 3]. In the case of thymidylate synthase, inhibition by H_2PteGlu$_2$ may indirectly control the availability of H_4PteGlu$_n$ [9, 10]. Similarly, the H_2PteGlu$_n$ inhibition of methylenetetrahydrofolate reductase may conserve 5,10-CH_2-H_4PteGlu$_n$ for DNA synthesis by limiting methyl group biogenesis [11] In *Neurospora*, 5-Me-H_4PteGlu$_n$ is an allosteric effector of cystathionine synthase [12] and its action is antagonized by *S*-adenosylmethionine. In bacteria, nutritional conditions regulate one-carbon metabolism by affecting the glutamyl chain lengths of endogenous folates [13], and in liver [14], the glutamyl chain length of 5,10-CH_2-H_4PteGlu$_n$ changes the flux of one-carbon units.

These basic metabolic roles have promoted work on polyglutamate biosynthesis *in vivo* [3, 15] and *in vitro* [3, 13] In this regard, bacterial and mammalian FPGS activities [3, 16–23] have been extensively purified, work has started on the plant enzyme [24] and two complementary FPGS activities have been reported for *Neurospora* [7] Work on porcine liver FPGS suggests that folate distributions are determined by the substrate specificity and the affinities of folates for the enzyme [25] In addition, feedback inhibition and physiological factors can modify the pools of C-1 substituted folates [25–27].

Folylpolyglutamate synthesis in *Neurospora* [28] is reduced by methionine supplements. The nature of this effect in the wild type [28] was however distinct from that exerted by *S*-adenosylmethionine on 5-MeH_4PteGlu$_n$ production [12]. Conceivably, methionine or its metabolic products may also modulate H_4PteGlu production and affect the subsequent conjugation reaction.

In the present work, we have examined the *met*-9 mutant to determine whether the lesion [29] affects the expression of FPGS activity. Evidence is presented for the occurrence of a protein inhibitor in *met*-9 and other *Neurospora* strains that has inhibitory action on FPGS

The abbreviations used for folate derivatives are those suggested by the IUPAC-IUB Commission e.g. H_4PteGlu = 5,6,7,8-tetrahydropteroylmonoglutamate, H_4PteGlu$_n$ = poly-γ-glutamyl derivatives where n = the number of L-glutamate moieties, 5-Me-H_4PteGlu$_n$ = 5-methyltetrahydropteroylpolyglutamate, FPGS = folylpolyglutamate synthetase, PMSF = phenylmethylsulphonyl fluoride

activities from prokaryotic and eukaryotic sources A preliminary report of this work has been published [30]

RESULTS

Folate pools and FPGS activity in the met-9 *mutant*

In *Neurospora*, mutation at the *met*-9 locus results in a requirement for methionine that cannot be replaced by homocysteine, cystathionine, or cysteine [31, 32] In the present work we found that *met*-9 did not respond to single supplements of glycine, serine or adenine (data not shown) Selhub [29] suggested that the *met*-9 gene may control the synthesis of enzymes needed to convert C-3 of serine to the methyl group of methionine. He also reported that *met*-9 produced less total and polyglutamyl folate than the wild type [29].

The data in Table 1 confirms this latter observation The concentration of total, *L casei*-active folates in unsupplemented cultures of *met*-9 was only 75% of that found in the Lindegren A wild type These folates were mainly conjugated derivatives in the wild type, whereas in *met*-9 the major folates were short-chained (Table 1) In both strains, methionine decreased folate concentration without altering the ratio of short-chained to polyglutamate derivatives Freshly harvested conidiospores of *met*-9 contained small polyglutamate concentrations and this was also apparent in the mycelia of cultures receiving a double supplement of adenine and methionine (Table 1).

Mycelial extracts were examined for FPGS activity as summarized in Table 2. Activity in crude extracts was very low (*ca* 36% of the wild type) and the major product after a 2 hr reaction was a triglutamate When the substrate was 5,10–CH_2–H_4PteGlu$_2$, *ca* 30% of the product was hexaglutamate. Salt fractionation gave protein with FPGS activity in the 45–60% range of saturation with $(NH_4)_2SO_4$. This fraction routinely contained 150–200% of the FPGS recovered in the crude mycelial extract (data not shown) The presence of an inhibitor in the crude extract was confirmed by combining protein fractions as shown in Table 2 In this regard, protein from the 30–45% fraction (step 2) inhibited polyglutamate synthesis and this was more pronounced when a 1 hr preincubation period was included

Occurrence of FPGS inhibition in other Neurospora *strains*

The FPGS activity of other *Neurospora* strains was also inhibited by combining protein from steps 2 and 3 of the fractionation procedure (Fig 1) In each case, preincubation of the protein fractions resulted in almost complete inhibition of polyglutamate formation The inhibitory fraction of each strain was also active against the FPGS of the wild type and other mutants Thus the inhibitor present in *met*-9 extracts (step 2 protein) inhibited the $Glu_2 \rightarrow Glu_6$ reaction of the *mac* mutant and the diglutamate-forming activity of *met*-6 (Table 3) On the other hand, mixing extracts that both contained FPGS activity (step 3 protein) resulted in an enhancement of polyglutamate synthesis.

The nature of FPGS inhibition

A series of further experiments provided evidence that the FPGS inhibitor was protein in nature In the assays shown in Fig 2, varying amounts of the inhibitor (*met*-9, step 2 protein) were added to reactions systems containing *met*-9 protein from step 3 of the fractionation procedure Inhibition of FPGS activity occurred in all cases. This was related to the amount of inhibitor added and affected the incorporation of glutamate into di- and triglutamate products

In contrast, inhibition did not occur when step 2 protein was boiled prior to use in the FPGS reaction system (Fig 2) The data in Fig 3 also provides evidence that the inhibitor was thermolabile In these studies, the

Table 1 Effect of L-methionine supplements on the growth and folate contents of *N crassa* wild type and *met*-9 mutant

Strain	L-Methionine added (mM)	Growth (mg dry wt/ 100 ml culture	Folate contents (ng PteGlu equiv/mg dry wt) Short-chained folates	(%)	Polyglutamyl folates	(%)	Total *L casei* folates
1 Wild type mycelium	none	137	21 0	(17 5)	99	(82 5)	120
	1 mM	135	11 0	(18 0)	50	(81 9)	61
2 *met*-9 mycelium	none	30 0	70 5	(78 3)	19 5	(21 6)	90
	0 5 mM	71 6	10 0	(76 9)	3 0	(23 1)	13
	0 5 mM + 0 5 mM adenine	96 7	23 0	(76 7)	7 0	(23 0)	30
3 *met*-9 conidiospores	0 5 mM	—	6 8	(79 6)	1 7	(20 3)	8 6

After 24 hr culture in Vogel's minimal medium (with supplements where indicated), folates were extracted and assayed using *L casei* The data are averages of two separate determinations and values in brackets are percentages of total *L casei*-active folates after γ-glutamylcarboxypeptidase treatments

Table 2. FPGS activities of *met*-9 extracts

Fractionation step and folate substrates	Polyglutamate products formed (pmol folate incorporated/mg protein/hr) diglu	triglu	hexaglu	total
1 Crude extract				
5,10-CH_2-H_4PteGlu	52	140	18	210
5,10-CH_2-H_4PteGlu$_2$	—	170	98	268
2 30–45% $(NH_4)_2SO_4$				
5,10-CH_2-H_4PteGlu	n d	n d	n.d	n.d.
5,10-CH_2-H_4PteGlu$_2$	—	n.d.	n.d.	n.d
3 45–60% $(NH_4)_2SO_4$				
5,10-CH_2-H_4PteGlu	840	1200	159	2200
5,10-CH_2-CH_4PteGlu$_2$	—	1420	587	2010
4 Combined protein* from steps 2 and 3 then assayed				
5,10-CH_2-H_4PteGlu	242	318	54	614 (1320)†
5,10-CH_2-H_4PteGlu$_2$	—	305	157	462 (1200)†
5. Combined protein* from steps 2 and 3 incubated at 37°C for 1 hr then assayed				
5,10-CH_2-H_4PteGlu	12	4	4	20 (1320)†
5,10-CH_2-H_4PteGlu$_2$	—	28	11	67 (1200)†

*0 84 mg of protein from step 2 was mixed with 1.24 mg protein from step 3.
†Theoretical specific enzyme activities based on dilution of FPGS
n.d Radioactive products not detected

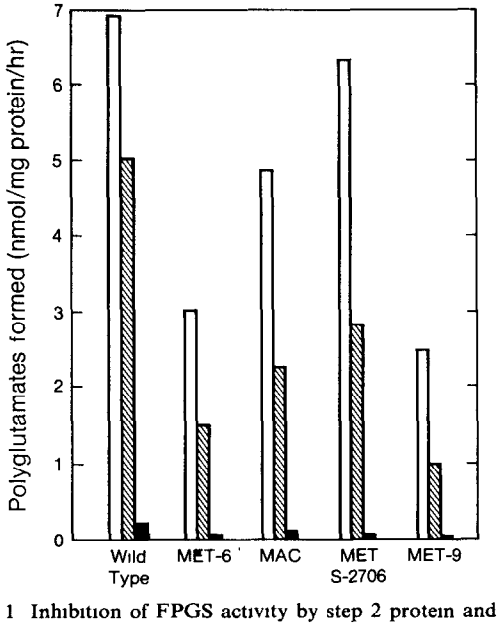

Fig. 1 Inhibition of FPGS activity by step 2 protein and the effect of preincubation. Enzyme activity of each strain (step 3 protein) was determined under standard conditions (□). Other assays were carried out with (■) and without (▨) preincubation in the presence of step 2 protein.

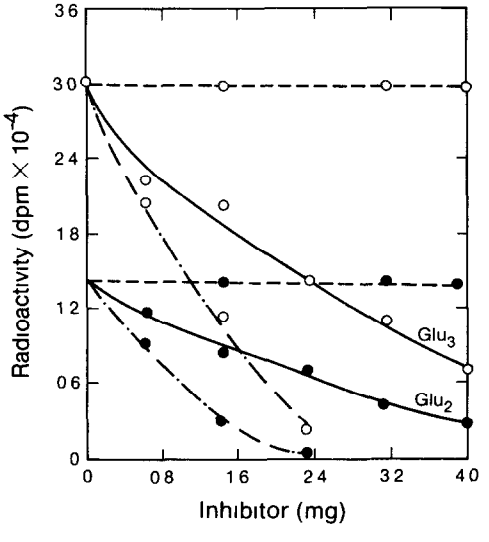

Fig 2 Effect of inhibitor concentraion on FPGS activity Enzyme and inhibitor fractions (step 2 and 3 protein) were isolated from the *met*-9 mutant. Glutamate incorporation into Glu_2 (●) and Glu_3 (○) was examined after elution of FPGS products from DEAE-cellulose. The reaction systems were: enzyme + boiled inhibitor (---), enzyme + inhibitor without preincubation (——); and enzyme + inhibitor, preincubated for 1 hr at 37° (– –).

inhibitor was preincubated at various temperatures, brought to 37° and then added to the reaction system. Potency was progressively lost at preincubation temperatures above 40°. In contrast, the inhibitor appeared to be slightly more effective than control samples, when preincubated in the 15–40° range (Fig. 3). Pretreatment of the inhibitor with urea or trypsin resulted in partial or complete loss of inhibitory ability (Table 4). In other

Table 3 Effect of *met*-9 extracts on *met*-6 and *mac* FPGS activity

Source of FPGS activity and reaction conditions	Polyglutamate products (pmol/mg protein/hr)			
	Glu_2	Glu_3	Glu_n	Total
met-6, control, 1 3 mg protein	700	n d	n d	700
+ *met*-9, crude extract, 0 68 mg protein	923	212	160	1300
+ *met*-9, step 2, 0.88 mg protein	27	n d	n d	27
+ *met*-9, step 3, 1 45 mg protein	1200	1320	675	3130
mac, control, 1 6 mg protein	–	140	140	280
+ *met*-9, crude extract, 0 68 mg protein	—	187	153	340
+ *met*-9, step 2, 0 88 mg protein	--	n d	n d	
+ *met*-9, step 3, 1 45 mg protein	—	902	1080	1980

Step 3 protein was used as a source of *met*-6 and *mac* FPGS activity The folate substrate for *met*-6 was 5,10–CH_2–H_4PteGlu, the corresponding diglutamate was employed in assays of *mac* FPGS The FPGS activities of the *met*-9 crude extract and step 3 protein were 237 and 2156 pmole 5,10-CH_2–H_4PteGlu incorporated/mg protein/hr respectively
n d Not detected

Table 4 The effect of urea and trypsin pretreatments on the FPGS inhibitor

Assay conditions	Wild type FPGS activity (% of controls)	
	Column A Urea-treated*	Column B Trypsin-treated†
1 Enzyme only (control)	100	100
2 Enzyme + FPGS inhibitor	6 5	35
3 Enzyme + pretreated FPGS inhibitor	72	96

Pretreatment conditions *25 mg of *met*-9 inhibitor fraction (step 2) were preincubated with 3 ml of 1 M urea solution for 1 hr at 30° The mixture was then dialysed against the extraction buffer (see Experimental) for 18 hr at 2°

†0 75 units of immobilized trypsin were incubated with 0 45 mg of inhibitor protein (recovered after gel filtration) for 5 hr at 37° Trypsin was then removed by centrifugation.

Assay conditions 1 Samples of FPGS protein (step 3) were assayed in the absence of inhibitor 2 FPGS inhibitor protein incubated in the extraction buffer for 1 hr at 30° then dialysed against this buffer for 18 hr at 2° FPGS and inhibitor preincubated for 1 hr prior to addition of FPGS substrates (see Experimental) 3 Inhibitor pretreated with urea or trypsin, then preincubated with FPGS protein and assayed as in 2

Data are derived from triplicate determinations Average control activities were 2512 pmol glutamate incorporated/mg protein/hr (column A) and 1980 pmol glutamate incorporated/mg protein/hr (column B)

experiments, gel filtration of step 2 protein (wild type extracts) gave a major peak of FPGS inhibition corresponding to a M_r of 150000, based on four separate determinations (Fig. 4) A smaller peak of inhibitory activity occurred later in the elution sequence. This protein had a M_r of about 70000, and on a protein basis, was considerably more potent as an FPGS inhibitor than peak I.

The loss of FPGS activity caused by incubation with step 2 protein could not be reversed by subsequent refractionation using ammonium sulphate (Table 5) Similarly, the initial levels of FPGS activity in step 3 protein could not be recovered following preincubation with the inhibitor and gel filtration (Table 5) A number of other experiments showed that inhibition was not due to protease or folate hydrolase activities Thus step 2 protein contained only minimal levels of protease at pH 8 5 and inhibition of FPGS was not affected by adding 0 5 mg of PMSF to the reaction (Table 6) Previous studies showed that PMSF is a strong inhibitor of alkaline protease in *Neurospora* [33] Step 2 protein also failed to hydrolyse yeast polyglutamates at pH 8 5 and addition of the inhibitor, after termination of the FPGS reaction did not alter product chain lengths (data not shown)

The assays shown in Table 7 were conducted to determine whether FPGS inhibition was associated with

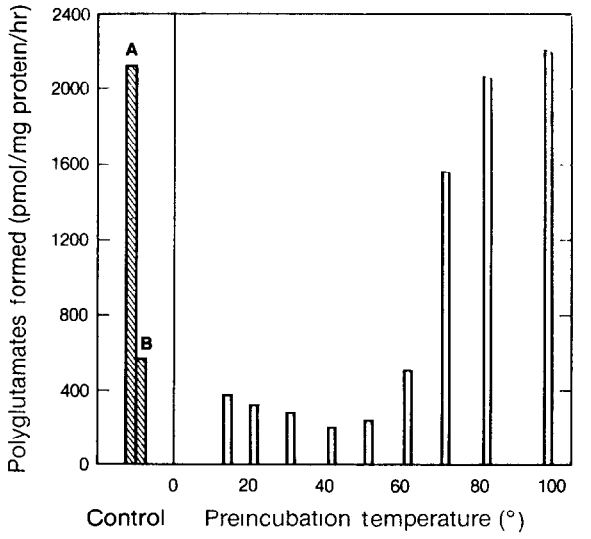

Fig 3 The effect of preincubation temperature on inhibitor potency Samples of the FPGS inhibitor (0 8 mg protein) were preincubated for 1 hr at the temperatures indicated. After cooling, the samples were mixed with FPGS (1.2 mg protein) and polyglutamate synthesis was measured after a 2 hr reaction period. Control systems (A) lacked the inhibitor or contained inhibitor that had not been preincubated (B).

the removal of ATP or glutamate. The standard reaction system, lacking folate and FPGS protein, was preincubated with the inhibitor for 1 hr and then boiled. Folate and FPGS were then added to initiate polyglutamate synthesis. It is clear from the data that comparable amounts of products were formed in the inhibitor-treated and control systems. The *Neurospora* FPGS inhibitor did not affect the activities of several other enzymes. These included serine hydroxymethyltransferase, 5,10-CH_2-H_4PteGlu dehydrogenase and isocitrate lyase of *N. crassa*, yeast alcohol dehydrogenase, beef heart lactate dehydrogenase, and wheat germ acid phosphatase. However, the FPGS activities of *E coli*, bovine liver and pea cotyledons were strongly inhibited when incubated with step 2 protein of *met*-9 extracts (Table 8)

DISCUSSION

The folate analyses summarized in Table 1 show that the *met*-9 mutant produces less folylpolyglutamate *in vivo* than the Lindegren A wild type. This difference was evident following culture in minimal or supplemented media, and crude mycelial extracts had little ability to form polyglutamates *in vitro* (Table 2) It is clear from the present and previous [31, 32] work that *met*-9 and the polyglutamate-deficient *met*-6 mutant have a requirement for methionine that cannot be replaced by homocysteine and serine However, mutation at the *met*-6 locus causes deletion of the $Glu_2 \rightarrow Glu_6$ FPGS activity [7] whereas the data in Table 2 shows that the Glu_3 and Glu_6 products were formed by *met*-9 extracts. This latter ability was enhanced by ammonium sulphate fractionation (Table 2) so mutation at the *met*-9 locus does not result in a deletion of FPGS expression. On the other hand, the presence of a protein inhibitor that appears to be specific in its action against FPGS raises the possibility that *met*-9

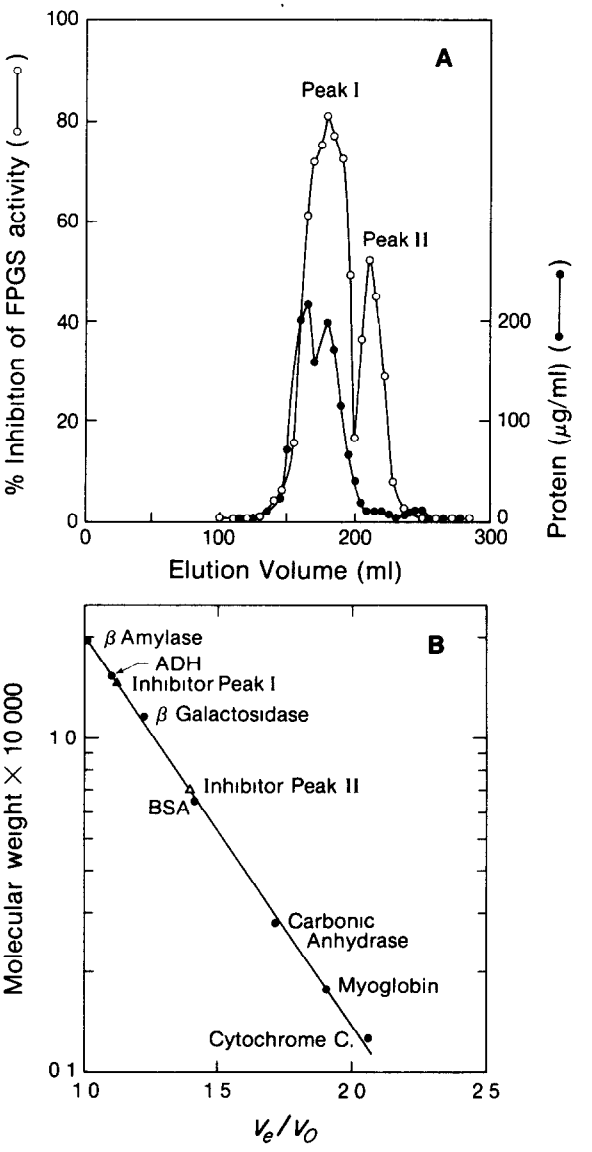

Fig 4. M_r of wild FPGS inhibitor as determined by gel filtration of step 2 protein. Samples (*ca* 350 mg protein) of the inhibitor fraction were applied to a column (80 × 2.6 cm) of Sephacryl S 200 Fractions of 6 ml were collected at a flow rate of 34 ml/hr. The inhibitor was localized by preincubating 0.2 ml aliquots of each fraction with FPGS (1.2 mg protein). Protein markers and Blue Dextran were used to determine values of V_e and V_0 respectively.

has an endogenous control of polyglutamate production that results in the observed folate deficiency.

It is not clear why the *met*-9 mutant has a partial deficiency in the folate-dependent enzymes for conversion of serine to methionine [29]. Serine hydroxymethyltransferase, the first enzyme in this pathway, was not affected by the FPGS inhibitor and Selhub [29] found no evidence for activators or inhibitors of these enzymes in *met*-9. By analogy with other species [2, 14], these reactions in *Neurospora* probably have a preference for polyglutamyl folates. As a result, control of the methionine pathway in

Table 5 Recovery of FPGS activity after incubation with inhibitor protein

Experimental conditions	Activity recovered (nmol product)	Specific enzyme activity (nmol/mg protein/hr)
1 FPGS protein, no preincubation	441 0	4 30
2 FPGS protein + inhibitor protein 1 hr preincubation	17 6	0 15
3 $(NH_4)_2SO_4$ fractionation of protein mixture in (2) Protein recovered in 45–60% range, dialyzed	32 4	2 80
4 Sephacryl S-200 chromatography of protein mixture in (2). Fractions 39–45 pooled	29 0	0 9

Samples of FPGS (*met*-6, step 3 protein, 2 4 mg) and the FPGS inhibitor (*met*-6, step 2 protein, 2 2 mg) were mixed and preincubated for 1 hr at 37° This mixture was then fractionated by addition of ammonium sulphate or applied to a column of Sephacryl S-200 In the absence of the inhibitor, the specific enzyme activity of FPGS after gel filtration was *ca* 85 nmol/mg protein/hr

Table 6 Protease activities and inhibition of FPGS in the presence of PMSF

N Crassa strain	Reaction conditions	Protease activity of step 2 protein (μmol hydrolysed/mg protein/hr) pH 6 8	pH 8 5	FPGS activity in presence of step 2 protein (μmol/mg protein/hr)	FPGS inhibition (%)
Wild type					
	Control	6 30	0 10	5 10	26 1
	+PMSF	0 70	n d	4 99	27 7
met-6 mutant					
	Control	39 00	0 20	1 50	51 7
	+PMSF	3 70	n d	1 52	51 0
mac mutant					
	Control	10 70	0 15	2 25	53 2
	+PMSF	0 75	n d	2 28	52.5
met-9 mutant					
	Control	18 50	0 15	1 0	60 0
	+PMSF	1 60	n d	0 9	64 0

Protease activity was assayed with L-leucyl-L-leucine substrate in the presence and absence of PMSF (0 5 mg/reaction system) Ability of step 2 protein to inhibit FPGS was determined without preincubation in the presence and absence of PMSF FPGS activities in the absence of step 2 protein were 6 9, 3 1, 4 8 and 2 5 nmol/mg protein/hr for the wild type, *met*-6, *mac* and *met*-9 respectively

n d · Protease activity not detected

met-9 could centre on the availability of these conjugated folates.

A number of criteria support the view that the *Neurospora* FPGS inhibitor is protein in nature. For example, the inhibitor was non-dialysable, thermolabile (Fig 3) and could be recovered by $(NH_4)_2SO_4$ precipitation and gel filtration (Table 2, Fig 4). Like other inhibitory proteins [34–38], the present factor from *Neurospora* is highly specific in its action, affecting several FPGS activities (Table 8) but having no effect on a variety of other enzymes. FPGS inhibition did not appear to involve product hydrolysis or destruction of the enzyme by alkaline protease activity The lack of inhibition of serine hydroxymethyltransferase and 5,10-methylenetetrahydrofolate dehydrogenase implies that the basis for FPGS inhibition is not removal of H_4PteGlu or 5,10-CH_2-H_4PteGlu by a folate binding mechanism Also, the finding that polyglutamate synthesis could not be restored by fractionating mixtures of FPGS and inhibitor suggests that the inhibitory process results in an irreversible inactivation of FPGS It is noteworthy that total FPGS activity was enhanced in *met*-9 extracts by salt fractionation Thus the inactivation process may be curtailed at 2° but accentuated when the enzyme and inhibitor are preincubated at physiological temperatures (Fig 3).

The activities of several plant and animal enzymes are now known to be inhibited or inactivated by specific inhibitory proteins [e.g 35–40] There is also strong evidence that many such proteins exert their effect by combining with and modifying the target enzyme [36, 40, 48, 49]. As a result of such work, data are accumulating to suggest that these proteins have physiological importance in metabolic regulation [36, 49, 50] On the basis, it seems

Table 7 FPGS inhibition is not due to the removal of ATP of L-glutamate in the wild type, *met*-6 and *met*-9

Reaction conditions	FPGS activity (nmol product formed)		
	wild type (0.85 mg prot)	*met*-6 (0.9 mg prot)	*met*-9 (1.1 mg prot.)
1. Standard reaction system, no FPGS inhibitor	16 5	5.1	2.9
2. Inhibitor + ATP Incubated 1 hr at 37° Boiled FPGS & other substrates added	13.4	3 7	2 1
3. ATP incubated 1 hr at 37° Boiled FPGS and other substrates added	16 7	5.9	3.7
4 ATP + inhibitor + FPGS incubated 1 hr at 37° Other substrates added	1 2	0.1	0.1
5. Inhibitor + L-glutamate incubated 1 hr at 37° Boiled. FPGS & other substrates added	14.5	5 0	3 1
6 L-Glutamate incubated 1 hr at 37° Boiled FPGS and other substrates added	14 8	4 8	2.8
7 L-Glutamate + inhibitor + FPGS incubated 1 hr at 37°. Other substrates added	1 0	<0 1	<0.1
8. Inhibitor + FPGS incubated 1 hr at 37° then other substrates added	1 2	n.d.	n d.

Polyglutamate synthesis was measured after a 2 hr reaction period at 37° FPGS inhibitor (step 2) protein, wild type, 0 7 mg, *met*-6, 0 8 mg and *met*-9, 0.85 mg was added as indicated.
n.d incorporation of ^3H not significantly above background

Table 8 Inhibition of *E coli*, bovine liver and pea cotyledon FPGS activities by *met*-9 protein

Source of FPGS and specific enzyme activity	*met*-9 inhibitor (mg/reaction)	Residual FPGS activity (%)
1 *E. coli* purified enzyme 3 8 nmol product/mg protein/hr	0	100
	0.12	36
	0 62	14
	1 24	9
2. Bovine liver partially purified enzyme. 11 18 nmol/mg protein/hr	0	100
	0 55	1
3 Pea cotyledon partially purified enzyme 2 1 nmol/mg protein/hr	0	100
	1.2	85
	2 0	60
	4 0	9
	5 0	0

Samples of the FPGS activities (*E. coli*, 5.0 μg protein, liver, 0.25 mg protein; pea cotyledon, 5.2 mg protein) were preincubated in the presence or absence of the *met*-9 inhibitor (step 2 protein) for 1 hr at 37°. The FPGS reaction components were then added and polyglutamate synthesis was terminated after 2 hr

likely that the FPGS inhibitor of *N crassa* could have some role in the control of folylpolyglutamate biosynthesis.

EXPERIMENTAL

Chemicals PteGlu was purchased from Sigma (St Louis) [U-^3H] Glutamate was supplied by Amersham-Searle and diluted with carrier L-glutamate to give final specific radioactivities of 2.5 μC/1.5 μmol. Tetrahydrofolate was generated from PteGlu by catalytic hydrogenation [52] and $H_4PteGlu_2$ was produced enzymically using the FPGS of *N. crassa*, *met*-6 [7]. Cellex-D (OH–form) was supplied by Bio–Rad. Samples of highly purified enzymes including immobilized trypsin and M_r marker protein were purchased from Sigma Partially purified pea cotyledon FPGS was prepared from 3-day-old tissues [24]. Samples of highly purified FPGS from *E coli* and bovine liver were donated by Dr A Bognar and Dr K G Scrimgeour (University of Toronto, Canada). All other chemicals were from either Fisher Scientific (Edmonton) or Sigma (St Louis).

Culture of Neurospora. The Lindegren A wild type (FGSC 853), *mac* mutant (FGSC 3609), *met*-6 mutant (FGSC 1330), *met*

S-2706 mutant (FGSC 4248) and the *met*-9 mutant (FGSC 552), allele C124) were cultured in defined media as described previously [7] Mycelia were grown with aeration in Vogel's liquid media at 30° [7] The cultures were harvested 22 hr after inoculation

Preparation of cell-free extracts The procedures for extraction of folate derivatives using ascorbate at pH 6 0 and for inactivation of endogenous folate hydrolase were as described previously [6] Extracts for assay of FPGS were prepared at 2° by grinding samples of mycelium in 20 mM Pi buffer (pH 7 4) containing 0 1 mM 2-mercaptoethanol [7]

Folate assay Mycelial extracts were assayed for short-chained and polyglutamyl folates using a standard microbiological method [6, 53] The assay organism was *Lactobacillus casei* (ATCC 7469) which responds quantitatively to Glu_1 and Glu_3 derivatives To measure folylpolyglutamate concentration the mycelial extracts were treated with plant carboxypeptidase [54] prior to microbiological assay of folate content

Assay of FPGS activity The standard assay system [7], depended on the incorporation of tritiated glutamate into folylpolyglutamates in the presence of either H_4PteGlu or 5,10-CH_2-H_4PteGlu or the corresponding diglutamate derivatives Control systems lacked folate substrate The reaction system, (1 ml), contained ATP (2 5 μmol), $MgCl_2$ (5 μmol), KCl (15 μmol), Tris–HCl pH 8 5 (200 μmol), L-glutamate (1 5 μmol, 2 5 μCi of ^{3}H), DL-5, 10-CH_2-H_4PteGlu$_n$ or DL-H_4PteGlu (0 1 μmol) and 1 2–2 0 mg of extract protein After purging the reaction tubes with H_2, polyglutamate synthesis was allowed to proceed for 2 hr at 37° Ascorbate (pH 6 0) was then added followed by boiling and removal of denatured protein [7] Labelled products were recovered by ion exchange cellulose by gradient [7] or batch [19] elution

Measurement of FPGS inhibition. The FPGS inhibitor was recovered from crude mycelial extracts by $(NH_4)_2SO_4$ fractionation (30–45% range of satn) After dialysis against 20 mM Pi buffer (pH 7 4) containing 0 1 mM 2-mercaptoethanol for 18 hr, this fraction was partially purified by passage through a column (80 × 2 6 cm) of Sephacryl S-200 Fractions of 6 ml were collected at a flow rate of 34 ml/hr In other studies, the 30–45% $(NH_4)_2SO_4$ fraction was used as a source of the inhibitor Inhibitor protein (*ca* 0 6–1 0 mg) was preincubated for 1 hr at 37° with 1 2–2 0 mg of FPGS protein and 200 μmol of Tris–HCl buffer (pH 8 5) in a total vol of 0 5 ml Reaction components of the FPGS assay system were then added After 2 hr the reaction was terminated by addition of ascorbate and boiling [7] The procedures for treatment of the inhibitor fraction with trypsin, urea and other experimental conditions are described in the Tables The presence of alkaline protease in the inhibitor fraction was assessed by a fluorescamine assay [33] Ability of the inhibitor fraction to hydrolyse polyglutamates was examined using yeast extract as a folate source [54]

Measurement of protein and radioactivity. Protein was determined by the Lowry method [55] or by the Bio-Rad Protein Assay ^{3}H was measured by liquid scintillation counting [56]

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