Phytochemistry, Vol. 23, No. 5, pp. 965–971, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00
Pergamon Press Ltd.

FOLYLPOLYGLUTAMATE SYNTHETASE ACTIVITIES OF *NEUROSPORA**

EDWIN A. COSSINS and PATRICK Y. CHAN

Department of Botany, University of Alberta, Edmonton, Alberta, T6G 2E9, Canada

(*Received* 11 *April* 1983)

Key Word Index—*Neurospora crassa* (FGSC 853) wild type, *met*-6 and *mac* mutants; folylpolyglutamate synthetase activity; partial purification; intracellular localization.

Abstract—The folylpolyglutamate synthetase (FPGS) activities of *Neurospora crassa*, wild type (FGSC 853) and two polyglutamate-deficient mutants, *met*-6, 35809 (FGSC 1330) and *mac*, 65108 (FGSC 3609), were examined after growth in defined media. Extracts of the wild type produced $H_4PteGlu_6$ (60%), $H_4PteGlu_3$ (35%) and $H_4PteGlu_2$ (15%). *Met*-6 extracts formed $H_4PteGlu_2$ but lacked the ability to utilize $H_4PteGlu_4$ or $H_4PteGlu_5$. The *mac* mutant failed to catalyse glutamate addition to $H_4PteGlu$ but $H_4PteGlu_2$ was an effective substrate for tri- and hexaglutamate synthesis. These polyglutamates were also formed by reaction systems containing mixtures of *met*-6 and *mac* protein or heterokaryon protein derived from mycelial fusions of *met*-6 and *mac*. Extract fractionations and heat treatments provided evidence for more than one FPGS activity in the wild type. A mitochondrial FPGS catalysed the $H_4PteGlu_2 \rightarrow H_4PteGlu_3$ reaction but a cytosolic fraction synthesized di-, tri- and hexaglutamates when incubated with $H_4PteGlu$ and glutamate. The latter system contained a temperature-sensitive diglutamate-forming activity and a relatively stable $H_4PteGlu_2 \rightarrow H_4PteGlu_6$ activity. Polyglutamate synthesis in *N. crassa* appears to involve more than one step, $H_4PteGlu \rightarrow H_4PteGlu_2$ followed by $H_4PteGlu_2 \rightarrow H_4PteGlu_6$, in addition to the mitochondrial activity. These partial activities are lacking in *mac* and *met*-6 respectively. Consequently, these mutants are unable to form the folylhexaglutamates that predominate the folate pool of the wild type.

INTRODUCTION

It is now well established [1–9] that the majority of folate-dependent enzymes have greater affinities for folylpolyglutamate derivatives than for the corresponding monoglutamates of tetrahydrofolate. Recognition of this general property has given impetus to recent studies [10] of folylpolyglutamate synthetase (FPGS), an enzyme catalysing the sequential addition of L-glutamate to $H_4PteGlu_n$ (equation 1). The synthetases of

$$H_4PteGlu_n + ATP + \text{L-glutamate} \rightarrow H_4PteGlu_{n+1} + ADP + P_i. \quad (1)$$

bacteria [11–15] and mammalian cells [16–19] have received detailed study but there is still relatively little information on the FPGS of lower eukaryotes.

Folylpolyglutamate synthesis accompanies growth in fungi, and as in other species, the folate pool contains methyl- and formyl-substituted derivatives of $H_4PteGlu_n$ [20–27]. In *Neurospora crassa*, preliminary reports from Sakami's laboratory [28–30] support the view that FPGS catalyses this synthesis but the nature of the reaction products has not been fully elucidated [26–30]. Despite this, it has been proposed [30, 31] that $H_4PteGlu_n$ biosynthesis in *N. crassa* involves two FPGS enzymes, one producing $H_4PteGlu_2$ and the other forming $H_4PteGlu_n$ from this diglutamyl substrate. Unfortunately, details of this system were not published and later work in Shane's laboratory [10] has clearly questioned the validity of this interesting proposal.

The work described in this paper was carried out as part of a continuing study of folate metabolism in *Neurospora* [24, 27]. Emphasis has been placed on an elucidation of FPGS activity in the wild type and in two mutants that show folylpolyglutamate deficiencies during growth [26, 27]. Preliminary reports of this work have been published recently [32, 33].

RESULTS

FPGS activities of dialysed, mycelial extracts

Initially, assays were conducted using extracts of the wild type and the *met*-6 mutant. In both cases, FPGS activity was found to be ATP- and $H_4PteGlu$-dependent, a finding that is in agreement with data for the *Corynebacterium* enzyme [13]. Unlike the latter bacterial enzyme, anaerobic conditions favoured glutamyl conjugation of $H_4PteGlu$ in both *N. crassa* strains. The products of FPGS activity for both strains are compared in Table 1. In the wild type, [^{3}H]-glutamate and [^{14}C]$H_4PteGlu$ were incorporated into three labelled products that were sequentially eluted from Cellex-D by increasing phosphate concentration. The $^3H:^{14}C$ ratios of these products suggested that they were $H_4PteGlu_2$, $H_4PteGlu_3$ and $H_4PteGlu_6$ respectively. This was verified by co-chromatography of each peak area with authentic folylpolyglutamate standards and by gel co-chromatography of azo dye derivatives (see Experimental). Distinct peaks, corresponding to $H_4PteGlu_4$, $H_4PteGlu_5$ or to

*The abbreviations used for derivatives of folic acid are those suggested by the IUPAC-IUB Commission listed in (1967) *Biochem. J.* **102**, 15, e.g. $H_4PteGlu$ = 5,6,7,8-tetrahydropteroylglutamate; $H_4PteGlu_n$ = poly-γ-glutamyl derivatives where n = the number of L-glutamate moieties; PteGlu = pteroylmonoglutamic acid; FPGS = folylpolyglutamate synthetase.

Table 1. Incorporation of radio-labelled glutamate and tetrahydrofolate into polyglutamates by wild type and *met*-6 extracts

Strain	Elution vol from Cellex-D (ml)	Glutamate-^{3}H incorporated (nmol)	H_4PteGlu-^{14}C incorporated (nmol)	Ratio ^{3}H:^{14}C
Wild type	43–47	0.40	0.38	1.05
	53–58	1.70	0.78	2.18
	65–74	5.40	1.06	5.10
Met-6 mutant	43–48	2.10	2.00	1.05
	53–58	n.d.	n.d.	—
	65–74	n.d.	n.d.	—

Values are derived from triplicate determinations.
n.d., Not detected.

products with polyglutamyl chain lengths of more than six were not detected even when the reaction time was extended to 6 hr. Furthermore, $H_4PteGlu_6$ remained the principal FPGS product when the concentration of H_4PteGlu was varied from 10–200 nmol/reaction system.

Reaction systems containing dialysed *met*-6 protein (Table 1) synthesized only one dual labelled product. The $^3H:^{14}C$ ratio of this folate averaged 1.05; indicative of a folyldiglutamate. This product co-chromatographed with authentic $H_4PteGlu_2$ and with the early eluting product of wild type FPGS activity. Longer reaction periods and changes in the H_4PteGlu concentration did not result in labelling of additional products in these *met*-6 assays. In this regard, the absence of tri- and hexaglutamate products could result from action of a non-dialysable inhibitor of $H_4PteGlu_2$ glutamyl conjugation or the presence of a hydrolase that cleaves products with glutamyl chain lengths of three or more. Both of these possibilities appear unlikely as mixing equal volumes of wild type and *met*-6 protein (Fig. 1) resulted in greater labelling of the tri- and hexaglutamate products. This stimulation in product formation was also observed when different ratios of wild type: *met*-6 protein were examined. From these assays it appears that *met*-6 extracts lacked the ability to add glutamate to $H_4PteGlu_2$. On the other hand, the wild type extracts appeared to contain a synthetase that preferentially utilized the diglutamate for $H_4PteGlu_3$ and $H_4PteGlu_6$ synthesis.

Dialysed extracts of the *mac* mutant were also examined for FPGS activity (Fig. 2A). With H_4PteGlu as folate substrate, [^{3}H]glutamate was not incorporated. However, when *mac* and *met*-6 extracts were mixed, substantial labelling of the di-, tri- and hexaglutamate products occurred. These latter polyglutamates were found when different ratios of *met*-6 : *mac* protein were assayed in the H_4PteGlu reaction system. In other experiments (data not shown), *met*-6 extracts were incubated with H_4PteGlu and [^{3}H]glutamate for 2 hr. The reaction was terminated by boiling, and the centrifuged supernatant used as a source of folate substrate in assays of *mac* FPGS. In these systems, large amounts of $H_4PteGlu_3$ and $H_4PteGlu_6$ were formed. Conceivably, the *mac* mutant lacks the ability to produce $H_4PteGlu_2$ but can generate higher polyglutamates when supplied with this diglutamate. This was subsequently confirmed in later assays (see below) using authentic $H_4PteGlu_2$.

Complementation of the mutant FPGS activities was also shown when extracts of the *met*-6 : *mac* heterokaryon were examined (Fig. 2B). In these studies, hyphal fusions were allowed to occur between *met*-6 and *mac* followed by growth in media lacking the usual L-methionine supplement (see Experimental). It is clear from Fig. 2B that di-, tri- and hexaglutamylfolates were formed when H_4PteGlu and [^{3}H]glutamate were incubated with these heterokaryon extracts.

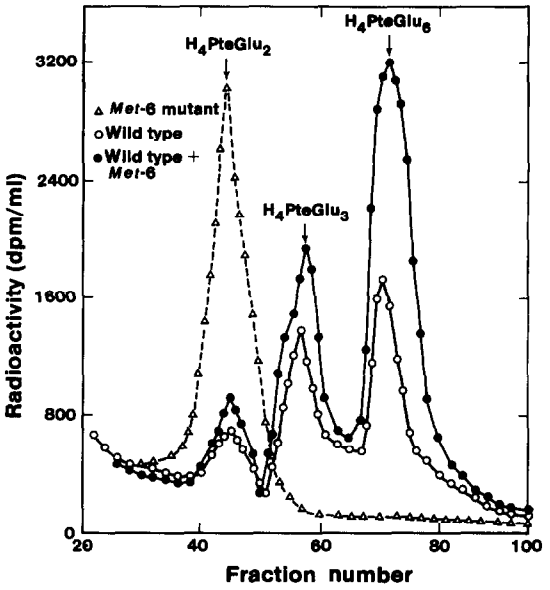

Fig. 1. Products of FPGS activity in the wild type and *met*-6 mutant. Reaction systems contained H_4PteGlu as folate substrate and [^{3}H]glutamate. ○, wild type (1.2 mg protein); △, *met*-6 mutant (0.8 mg protein); and ●, a mixture of 1.2 mg wild type protein and 0.8 mg *met*-6 protein.

Polyglutamate synthesis by $(NH_4)_2SO_4$*-fractionated extracts*

In the FPGS assays of Ritari *et al.* [29], *N. crassa* extracts were fractionated with $(NH_4)_2SO_4$. Activity was detected in protein precipitating in the 0–35% and 45–60% ranges of saturation respectively. These workers

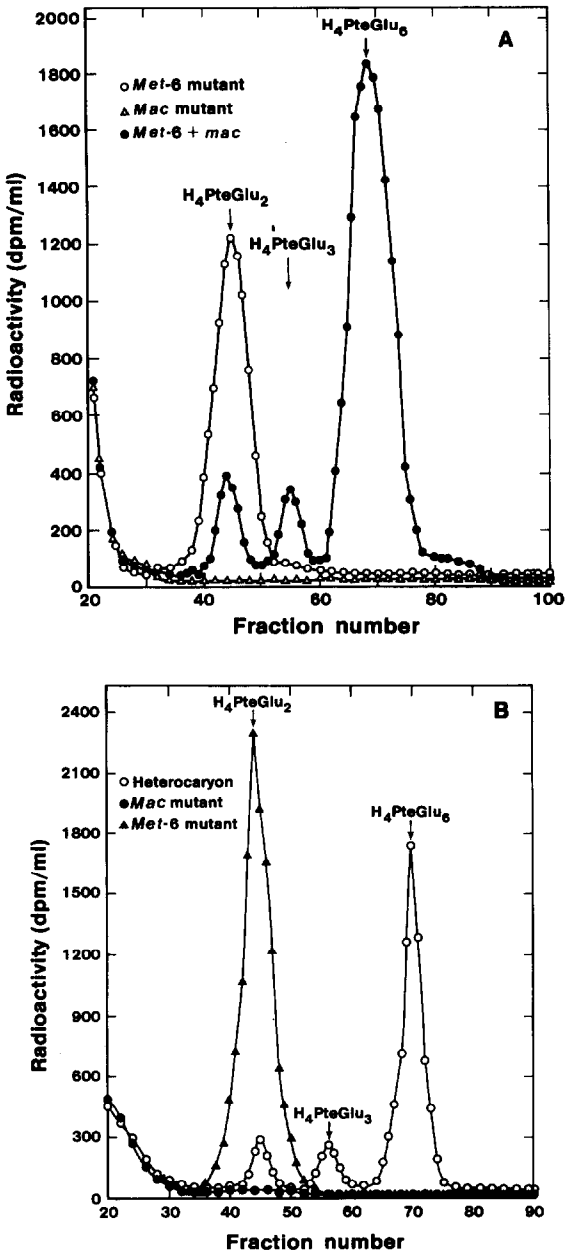

Fig. 2. Polyglutamate synthesis by mutant and heterokaryon extracts. Standard reaction systems contained $H_4PteGlu$ and [^{3}H]glutamate as in Fig. 1. A. Dialysed extracts of: ○, *met*-6 (0.72 mg protein); △, *mac* (0.6 mg protein); ●, mixture of *met*-6 (0.72 mg protein) and *mac* (0.6 mg protein). B. Protein recovered at 45–60% $(NH_4)_2SO_4$ saturation was dialysed and used in systems containing: ▲, *met*-6 protein (1 mg); ●, *mac* protein (1.5 mg); and ○, 1.6 mg protein extracted from the *met*-6:*mac* heterokaryon.

claimed that the first protein fraction catalysed glutamate additions to $H_4PteGlu_2$, $H_4PteGlu_3$ and $H_4PteGlu_4$ but that the monoglutamate was not an effective folate substrate. It was inferred that this fraction catalysed the reaction shown in equation 1. The folate reaction products were not characterized. Data for the 45–60% fraction [29] indicated FPGS activity that displayed some preference for the monoglutamate substrate (equation 2). The folate product in this case was

$$H_4PteGlu + ATP + \text{L-glutamate} \rightarrow H_4PteGlu_2 + ADP + P_i \quad (2)$$

assumed to be folyldiglutamate.

We were unable to confirm these findings when $(NH_4)_2SO_4$ fractionated extracts of the wild type (FGSC 853) were assayed with mono-, di-, tetra- and pentaglutamyl folate substrates (Table 2). The 0–35% fraction had FPGS activity that favoured $H_4PteGlu_3$ formation from the diglutamate. In contrast, the 0–35% fraction did not utilize $H_4PteGlu$ or the corresponding pentaglutamate. The 45–60% fraction (Table 2) added [^{3}H]glutamate to all four unsubstituted folate substrates. In each case, $H_4PteGlu_6$ was the principal product.

Assay of $(NH_4)_2SO_4$-fractionated extracts prepared from *met*-6 and *mac* revealed some striking differences (Table 2). The 0–35% fraction of both mutants assayed separately or combined, had no detectable FPGS activity despite use of four different folate substrates. However, the 0–35% fractions of the heterokaryon catalysed a reaction similar to the wild type (data not shown). The 45–60% fraction of *met*-6 catalysed an $H_4PteGlu \rightarrow H_4PteGlu_2$ reaction but no other products were formed with this or other folate substrates. In the case of *mac*, the 45–60% fraction converted di-, tetra- and pentaglutamyl folates to $H_4PteGlu_6$. The monoglutamate was not utilized.

FPGS activities of cytosolic and mitochondrial fractions

When mycelial extracts were prepared in isotonic media and subjected to differential centrifugation (Table 3) evidence was obtained for soluble and particulate FPGS activities. In the wild type, this latter activity was associated with mitochondria that were characterized by standard biochemical criteria [34]. The mitochondrial fraction produced $H_4PteGlu_3$ from the diglutamate but $H_4PteGlu$ was apparently not utilized. The specific enzyme activity of this fraction was only slightly lower than the 0–35% $(NH_4)_2SO_4$ fraction, prepared from the initial mycelial homogenate. These experiments showed that the cytosolic activity catalysed a different reaction, namely the conversion of $H_4PteGlu$ to a mixture of di-, tri- and hexaglutamates (Table 3). $(NH_4)_2SO_4$ fractionation of the mycelial homogenate yielded a 0–35% fraction with negligible FPGS activity. Mixing cytosolic and mitochondrial fractions resulted in FPGS activity that was similar to that of the initial homogenate.

When mutant extracts were fractionated (data not shown) mitochondria had no ability to add glutamate to $H_4PteGlu_n$ (where $n = 1, 2, 4$ and 5). However, in *met*-6 and *mac*, the cytosolic fraction had FPGS activity like that of the corresponding 45–60% $(NH_4)_2SO_4$ fractions (Table 2).

FPGS activities after heat treatment of extracts

From the above data, it follows that mutations at the *met*-6 and *mac* loci result in the expression of markedly different FPGS activities. Besides the already noted differences in reaction products (Figs 1 and 2), the mutant FPGS activities differed in their sensitivity to inactivation at 50° (Fig. 3). Preincubation of *met*-6 protein (40–65% $(NH_4)_2SO_4$ fraction) for 1 min at 50° resulted in an 80%

Table 2. Folylpolyglutamate synthetase activities after $(NH_4)_2SO_4$ fractionation

Folate substrate	Wild type						*met*-6			*mac*		
	0–35% sat.			45–60% sat.			45–60% sat.			45–60% sat.		
	diglu	triglu	hexaglu	diglu	triglu	hexaglu	diglu	triglu	hexaglu	diglu	triglu	hexaglu
$H_4PteGlu$	n.d.	n.d.	n.d.	0.10	0.04	0.35	0.70	n.d.	n.d.	n.d.	n.d.	n.d.
$H_4PteGlu_2$	—	0.68	0.09	—	0.07	0.17	—	n.d.	n.d.	—	0.14	0.14
$H_4PteGlu_4$	—	—	0.03	—	—	0.34	—	—	n.d.	—	—	0.50
$H_4PteGlu_5$	—	—	n.d.	—	—	0.38	—	—	n.d.	—	—	0.12

Data are expressed in nmol folate incorporated/mg protein/hr. The folate substrates were supplied at 100 nmol/reaction system except for $H_4PteGlu_2$, supplied at 2.5 nmol/reaction system. Enzyme activity was not detected in *met*-6 or *mac* protein precipitated between 0–35% saturation with $(NH_4)_2SO_4$.

n.d., Not detected.

Table 3. Association of folylpolyglutamate synthetase activity with soluble and particulate fractions in the wild type

Fractionation step and folate substrate	Polyglutamate products formed (pmol $H_4PteGlu$ or $H_4PteGlu_2$ incorporated/mg protein/hr)			
	diglu	triglu	hexaglu	Total
1. Crude extract				
$H_4PteGlu$	165	95	210	470
2. 0–35% $(NH_4)_2SO_4$				
$H_4PteGlu$	n.d.	n.d.	6	6
$H_4PteGlu_2$		360	n.d.	360
3. Mitochondrial pellet				
$H_4PteGlu$	n.d.	n.d.	n.d.	
$H_4PteGlu_2$		270	n.d.	270
4. Cytosolic				
$H_4PteGlu$	200	30	120	350
5. Cytosolic 0–35% $(NH_4)_2SO_4$				
$H_4PteGlu$	n.d.	n.d.	10	10
$H_4PteGlu_2$		n.d.	12	12
6. Mitochondrial + cytosolic (3 + 4)				
$H_4PteGlu$	185	120	200	505

Mycelia were homogenized in isotonic media as described in the text. Isolated fractions were dialysed against hypotonic buffer prior to assay. In step 6, equal volumes (0.2 ml) of mitochondrial (1.5 mg) and cytosolic (0.7 mg) protein, from steps 3 and 4 respectively, were mixed prior to assay.

n.d., Not detected. The folate substrates were provided as noted in Table 2.

loss of FPGS activity (Fig. 3A). Similar heat treatment of *mac* protein (Fig. 3B) gave losses of only 10%. With 2 min preincubations, the losses of FPGS activity were 90% and 30% for *met*-6 and *mac* respectively. The sensitivity of wild type FPGS (40–65% fraction) to these 50° pretreatments was more complex (Fig. 4). A marked temperature sensitivity was noted when preincubated protein was subsequently assayed at 37° with glutamate and $H_4PteGlu$ (Fig. 4A). Under these assay conditions, a 5 min preincubation at 50° resulted in complete loss of FPGS activity (data not shown). If these 50°-treated extracts were however assayed at 37° with $H_4PteGlu_2$, polyglutamate synthesis was not affected (Fig. 4B). These data suggest that the wild type, 40–65% $(NH_4)_2SO_4$ fractions, contain a temperature-sensitive FPGS activity that catalyses folyldiglutamate synthesis and a relatively stable FPGS activity that uses $H_4PteGlu_2$ for tri- and hexaglutamate formation. Figure 3B also shows that the mitochondrial FPGS of the wild type was progressively denatured when preincubated at 50°.

DISCUSSION

In earlier studies [27, 33], we characterized the native folylpolyglutamates of *Neurospora* using a variety of methods including cleavage to *p*-aminobenzoylpoly-

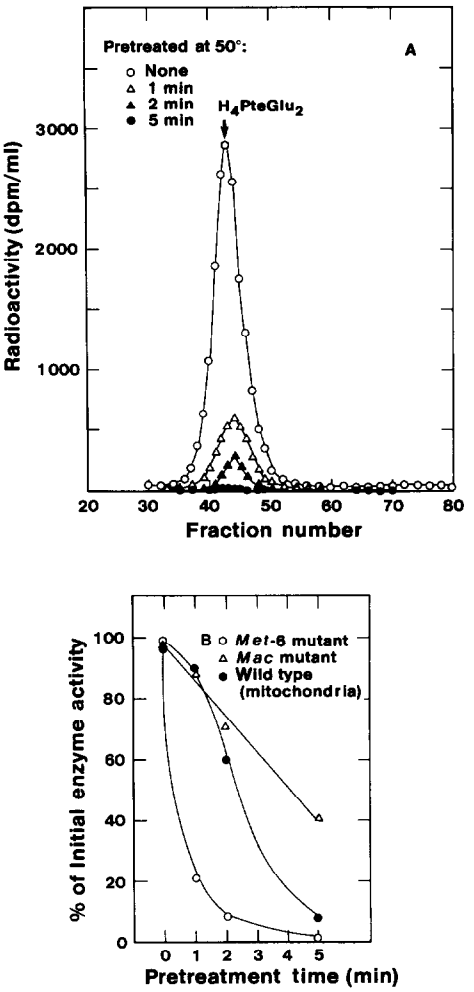

Fig. 3. Effect of 50° pretreatments on FPGS activity. A. Samples (5.6 mg protein) of *met*-6 FPGS [45–60% $(NH_4)_2SO_4$ fraction] were preincubated at 50° for 1 (△), 2 (▲), and 5 (●) mins. After cooling to 5°, FPGS activity was determined in reaction systems containing $H_4PteGlu$ and [^{3}H] glutamate. Folyldiglutamate was separated on Cellex-D. ○, Control system, lacking the 50° pretreatment. B. Effect of 50° pretreatment time on total incorporation of [^{3}H]glutamate into folylpolyglutamate. ○, 5.6 mg *met*-6 protein, $H_4PteGlu$ as folate substrate; △ 0.86 mg *mac* protein, $H_4PteGlu_2$ as folate substrate; ●, 0.80 mg wild type mitochondrial protein, $H_4PteGlu_2$ as folate substrate.

glutamates and isolation of diazo dye derivatives. From such work it is clear that hexaglutamates are the principal conjugated folates of the wild type (FGSC 853) but these derivatives are completely lacking in the *met*-6 and *mac* mutants. In these latter strains, monoglutamates account for 35% and 100% of the folate pool respectively. About 65% of the *met*-6 pool occurs as $H_4PteGlu_2$ derivatives. The present work with crude, dialysed extracts (Table 1) shows that the principal native γ-glutamyl chain length can be generated *in vitro* from $H_4PteGlu$ and glutamate. In contrast to work on bacterial and mammalian FPGS [14, 19] the activity in *Neurospora* was altered when mycelial extracts were fractionated (Tables 2 and 3). These treatments affected the polyglutamyl chain length of the major product and the relative effectiveness of $H_4PteGlu$ in the reaction catalysed. It is clear that studies of folate specificity will have to be carried out before these FPGS activities can be fully elucidated. Despite this, the present data shows that the wild type possesses cytosolic and mitochondrial enzymes (Table 3) and that the activities of *met*-6 and *mac* are complementary.

The mitochondrial FPGS of the wild type (Table 3) was also associated with protein that precipitated in the 0–35% range of saturation with $(NH_4)_2SO_4$. Ritari *et al.* [30] reported that this fraction could catalyse synthesis of higher polyglutamates but we were unable to confirm this finding using various folate substrates (Table 2). Considering the mitochondrial localization of this triglutamate-forming enzyme and the $H_4PteGlu \rightarrow H_4PteGlu_6$ reaction catalysed by the cytosolic FPGS (Table 3) it seems unlikely that these separate fractions represent mandatory parts of a hexaglutamate generating system. Conceivably, the mitochondrial FPGS has physiological importance in the generation of a spatially distinct pool of folylpolyglutamates. In this regard, it is well documented that mitochondria contain conjugated folates as well as several folate-dependent enzymes that have key roles in C_1 metabolism [35, 36]. The apparent absence of mitochondrial FPGS activity in *met*-6 and *mac* mutants requires elucidation as this deficiency can not solely account for the lack of folylpolyglutamates in these strains. It seems more likely that the mitochondrial folate pool has significance in methionine biosynthesis which in this species is preferentially mediated by polyglutamates of $5CH_3H_4$ PteGlu [26, 37].

In the wild type, the cytosolic FPGS activity formed di-, tri- and hexaglutamates in a reaction that could utilize several different unsubstituted tetrahydrofolates (Table 2). This activity was recovered in protein precipitating in the 45–60% range of saturation with $(NH_4)_2SO_4$. Data for *met*-6 and *mac* (Table 2) show that this activity was altered by mutation. The observed changes could result from either modification of a single, synthetase enzyme or from lesions affecting production of more than one type of FPGS molecule. The first possibility has precedent from the work of Taylor and Hanna [18] where mutations in Chinese hamster ovary cell lines altered the properties of FPGS. Furthermore, folylpolyglutamate synthesis in mammalian and bacterial cells appears to be catalysed by a single FPGS [10]. The second possibility regarding lesions affecting enzyme production is supported by genetic evidence in *Neurospora*. Murray [38] analysed the *met*-6 and *mac* loci, concluding that they were allelic and in close proximity. She also analyzed methionine prototrophs derived from *met*-6 × *mac* crosses and obtained evidence for complementation. Support for this second possibility was also obtained in the present work. For example, the *met*-6 and *mac* FPGS activities were complementary so that mixing extracts of both strains resulted in products that were typical of the wild type (equation 3). Mycelial extracts

$$H_4PteGlu + Glu \xrightarrow[\text{locus}]{mac} H_4PteGlu_2 + 4Glu \xrightarrow[\text{locus}]{met\text{-}6} H_4PteGlu_6 \quad (3)$$

of the *met*-6:*mac* heterokaryon (Fig. 2B) also had the ability to generate folylhexaglutamate from $H_4PteGlu$ and glutamate. The effects of 50° pretreatments on wild type FPGS activity (Fig. 4) can also be interpreted in terms of two partial activities of differing temperature sen-

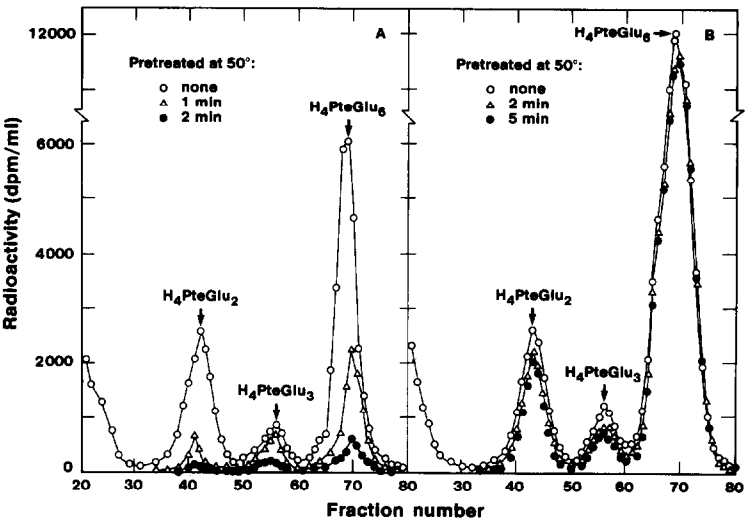

Fig. 4. Effect of 50° pretreatments on cytosolic FPGS activity of the wild type. Extracts were $(NH_4)_2SO_4$-fractionated (45–60% range of saturation) and dialysed. A. Reaction systems contained 5.8 mg protein, pretreated at 50° for 1 (△) and 2 (●) min followed by assay at 37° with H_4PteGlu as folate substrate. B. Samples (5.8 mg protein) were preincubated at 50° for 2 (△) and 5 (●) min. FPGS assay was carried out with $H_4PteGlu_2$ as folate substrate. Data are elution sequences of ^{3}H-labelled polyglutamates. ○, Product formation in systems not receiving the 50° pretreatment and with H_4PteGlu (A) or $H_4PteGlu_2$ (B) as folate substrate.

sitivities. We therefore conclude that folylpolyglutamate synthesis in *N. crassa* is not mediated by a single FPGS enzyme, but by at least two soluble enzymes which are products of the *mac* and *met*-6 loci respectively. Thus mutation at the *mac* locus results in a folate pool that contains no polyglutamyl folates. However, addition of $H_4PteGlu_2$ to extracts of the *mac* mutant will result in hexaglutamate (Fig. 2A) as the FPGS coded for by the *met*-6 locus, will still be synthesized. Mutation at the *met*-6 locus will result in a diglutamyl folate pool. As methyl group biogenesis in *Neurospora* involves a polyglutamate-requiring 5,10-methylene tetrahydrofolate reductase [26] mutations at both loci will result in methionine auxotrophy.

We are currently examining the cytosolic FPGS fraction using chromatographic methods to determine whether these partial activities can be resolved into different protein fractions.

EXPERIMENTAL

Chemicals. PteGlu and H_4PteGlu were obtained from Sigma, St. Louis, Mo. [U-^{3}H]glutamate and [2-^{14}C]PteGlu were supplied by Amersham-Searle and diluted with carrier to give final specific radioactivities of 25 μCi/μmol. Samples of $PteGlu_4$ and $PteGlu_5$ were kindly provided by Dr. Barry Shane, Department of Biochemistry, Johns Hopkins University. Tetrahydrofolate derivatives of these folates were generated by catalytic hydrogenation [39]. $H_4PteGlu_2$ was synthesized biologically using a partially purified FPGS of the *N. crassa*, *met*-6, 35809 (FGSC 1330) mutant (see below). Cellex-D (OH^+ form) was purchased from Bio-Rad Laboratories. All other chemicals were obtained from Sigma or from Fisher Scientific.

Culture of N. crassa. The Lindegren A wild type (FGSC 853), a methionine-requiring mutant (*met*-6, 35809, FGSC 1330) and a methionine-adenine mutant (*mac*, 65108, FGSC 3609) were maintained and cultured in defined media [24]. Both mutants received 0.2 mM L-methionine supplements. Cultures of *mac* also received 0.2 mM adenine. Mycelia were generated in liquid culture at 30° with samples being removed for enzyme assays at 22 hr when growth was exponential [24]. *Met*-6: *mac* heterokaryon cultures [40] were established by mixing a dense suspension of mutant conidiospores in Vogel's minimal medium [24]. After 48 hr culture at 30°, the resulting mycelium was washed to remove ungerminated spores and examined for FPGS activity.

Preparation of cell-free extracts. Procedures were conducted at 2°. 3–4 g fr. wt of mycelium were ground in 25 ml of 20 mM Pi buffer (pH 7.4) containing 0.1 M 2-mercaptoethanol. After centrifugation (25000 *g* for 20 min) the supernatant was dialysed against the extraction buffer for 12 hr. In other studies, the supernatant was fractionated by addition of solid $(NH_4)_2SO_4$. Protein precipitating at 0–35% and 45–60% of saturation was redissolved in 3–4 ml of the extraction buffer and then dialysed as above. Mitochondrial and cytosolic fractions were prepared in sucrose-buffered media [41] and characterized by examination of marker enzymes [34].

FPGS assay. Optimal activities with respect to pH and substrate concentrations were determined, based on the incorporation of [^{3}H]glutamate into $H_4PteGlu_n$ using DL-H_4PteGlu as folate substrate. The reaction system (final vol. 0.6 ml) contained: ATP (2.5 μmol), $MgCl_2$ (5 μmol), KCl (15 μmol), Tris–HCl, pH 8.5 (100 μmol), L-glutamate (1.5 μmol, 2.5 μCi of ^{3}H), DL-H_4PteGlu (0.1 μmol) and 1.2–1.5 mg of extract protein. The tubes were H_2-purged and incubated for 2 hr at 37°. The reaction was terminated by adding 0.1 ml of 6% ascorbic acid and heating at 95° for 5 min [17]. In assays involving ^{14}C and ^{3}H-labelled substrates, [2-^{14}C]H_4PteGlu (0.1 μmol, 2.5 μCi of ^{14}C) and [^{3}H]glutamate (0.1 μmol, 2.5 μCi of ^{3}H) were added to the standard system. [^{3}H]$H_4PteGlu_2$ was generated by incubating *met*-6 protein [45–60% $(NH_4)_2SO_4$ fraction] with components of the standard reaction system. The diglutamyl product, formed in five replicate incubations, was pooled and isolated by gradient

elution from Cellex-D (see below). The data presented in the tables are average values, derived from at least three separate determinations.

Separation and characterization of $H_4PteGlu_n$ *derivatives.* The chromatographic method was essentially that of Taylor and Hanna [17] involving elution of $H_4PteGlu_n$ derivatives from Bio-Rad Cellex-D by a linear gradient of K-Pi (pH 7). Glutamate was eluted prior to fraction 15; $H_4PteGlu$ was recovered in fractions 25–35. The phosphate gradient was accentuated after collection of fraction 60 to accelerate collection of $H_4PteGlu_6$. Folate polyglutamate products were identified by reference to their $^{14}C:^{3}H$ ratios and by co-chromatography with authentic $H_4PteGlu_n$ standards, the latter being detected by their absorbance at 296 nm. Further characterization of polyglutamate chain lengths involved reductive cleavage to *p*-aminobenzoylpolyglutamates, conversion to azo dye derivatives and chromatography on Bio-Gel P4 [42]. In these separations, additional standards (Glu = 6 and 7), were employed [27]. When the principal product of wild type FPGS activity was examined in this way, 95% of the radioactivity was recovered with an azo dye derivative that eluted in the position of authentic $AzoGlu_6$.

Measurements of protein and radioactivity. Protein was determined by the method of Lowry *et al.* [43] after dialysis to remove mercaptoethanol. ^{3}H and ^{14}C were determined by liquid scintillation counting [27].

Acknowledgements—This work was supported by a grant-in-aid of research awarded to one of us (E.A.C.) by the Natural Sciences and Engineering Research Council of Canada. The authors thank Dr. Barry Shane for providing samples of $PteGlu_4$ and $PteGlu_5$ that were used as precursors of $H_4PteGlu_4$ and $H_4PteGlu_5$ respectively.

REFERENCES

1. Curthoys, N. P. and Rabinowitz, J. S. (1972) *J. Biol. Chem.* **247**, 1965.
2. Coward, J. K., Parameswaran, K. N., Cashmore, A. R. and Bertino, J. R. (1974) *Biochemistry* **13**, 3899.
3. Kisliuk, R. L., Gaumont, Y. and Baugh, C. M. (1974) *J. Biol. Chem.* **249**, 4100.
4. Cheng, F. W., Shane, B. and Stokstad, E. L. R. (1975) *Can. J. Biochem.* **53**, 1020.
5. Baggott, J. E. and Krumdieck, C. L. (1979) *Biochemistry* **18**, 1036.
6. MacKenzie, R. E. and Baugh, C. M. (1980) *Biochim. Biophys. Acta* **611**, 187.
7. Matthews, R. G. and Baugh, C. M. (1980) *Biochemistry* **19**, 2040.
8. Kisliuk, R. L. Gaumont, Y., Lafer, E., Baugh, C. M. and Montgomery, J. A. (1981) *Biochemistry* **20**, 929.
9. Kisliuk, R. L. (1981) *Mol. Cell. Biochem.* **39**, 331.
10. Cichowicz, D. J., Foo, S. K. and Shane, B. (1981) *Mol. Cell. Biochem.* **39**, 209.
11. Masurekar, M. and Brown, G. M. (1975) *Biochemistry* **14**, 2424.
12. Shane, B., Brody, T. and Stokstad, E. L. R. (1979) in *Chemistry and Biology of Pteridines* (Kisliuk, R. L. and Brown, G. M., eds) p. 341. Elsevier/North-Holland, New York.
13. Shane, B. (1980) *J. Biol. Chem.* **255**, 5655.
14. Shane, B. (1980) *J. Biol. Chem.* **255**, 5663.
15. Shane, B., Bognar, A. L., Goldfarb, R. D. and LeBowitz, J. H. (1983) *J. Bacteriol.* **153**, 316.
16. Gawthorne, J. M. and Smith, R. M. (1973) *Biochem. J.* **136**, 295.
17. Taylor, R. T. and Hanna, M. L. (1977) *Arch. Biochem. Biophys.* **181**, 331.
18. Taylor, R. T. and Hanna, M. L. (1979) *Arch. Biochem. Biophys.* **197**, 36.
19. McGuire, J. J., Kitamoto, Y., Hsieh, P., Coward, J. K. and Bertino, J. R. (1979) in *Chemistry and Biology of Pteridines* (Kisliuk, R. L. and Brown, G. M., eds) p. 471. Elsevier/North-Holland, New York.
20. Schetel, M. F., Boehue, J. W. and Libby, D. A. (1965) *J. Biol. Chem.* **240**, 3154.
21. Lor, K. L. and Cossins, E. A. (1972) *Biochem. J.* **130**, 77.
22. Baugh, C. M., Braverman, E. and Nair, M. G. (1975) in *Chemistry and Biology of Pteridines* (Pfleiderer, W., ed.) p. 465. Walter de Gruyter, Berlin.
23. Bassett, J. R., Weir, D. G. and Scott, J. M. (1976) *J. Gen. Microbiol.* **93**, 169.
24. Cossins, E. A., Chan, P. Y. and Combepine, G. (1976) *Biochem. J.* **160**, 305.
25. Cossins, E. A. and Chan, P. Y. (1980) *Plant Biochem. J.*, S. M. Sircar Memorial Vol. 53.
26. Selhub, J. (1970) Ph.D. thesis, Case Western Reserve University, University Microfilms International, Ann Arbor, Michigan.
27. Chan, P. Y. and Cossins, E. A. (1980) *Arch. Biochem. Biophys.* **200**, 346.
28. Sakami, W., Ritari, S. J., Black, C. W. and Rzepka, J. (1973) *Fed. Proc.* **32**, 471.
29. Ritari, S. J., Sakami, W., Black, C. W. and Rzepka, J. (1975) *Analyt. Biochem.* **63**, 118.
30. Ritari, S. J., Sakami, W., Black, C. W. and Rzepka, J. (1973) *Neurospora Newsletter* **20**, 26.
31. Ritari, S. J., Sakami, W. and Black, C. W. (1973) *Neurospora Newsletter* **20**, 27.
32. Cossins, E. A. and Chan, P. Y. (1983) in *Folyl and Antifolylpolyglutamates* (Goldman, D., Chabner, B. and Bertino, J. R., eds) p. 183. Plenum Press, New York.
33. Cossins, E. A. and Chan, P. Y. (1983) in *Chemistry and Biology of Pteridines* (Blair, J. A., ed.). Walter de Gruyter, Berlin (in press).
34. Cossins, E. A., Pang, S. H. Y. and Chan, P. Y. (1980) *Plant Cell Physiol.* **21**, 719.
35. Cossins, E. A. (1983) in *Chemistry and Biochemistry of Folates* (Blakley, R. L. and Benkovic, S., eds) Vol. 1B, p. 251. John Wiley, New York.
36. Cossins, E. A. (1980) in *The Biochemistry of Plants* (Davies, D. D., ed.) Vol. 2, p. 365. Academic Press, New York.
37. Burton, E. G., Selhub, J. and Sakami, W. (1969) *Biochem. J.* **111**, 793.
38. Murray, N. E. (1969) *Genetics* **61**, 67.
39. Reid, V. E. and Friedkin, M. (1973) *Mol. Pharmacol.* **9**, 74.
40. Fincham, J. R. S. (1966) *Genetic Complementation*, W. A. Benjamin, New York.
41. Kuntzel, H. and Noll, H. (1967) *Nature* **215**, 1340.
42. Brody, T., Shane, B. and Stokstad, E. L. R. (1979) *Analyt. Biochem.* **92**, 501.
43. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.