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Synchronization of Converging Metabolic Pathways: Activation of the Cystathionine γ-Synthase of *Neurospora crassa* by Methyltetrahydrofolate

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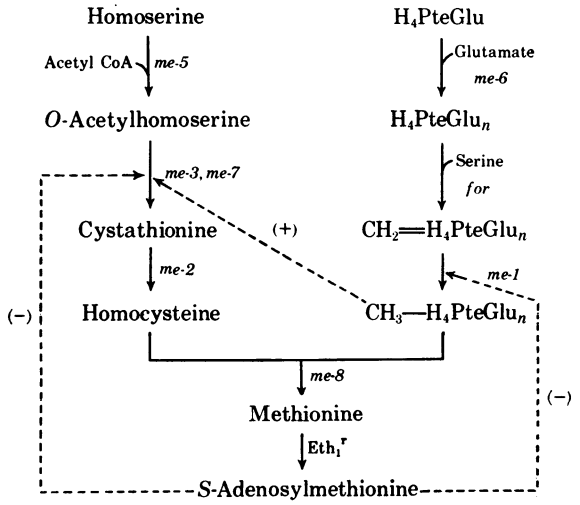
ABSTRACT **Methyltetrahydrofolate synchronizes the activities of the two branches of the pathway of methionine biosynthesis in *Neurospora crassa* by serving as an essential activator of cystathionine γ-synthase and antagonizing the feedback inhibition of this enzyme by S-adenosylmethionine. Activation is specific for the methylated form of folate and increases with increasing glutamate content. The inability of extracts of *me-1* and *me-6* mutants to form cystathionine that has been previously reported is due to the absence of N^5-methyltetrahydrofolate from these preparations. Extracts of *me-1* mutants lack methyltetrahydrofolate because the organisms are deficient in methylenetetrahydrofolate reductase, and those of *me-6* because their methyltetrahydrofolate is quantitatively removed by the procedure employed in the preparation of extracts. The folate of the *me-6* organisms differs from that of wild type strains in consisting largely of the monoglutamate rather than higher conjugates.**

The pathway of methionine biosynthesis in *Neurospora crassa*, shown in Scheme 1, consists of two converging branches. In one, CH_3—THF is formed, and in the other, homocysteine. The two branches are joined with the methylation of homocysteine by CH_3—THF to produce methionine. Studies of genes specifying enzymes of the pathway (Scheme I) have produced intriguing results. Mutants of *me-1* and *me-6*, which were found to be deficient in CH_2=THF reductase and folyl polyglutamate respectively (1, 2), were later found to be lacking also in the activity of an enzyme coded by the *me-3* and *me-7* genes, cystathionine γ-synthase[3], an enzyme that catalyzes the replacement of the acetyl group of *O*-acetyl homoserine by cysteine. The present communication describes a study of the relationship between the *me-1* and *me-6* genes and cystathionine formation. One possible explanation of the failure of extracts of *me-1* mutants to synthesize cystathionine was that the type of folate that is missing from these strains, CH_3—THF, is required by the enzyme catalyzing this process. This explanation was suggested by the observations that *a*) cystathionine γ-synthase activity was reconstituted by pairwise mixing of sephadex-filtered extracts of *me-1*, *me-3*, *me-6*, and *me-7* mutants in all combinations *except me-1* + *me-6* (3), and *b*) trypsin inactivated the components needed to reactivate *me-3* and *me-7*, but not *me-1*. In the case of the *me-6* mutants, it was necessary to assume that their CH_3—THF was removed during the preparation of the extracts (crude extracts were filtered through Sephadex G-25 to remove *S*-adenosylmethionine, a feedback inhibitor of cystathionine γ-synthase). The folate of the *me-6* organisms differs from that of other strains, in that it consists largely of the monoglutamate rather than higher (5 or more glutamic acid residues) polyglutamates (2). The results of this investigation demonstrate that the cystathionine γ-synthase of *N. crassa* has an absolute requirement for CH_3—THF that is satisfied by either the monoglutamate or polyglutamate forms.

Abbreviations: THF, tetrahydrofolate (number of glutamic acid residues unspecified); $H_4PteGlu_n$, tetrahydrofolate (number of glutamic acid residues specified by the subscript); CH_3—, CH_2=, and 5-CHO, the N^5-methyl, $N^{5,10}$-methylene, and N^5-formyl derivatives of the above compounds, respectively. The designations *dl* and *l* refer to the configuration of the tetrahydropteridine ring at *C*-6. The naturally-occurring folates are *l*-forms.



Scheme 1. Pathway of methionine biosynthesis in *N. crassa*. The genes are juxtaposed with the steps in which the respective mutants are defective. Activation and inhibition are indicated by (+) and (−), respectively.

MATERIALS AND METHODS

With the exception of *l*-CH_3—$H_4PteGlu_7$, the folates used in this study were synthesized by procedures that have been described (4, 5) and were purified by anaerobic chromatography using TEAE-cellulose (Cellex-T, Bio-Rad Laboratories) (6, 7). Yeast *l*-CH_3—$H_4PteGlu_7$ (70–80% pure) was a very generous

gift of Drs. Bernard T. Kaufman and Howard Bakerman. The sources of other materials have been reported previously (3).

Folates were determined spectrophotometrically using the extinction coefficients: THF, 22 × 10^6 M^{-1} cm^{-1} at 297 nm, pH 7.0 (8); CH_3-THF, 32 × 10^6 M^{-1} cm^{-1} at 290 nm, pH 7.0 (6); and 5-CHO-THF, 32.6 × 10^6 M^{-1} cm^{-1} at 282 nm, pH 13 (9). Protein was determined by the procedure of Layne (10).

N. crassa 74-OR8-1a (wild type) and 74-OR-23-1a (wild type) were provided by Dr. Robert L. Metzenberg and Dr. David Perkins, respectively. The 38706a (*me-1*) and 35809A (*me-6*) mutants were obtained from the Fungal Genetic Stock Center, Dartmouth College.

Procedures for the growth of *N. crassa* cultures have been described (3). Methods employed in the preparation of enzyme extracts and the assay of cystathionine γ-synthase, which are summarized in the tables, are described in detail in the publication of Kerr and Flavin (3).

RESULTS

In the preparation of extracts of *Neurospora* for the assay of cystathionine γ-synthase, the enzyme inhibitor *S*-adenosylmethionine has been removed by filtration through Sephadex G-25. As shown in Fig. 1, this process also removes some folates, including CH_3—H_4PteGlu, the principal methylated folate of *me-6*. The triglutamate, CH_3—H_4PteGlu$_3$, is partially retained indicating that a substantial part of the hexaglutamate and other higher conjugate forms of CH_3—THF that characterize *N. crassa* (2) are eluted with the protein fraction.

When extracts of *me-1* and *me-6* possessing negligible cystathionine γ-synthase activity were supplemented with CH_3—H_4PteGlu$_7$ they synthesized cystathionine at rates similar to that of an extract of the wild type organism (Table 1). This result clearly demonstrates that CH_3—THF is an essential activator of the cystathionine γ-synthase of *Neurospora* and that the inability of extracts of *me-1* and *me-6* to form cystathionine is due to their lack of this folate. The ability of fresh extracts of the wild type organism to form cystathionine without the addition of CH_3—THF (3) is accounted for by the presence of endogenous folate in this preparation. From what is known concerning the properties of methylated folates (11), it would be expected that the exposure of extracts to air for any considerable time would result in the destruction of these forms. Therefore, the finding that a preparation of wild type extract had lost most of its activity on standing in the freezer for 15 days with occasional thawing and freezing, and had regained activity on the addition of CH_3—H_4PteGlu$_7$ (Table 1) was not unexpected.

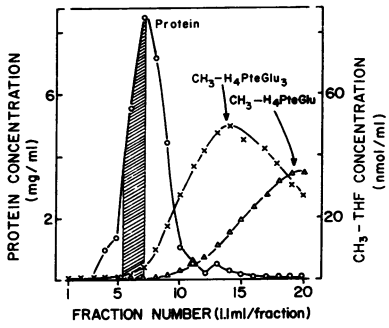


FIG. 1. Chromatography of mixtures of [^{14}C]CH_3—H_4PteGlu and [^{14}C]CH_3—H_4PteGlu$_3$ with a crude extract of *N. crassa* 38706a *me-1* on Sephadex G-25. *Neurospora* mycelia were sonicated in two volumes of a solution consisting of potassium phosphate, (pH 7.3) 50 mM; pyridoxal phosphate, 0.05 mM; EDTA, 10 mM, and dithiothreitol, 10 mM. The homogenate was centrifuged for 5 min at 15,000 × *g*, and a portion of the supernatant solution (2 ml) was mixed with 0.55 μmol [^{14}C]CH_3-H_4-PteGlu in 0.038 ml (880,000 cpm/μmol) or 0.5 μmol [^{14}C]CH_3-H_4-PteGlu$_3$ in 0.05 ml (1,730,000 cpm/μmol) and passed through a column of Sephadex G-25 coarse (15-ml bed volume, equilibrated with the solution used in the sonication as in the preparation of extracts for the determination of cystathionine γ-synthase). Fractions were analyzed for protein and radioactivity. Since the two protein curves were similar, only the one obtained with the triglutamate is shown. The portion of the protein used in the assay of cystathionine γ-synthase is indicated by the shaded portion of the curve.

TABLE 1. *Effect of* l-CH_3—H_4PteGlu$_7$ *on the cystathionine γ-synthase activities of* Neurospora *extracts*

N. crassa extract	Cystathionine γ-synthase activity (nmol min^{-1} mg^{-1}) Without CH_3—THF	With CH_3—THF	Increase in activity factor
1 Wild type (old)*	0.84	3.08	3.7
2 " (fresh)	7.97	10.40	1.3
3 *me-1*	0.37	7.14	19.3
4 *me-6*	0.05	5.79	116

* Stored frozen for 15 days with occasional thawing for sampling. Original specific activity 5 nmol min^{-1} mg^{-1}.

The incubation mixture contained in 1.0 ml: protein, as noted below; *O*-acetyl-L-homoserine, 4 μmol; [^{34}S]L-cysteine-HCl, 3.1 μmol (2 μCi); potassium phosphate buffer (pH 7.3), 125 μmol; pyridoxal phosphate, 0.23 μmol; dithiothreitol, 15 μmol; EDTA, 5 μmol; and *l*-CH_3—H_4PteGlu$_7$, when added, 0.015 μmol. The amounts of protein in the incubations were: 74-OR8-1a, wild type (old), 4.4 mg; 74-OR-23-1a wild type (fresh), 2.1 mg; 38706a, *me-1*, 1.3 mg; and 35809A, *me-6*, 3.9 mg. Incubation was for 30 min at 30°C under nitrogen. The reaction was terminated with $HClO_4$, and the [^{35}S]cystathionine that was formed was separated from other ^{35}S-containing amino acids by high voltage electrophoresis on paper. The [^{35}S]cystathionine spots were located by radioautography and counted with a liquid scintillation counter. Under these conditions, no methionine and negligible homocysteine was formed. A detailed description of the experimental procedure has been reported earlier.[3]

Addition of CH_3—THF to extracts of *me-3* or *me-7* had no effect; a mixture of the 2 extracts responded in the same way as wild-type extract.

Some of the characteristics of the effect of CH_3—THF have been established. The extent of the activation was found to be determined by the type as well as the concentration of CH_3—THF (Fig. 2). At equivalent concentrations the monoglutamate is considerably less active than the polyglutamate forms.

The effect of THF appears to be specific for the methyl form, since neither H_4PteGlu$_3$ nor 5-CHO-H_4PteGlu$_3$ activate the enzyme (Table 2).

In addition to activating cystathionine γ-synthase, CH_3—THF also antagonizes feedback inhibition of the enzyme by *S*-adenosylmethionine (3, 12). As shown in Table 3, when the concentration of CH_3—H_4PteGlu$_2$ is increased from 1 to 15 μM, the inhibition produced by a 30 μM concentration of *S*-

TABLE 2. *Effect of various folates on cystathionine γ-synthase activity*

Folate	Cystathione γ-synthase activity (nmol min^{-1} mg^{-1})
dl-CH_3—$H_4PteGlu_3$	2.4
dl-5-CHO—$H_4PteGlu_5$	0
dl-$H_4PteGlu_3$	0

The experimental procedure was the same as that described in Table 1 except that the protein, 2 mg, was supplied by an extract of *N. crassa* 35809A (*me-6*) and the folate, 30 μM (*dl*), was as specified above.

adenosylmethionine is decreased from 57 to 15%. The antagonizing effect of CH_3—THF increases with its glutamate content: inhibition of the enzyme by 30 μM *S*-adenosylmethionine was 57% with 1 μM CH_3—$H_4PteGlu_2$ but only 15% when the folate was CH_3—$H_4PteGlu_7$ in the same concentration. More precise kinetic studies are precluded at present by the limitations of the assay procedure, and by the fact that initial lags are observed in the onset both of the inhibition caused by *S*-adenosylmethionine (3), and the activation by CH_3—THF.

DISCUSSION

Previous studies from these laboratories have demonstrated that the formation of homocysteine (3, 12) and CH_3—THF (Selhub, Burton, and Sakami, unpublished data) in *N. crassa* is regulated by the inhibition of cystathionine γ-synthase and CH_2=THF reductase by *S*-adenosylmethionine (Scheme 1). In the absence of other controls, this feedback mechanism would modify the rates of CH_3—THF and homocysteine formation to meet changing requirements for *S*-adenosylmethionine, but would not assure the synthesis of these intermediates in equal amounts. The production of CH_3—THF in excess of that of homocysteine would be highly undesirable since the accumulation of THF in methylated form would reduce the amount that is available for purine nucleotide and thymidylate formation. The results of the present investigation indicate that overproduction of CH_3—THF and of homocysteine does not occur in *Neurospora*. The endowment of cystathionine γ-synthase with an absolute requirement for CH_3—THF, established by this study, allows methylated folate to control the synthesis of the methyl acceptor. Under these conditions an increase or decrease in the concentration of CH_3—THF directs a corresponding change in the rate of cystathionine, and therefore of homocysteine, biosynthesis.

TABLE 3. *Antagonizing effect of CH_3—THF on the inhibition of cystathionine γ-synthase by S-adenosylmethionine*

Folate added	*l*-Folate concentration (μM)	Cystathione γ-synthase activity (nmoles min^{-1} mg^{-1}) Without *S*-adenosylmethionine	With *S*-adenosylmethionine	Inhibition by *S*-adenosylmethionine (%)
CH_3—$H_4PteGlu_2$	15	8.70	7.41	15
	1	3.71	1.59	57
CH_3—$H_4PteGlu_7$	15	8.30	7.25	12.5
	1	6.99	5.96	15

The experimental procedure was the same as that of Table 1 except that the protein was provided by an extract of *N. crassa* 74-OR8-1a, wild type, that had been treated to remove folate, i.e., the crude extract had been passed through a column of Dowex-1X8, chloride form (100–200 mesh 1/3 bed volume of resin per volume of protein solution[13]) before Sephadex filtration. Also, a mixture of extract, 0.5 ml (3.45 mg protein); pyridoxal phosphate, 0.2 μmol; folate, as shown in the table, and potassium phosphate, (pH 7.3) 100 μmol; with or without *S*-adenosylmethionine, 0.03 μmol, in a total volume of 0.93 ml, were incubated for 10 min at 30°C before the addition of substrates. The substrates were present in a mixture, 0.07 ml, containing in addition, 10 μmol dithiothreitol.

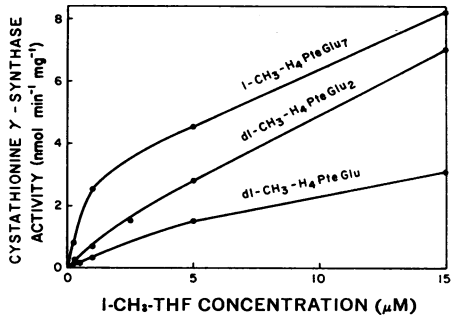


FIG. 2. Dependence of the activity of cystathionine γ-synthase on the concentration and form of the CH_3-THF. The experimental procedure was the same as that described in Table 1 except that the protein, provided by *N. crassa* 38706a, *me-1*, was 2 mg.

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