Synchronization of Converging Metabolic Pathways: Activation of the Cystathionine \( \gamma \)-Synthase of *Neurospora crassa* by Methyltetrahydrofolate

J. SELHUB*, M. A. SAVIN†, W. SAKAMI*, AND M. FLAVIN†

* Department of Biochemistry, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106 and †Laboratory of Biochemistry, Section on Enzymes, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

Communicated by Harland G. Wood, November 12, 1970

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 \( \gamma \)-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\(-\)-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 by 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\(_3\)-THF reductase and formyl polyglutamate respectively (1, 2), were later found to be lacking also in the activity of an enzyme coded by the me-5 and me-7 genes, cystathionine \( \gamma \)-synthase\(^4\), 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 \( \gamma \)-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 \( \gamma \)-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 \( \gamma \)-synthase of *N. crassa* has an absolute requirement for CH\(_3\)-THF that is satisfied by either the monoglutamate or polyglutamate 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.](image)

**MATERIALS AND METHODS**

With the exception of l-CH\(_3\)-H\(_4\)PteGlu\(_7\), the folates used in this study were synthesized by procedures that have been described (4, 5) and were purified by anaerobic chromatography using TBEA-cellulose (Cellex-T, Bio-Rad Laboratories) (6, 7). Yeast l-CH\(_3\)-H\(_4\)PteGlu\(_7\) (70–80% pure) was a very generous
Folates were determined spectrophotometrically using the extinction coefficients: THF, $22 \times 10^6 \text{ M}^{-1} \text{ cm}^{-1}$ at 297 nm, pH 7.0 (8); CH$_2$THF, $32 \times 10^6 \text{ M}^{-1} \text{ cm}^{-1}$ at 290 nm, pH 7.0 (6); and 5-CHO-THF, $32.6 \times 10^6 \text{ M}^{-1} \text{ cm}^{-1}$ 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 $\gamma$-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 $\gamma$-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$_4$–H$_4$PteGlu, the principal methylated folate of me-6. The trigitamate, CH$_4$–H$_4$PteGlu$_0$, is partially retained indicating that a substantial part of the hexaglutamate and other higher conjugate forms of CH$_4$–THF that characterize *N. crassa* (2) are eluted with the protein fraction.

When extracts of me-1 and me-6 possessing negligible cystathionine $\gamma$-synthase activity were supplemented with CH$_4$–H$_4$PteGlu$_0$, they synthesized cystathionine at rates similar to that of an extract of the wild type organism (Table 1). This result clearly demonstrates that CH$_4$–THF is an essential activator of the cystathionine $\gamma$-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$_4$–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$_4$–H$_4$PteGlu$_0$ (Table 1) was not unexpected.

Addition of CH$_4$–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$_4$–THF have been established. The extent of the activation was found to be determined by the type as well as the concentration of CH$_4$–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$_4$PteGlu$_3$ nor 5-CHO-H$_4$PteGlu$_4$ activate the enzyme (Table 2).

In addition to activating cystathionine $\gamma$-synthase, CH$_4$–THF also antagonizes feedback inhibition of the enzyme by S-adenosylmethionine (3, 12). As shown in Table 3, when the concentration of CH$_4$–H$_4$PteGlu$_0$ is increased from 1 to 15 $\mu$s, the inhibition produced by a 30 $\mu$s concentration of S-

**TABLE 1. Effect of 1-CH$_4$–H$_4$PteGlu$_0$ on the cystathionine $\gamma$-synthase activities of Neurospora extracts**

<table>
<thead>
<tr>
<th>N. crassa</th>
<th>Cystathionine $\gamma$-synthase activity (nmol min$^{-1}$ mg$^{-1}$)</th>
<th>Increase in activity factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>extract</td>
<td>Without CH$_4$–THF</td>
<td>With CH$_4$–THF</td>
</tr>
<tr>
<td>1 Wild type (old)*</td>
<td>0.94</td>
<td>3.08</td>
</tr>
<tr>
<td>2 me-1 (fresh)</td>
<td>7.97</td>
<td>10.40</td>
</tr>
<tr>
<td>3 me-1</td>
<td>0.37</td>
<td>7.14</td>
</tr>
<tr>
<td>4 me-6</td>
<td>0.05</td>
<td>5.79</td>
</tr>
</tbody>
</table>

* 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 ml): protein, as noted below; G-acetyl-1-homoserine, 4 $\mu$mol; $[^{35}S]$-cysteine–HCl, 3.1 $\mu$mol (2 $\mu$Ci); potassium phosphate buffer (pH 7.3), 125 $\mu$mol; pyridoxal phosphate, 0.23 $\mu$mol; dithiothreitol, 15 $\mu$mol; EDTA, 5 $\mu$mol; and 1-CH$_4$–H$_4$PteGlu$_0$, when added, 0.015 $\mu$mol. 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.4
Table 2. Effect of various folates on cysathionine γ-synthase activity

<table>
<thead>
<tr>
<th>Folate</th>
<th>Cystathionine γ-synthase activity (nmol min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dL-CH₃-H₄PteGlu₂</td>
<td>2.4</td>
</tr>
<tr>
<td>dL-5-CHO-H₄PteGlu₃</td>
<td>0</td>
</tr>
<tr>
<td>dL-H₂PteGlu₃</td>
<td>0</td>
</tr>
</tbody>
</table>

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-θ) and the folate, 30 μM (dl), was as specified above.

Adenosylmethionine is decreased from 57 to 15%. The antagonizing effect of CH₃-THF increases with its glutamate content: inhibition of the enzyme by 30 μM S-adenosylmethionine was 57% with 1 μM CH₃-THPteGlu₂ but only 15% when the folate was CH₃-H₂PteGlu₃ 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 CH₃-THF.

DISCUSSION

Previous studies from these laboratories have demonstrated that the formation of homocysteine (3, 12) and CH₃-THF (Selhub, Burton, and Sakami, unpublished data) in *N. crassa* is regulated by the inhibition of cysathionine γ-synthase and CH₃-THF reductase by S-adenosylmethionine (Scheme 1). In the absence of other controls, this feedback mechanism would modify the rates of CH₃-THF and homocysteine formation to meet changing requirements for S-adenosylmethio-

Table 3. Antagonizing effect of CH₃-THF on the inhibition of cysathionine γ-synthase by S-adenosylmethionine

| Folate added | Cystathionine γ-synthase activity (nmol min⁻¹ mg⁻¹) | Inhibition by S-adenosylmethionine (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃-H₂PteGlu₃</td>
<td>15</td>
<td>8.70</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.71</td>
</tr>
<tr>
<td>CH₃-H₂PteGlu₃</td>
<td>15</td>
<td>8.30</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6.99</td>
</tr>
</tbody>
</table>

The experimental procedure was the same as that described in Table 1 except that the protein was provided by an extract of *N. crassa* 74-OB8-1a, 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 1/8 bed volume of resin per volume of protein solution) 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 reincubated 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.

The authors wish to express their appreciation to Dr. Bernard T. Kaufman and Mr. Howard Bakerman for their very generous gift of the yeast l-methyltetrahydropteroylglutaminate that was employed in this investigation, and to Dr. Harland G. Wood for his valuable suggestions in connection with the preparation of the manuscript.

The portion of this work carried out at Case Western Reserve University was supported by Grant 5-R01-AO-08089 from the National Institutes of Health.

2. Selhub, J., Methionine Biosynthesis in Neurospora crassa (Doctoral Thesis, Case Western Reserve University, Cleveland, Ohio, 1970).