SYNTHESIS OF CYSTATHIONINE FROM O-ACETYLHOMOSERINE IN NEUROSPORA: A STEP IN METHIONINE BIOSYNTHESIS

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This report confirms the recently disputed role of cystathionine as an intermediate in methionine biosynthesis in Neurospora. Rapid synthesis of cystathionine is catalyzed by fresh sonic extracts. This activity is very unstable, and is inhibited by growth on methionine. Mutation in either of two unlinked genes, me-3 or me-7, yields strains which are deficient in the synthase activity; however when fresh extracts of me-3 and me-7 mutants are mixed activity is restored.

In bacterial species methionine synthesis involves formation of cystathionine from cysteine and O-succinylhomoserine by a cystathionine γ-synthase (Kaplan and Flavin, 1966), followed by liberation of homocysteine from cystathionine by a β-cystathionase (Delavier-Klutchko and Flavin, 1965). The cystathionine synthase can also utilize sulfide in place of cysteine, forming homocysteine directly, but this pathway, bypassing cystathionine, seems to be of minor importance since mutants lacking only β-cystathionase still require methionine for rapid growth (Flavin and Slaughter, 1967).

Neurospora has been shown to form O-acetyl- instead of O-succinylhomoserine as an intermediate in methionine biosynthesis (Nagai and Flavin, 1967), but results of studies of the utilization of this ester have been somewhat divergent from those obtained with bacteria in that cystathionine γ-synthase was: a) present at very low levels in extracts of Neurospora and not clearly specific for the acetyl ester; and b) not absent from mutants expected to lack this reaction. Furthermore, in Neurospora extracts and those of other fungi the reaction of acetylhomoserine with sulfide was very much faster than that with cysteine. This sulfhydrylase, when purified, could not utilize cysteine at all (Kerr and Nagai, 1967).

Wiebers and Garner (1967) and Giovanelli and Mudd (1967) have also reported that extracts of Neurospora, yeast and spinach leaf form homocysteine from sulfide and acetylhomoserine, and have proposed this to be on the major path for methionine

biosynthesis. We had favored the view that the sulfhydrylase reaction was not on the major path, because β-cystathionaseless mutants of Neurospora, like those of Salmonella, required methionine for rapid growth (Horowitz, 1947; Flavin and Slaughter, 1964; Nagai and Flavin, 1967). We now report more direct evidence that cystathionine is an intermediate in the major fungal pathway of methionine synthesis.

Recently we have obtained extracts of Neurospora with at least 50 times higher cystathionine γ-synthase activity than was previously observed.[1] At least part of the

TABLE I: Specificity of Neurospora cystathionine γ-synthase for acetylhomoserine

Homoserine derivative added	Rate of cystathionine formation ($m\mu moles\ min^{-1}\ mg^{-1}$)
None	0.09
O-Acetyl-DL-homoserine	3.2
O-Succinyl-DL-homoserine	0.06
DL-Homoserine	0.05

Wild type Neurospora crassa (EM 5297a) was grown in shake cultures for 24 hours at 30° in 500 ml of minimal medium (Vogel, 1955). Mycelium (about 10 g wet weight) was filtered out and rinsed 3 times with cold 0.05 M potassium phosphate, pH 7.3, containing 10^{-3} M dithiothreitol and 10^{-4} M each of EDTA and pyridoxal-P. A portion of 6 g was cut into small pieces and suspended in 12 ml of the same buffer in a 30 ml stainless steel Spinco tube. While rotating the tube in an ice bath, the suspension was treated with ultrasound for 3 one-minute periods, with a Branson L-S75 sonifier at maximum power setting. The protein concentration of the sonic homogenate was 30 mg/ml, measured by the biuret method. The assay mixtures included, in 1 ml volume: potassium phosphate, pH 7.3, 100 μmoles; dithiothreitol, 1 μmole; ^{35}S-L-cysteine (Nuclear Chicago), 10^6 cpm, 1 μmole; and other amino acid, as indicated, 20 μmoles. Reactions were started 5 minutes after completing sonication by adding 15 mg of homogenate protein. Duplicate tubes were incubated for 4 and 8 minutes, respectively, at 30°. Reactions were stopped by adding 0.2 ml of 1.5 M $HClO_4$. To convert O-acylhomoserines (which interfere with electrophoresis of cystathionine) to N-acylhomoserines, a 0.4 ml aliquot of supernatant was heated for 1 minute at 100° with 0.2 ml of 1 M KOH. The chilled mixture was then adjusted to pH 5 with 0.07 ml of 1.5 M $HClO_4$, and precipitated $KClO_4$ was discarded. Cystathionine was isolated from a 0.2 ml aliquot by paper electrophoresis at pH 2 for 4 hours at 5000 volts, and measured by radioassay, as previously described (Nagai and Flavin, 1967).

1 Homoserine acetylase was also present at suprisingly low levels, compared with the bacterial succinylase, in earlier extracts. The new preparation has a 10 times higher level of this enzyme, 0.6 $m\mu mole\ min^{-1}\ mg^{-1}$, as measured by the assay for net synthesis from acetyl CoA and ^3H-homoserine (Nagai and Flavin, 1967). However, in this case the difference from earlier results does not seem to be due to the same marked instability toward storage or dilution.

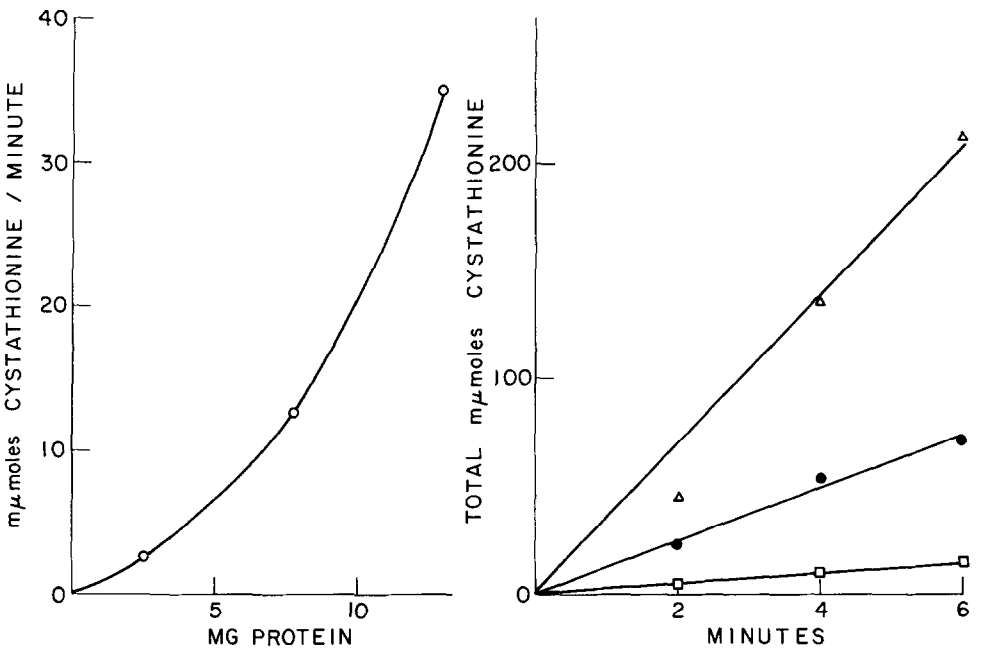

FIG. 1. Effect of protein concentration on rate of cystathionine synthesis. Methods were as in Table 1 except that sonication was for 4 minutes, the homogenate was centrifuged for 10 minutes at 15,000 x g, and the supernatant fraction was assayed 15 minutes after sonication. The rates indicated at various protein concentrations, on the left, were obtained from the data shown on the right where the protein concentration was 2.6 (□), 7.8 (●), and 13.0 (Δ) mg/ml.

reason for the difference from earlier results is the extreme instability of the activity, which decays 50% in 1 hour. There are also differences in the reaction conditions and in the method of extracting by brief sonication (Table 1). The activity decayed very rapidly during sonication, but could partly be liberated in a form which remained in the supernatant after centrifugation for 15 minutes at 100,000 x g. As shown in Table 1, the reaction catalyzed by a sonic homogenate was specific for acetylhomoserine. Figure 1 shows that cystathionine synthesis by a low speed supernatant fraction was proportional to reaction time for periods less than 10 minutes, with any given protein concentration, but that the reaction rate was not proportional to protein concentration. In repeated experiments the specific activity was markedly higher at higher extract concentrations. This loss of activity with dilution persisted after passage through Sephadex G-25, or addition of bovine serum albumin, 10 mg/ml, to reaction mixtures. Thus the activities of different extracts could be roughly compared only when

TABLE II: Cystathionine γ-synthase activity after growth of wild type on methionine

Expt.	DL-Methionine added to medium	Enzyme Preparation	Cystathionine γ-synthase specific activity (μmoles min^{-1} mg^{-1})
1	None	Sonic homogenate	3.2
	5×10^{-4}	" "	2.4
	5×10^{-3}	" "	0.14
2	None	Supernatant	1.56
	"	" , after Sephadex	1.07
	5×10^{-3}	Supernatant	0.11
	"	" , after Sephadex	0.87

Methods were as in Table 1 except that methionine was added to growth media as indicated, and in expt. 2 sonication was for 4 minutes, the sonicate was centrifuged at 15,000 x g for 5 minutes, part of the supernatant was passed through 5 volumes of Sephadex G-25 in the original buffer, and assays were started 25 minutes after sonication.

they had been prepared under identical conditions and added to assay mixtures in the same proportions. The reaction product was identified as L-cystathionine by paper electrophoresis and chromatography, by column chromatography with an amino acid analyzer, and by identifying the products formed after digestion with a γ-cystathionase from Neurospora (Flavin and Segal, 1964) and a β-cystathionase from Salmonella (S. Guggenheim, unpublished purification method).

Growth of wild-type Neurospora on levels of DL-methionine sufficient to support maximal growth of auxotrophs (5×10^{-4} M) yielded slightly lower cystathionine γ-synthase activity in homogenates, but negligible activity was present after growth in the presence of 10 times this much methionine (Table II, expt. 1). In the latter case activity was restored by passing the supernatant through Sephadex G-25 (Table II, expt. 2) which presumably removed a small inhibitor. No inhibition was observed on adding 10^{-2} M DL-methionine directly to reaction mixtures. It has not yet been determined what the inhibitory compound is responsible for the low activity of cells grown on high methionine.[2]

2 Neurospora β-cystathionase activity is probably also reduced after growth on 5×10^{-3} M methionine (Flavin and Slaughter, 1967)

Further evidence that cystathionine γ-synthase actually functions in methionine biosynthesis came from a study of mutants. Neurospora contains two unlinked genes (Murray, 1960), designated me-3 and me-7, mutation in either of which results in a positive growth response to cystathionine and negative response to acetylhomoserine (Nagai and Flavin, 1967). Until now it had not been possible to show that the traces of cystathionine synthase measurable in wild type were absent from these mutants, or to identify any other metabolic defect in them (Nagai and Flavin, 1967). When sonic homogenates were prepared and assayed by the new procedure, both me-3 and me-7 mutants were found to be essentially devoid of cystathionine synthase activity (Table III, expt. 1). Normal activity was found in an me-2 mutant lacking β-cystathionase (Flavin

TABLE III: Cystathionine γ-synthase in methionine auxotrophs

Expt.	Neurospora strain: Gene	Mutant	Cystathionine γ-synthase specific activity ($m\mu moles\ min^{-1}\ mg^{-1}$)
1	-	Wild type	2.4
	me-2	P162	2.2
	me-3	36104	0.14
	me-5	9666	5.0
	me-7	K79	0.15
	me-7	NM297	0.17
2	me-3	36104	0.08
	me-7	K79	0.08
	Extracts mixed: me-3 + me-7	36104 K79	1.75

Methods were as in Table I except that DL-methionine, 0.5 μmole/ml, was added to all growth media. In expt. 2, the extracts were prepared as in Table II, expt. 2, and after passage through Sephadex assayed separately or mixed in equal portions and assayed after waiting 10 min. The identity of the product in expt. 2 was confirmed by repeated electrophoresis of a small radioactive aliquot with excess carrier cystathionine. We are indebted to N.E. Murray and the Dartmouth Fungal Genetics Stock Center for the mutants.

and Slaughter, 1964) and a slightly elevated level in an me-5 mutant lacking homoserine acetylase (Nagai and Flavin, 1967).

Homogenates of me-3 and me-7 mutants, while defective separately, showed activity when promptly mixed together or when supernatant fractions from each mutant extract were mixed after being passed separately through Sephadex G-25 (Table III, expt. 2). This result indicates that each gene orders a macromolecular cytoplasmic product and that the products can reconstitute functional cystathionine synthase when mixed in vitro.

The acetylhomoserine sulfhydrylase of Neurospora is present in fresh crude extracts at about the same specific activity which we now observe for cystathionine synthase.[3] But in contrast, the sulfhydrylase is very stable, undergoing neither decay nor unmasking after extraction. The enzyme has been purified 500 fold and has some similarities to O-acetyl serine sulfhydrylase of Salmonella (Kredich and Tompkins, 1966). The purified enzyme can not utilize cysteine, but can utilize CH_3SH in place of sulfide.[4] The V_{max} for methionine synthesis is twice that for homocysteine synthesis. The enzyme is specific for acetylhomoserine, the rate being less than 2% with homoserine, O-succinyl-homoserine, O-phosphorylhomoserine, serine, O-acetylserine, or O-phosphorylserine. Activity is lost after dialysis against cysteine, and can then be restored by adding pyridoxal-P.

In Salmonella one gene orders the structure of an enzyme which is composed of four identical subunits (Kaplan and Flavin, 1966), and which catalyzes all three reactions discussed above: synthesis of cystathionine, homocysteine, and methionine (Flavin and Slaughter, 1967). In Neurospora two genes are involved in the formation of cystathionine γ-synthase, which may thus be a complex of two unlike polypeptide chains. Analogy to Salmonella suggests that the acetylhomoserine sulfhydrylase might be one of the polypeptide components, but at present there is no direct evidence to support this. The sulfhydrylase is present at about wild type levels in me-3 and me-7 mutants. At least one mutant from every other known methionine locus (Murray, 1960) has also been tested and found to have a normal level. Addition of purified sulfhydrylase to a fresh

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3 A sulfhydrylase specific for acetylhomoserine is also present at about the same level of activity in extracts of Saccharomyces cerevisiae; Candida utilis, and Cunninghamella blakesleeana. The four fungi are also similar in their levels of homoserine acetylase, and in the absence of a homoserine succinylase.

4 This direct synthesis of methionine was discovered in Salmonella, where it is catalyzed by cystathionine γ-synthase (Flavin and Slaughter, 1967). It was postulated that a similar reaction in Neurospora might account for the ability of methionine auxotrophs blocked after acetylhomoserine to respond to S-methylcysteine. Moore and Thompson (1967) have shown that extracts of Neurospora will form methionine from CH_3SH and acetylhomoserine.

extract of either me-3 or me-7 did not restore cystathionine synthase activity.

In conclusion, the following evidence, besides the genetic argument mentioned earlier, indicates that cystathionine synthase functions in the major pathway of methionine biosynthesis in fungi; a) the specific rate of this reaction is now shown to be reasonably high; b) the reaction is virtually absent from me-3 and me-7 mutants, whereas acetylhomoserine sulfhydrylase has not been found defective in any methionine auxotroph; c) the reaction is subject to end-product regulation. The sulfhydrylase reaction may provide an inefficient alternate path, and could account for the leakiness of methionine auxotrophs blocked after acetylhomoserine.

It is difficult to visualize the cystathionine γ-synthase reaction as occuring in more than one step, i.e., as involving a free intermediate which could allow for the sequential participation of two enzymes. It is easier to believe that Neurospora cystathionine γ-synthase may be a labile complex of at least two different polypeptides specified by the me-3 and me-7 genes (possibly also associated with homoserine acetylase[1] as well as other components). There is so far no direct evidence for such a complex, although the effect of dilution (Fig. 1) is suggestive.

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