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THE DEREPRESSION AND FUNCTION OF ENZYMES OF REVERSE *TRANS*-SULFURATION IN NEUROSPORA

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SUMMARY

Sulfur transfer from homocysteine to cysteine is mediated by 2 enzymes, cystathionine β-synthase and γ-cystathionase, which are present in animals and fungi, but absent from bacterial species so far examined. It has now been found that when nutrient sulfur limits the growth of Neurospora, the level of γ-cystathionase is increased 30-fold. Derepression is not coordinate, since the level of cystathionine β-synthase is not changed. β-Cystathionase is also unaffected. Non-coordinate derepression suggests that γ-cystathionase might have a more general function in mobilizing sulfur; the enzyme is shown able to rapidly decompose a wide variety of β- or γ-substituted amino acids besides cystathionine. The derepression was shown not to be brought about by starvation in general, and may therefore result from depletion of a low molecular weight, sulfur-containing corepressor.

INTRODUCTION

In *trans*-sulfuration (the transfer of sulfur between cysteine and homocysteine) fungi combine processes which are found separately in animals and bacteria[1]. In the forward direction, of methionine biosynthesis, *O*-acetyl-homoserine appears to play the same role in Neurospora[2] that *O*-succinyl-homoserine does in bacterial species[3], although the condensation reaction with cysteine has not yet been established (Scheme I). A β-cystathionase (forming pyruvate and homocysteine) is present in both types of organism[1]. However, unlike the bacteria, Neurospora also contains 2 enzymes similar to those mediating reverse *trans*-sulfuration in animal liver: cystathionine β-synthase (EC 4.2.1.13, from serine and homocysteine) and γ-cystathionase (EC 4.2.1.15, forming cysteine and α-ketobutyrate)[1].

The questions arose as to the functions of reverse *trans*-sulfuration in fungi, and why the bacteria had dispensed with this process. A possible function was to conserve sulfur efficiently at times when nutritional deprivation required the utilization of endogenous materials for the synthesis of new structures, for example during sporula-

tion[1]. It was from this viewpoint that we examined the effect of sulfur deprivation on the levels of various enzymes of *trans*-sulfuration in Neurospora[4,5].

MATERIALS AND METHODS

Vogel's medium, which normally contains 0.85 μmole of sulfate per ml (ref. 6), was modified by replacing all of the sulfate with the corresponding chloride salts, and this medium was then supplemented with various amounts of sulfur; the same results were obtained in the following experiments whether the latter was provided in the form of sulfate, cysteine, or methionine. 500-ml erlenmeyer flasks containing 100 ml of medium were inoculated with conidia, shaken for 3 days at 30°, and mycelia were extracted by grinding with alumina[6]. The extracts were passed over Sephadex[3] and assayed by the following procedures. γ-Cystathionase was assayed by the rate of liberation of mercaptan in the presence of 5,5′-dithiobis(2-nitrobenzoic acid)[6]; the aromatic disulfide inhibits β-cystathionase[6]. Cystathionine β-synthase was assayed by the rate of serine-dependent* disappearance of homocysteine, in a manner similar to that used for cystathionine γ-synthase of Salmonella[3], except that because of lower activity much longer anaerobic incubations were required, resulting in less accuracy. Reaction mixtures were incubated at 37° under helium and contained, in 1 ml: potassium phosphate, pH 7.0, 100 μmoles; pyridoxal phosphate, 0.1 μmole; L-homocysteine, 2 μmoles; L-serine, 10 μmoles (omitted from controls); and 0.5 to 2 mg of extract protein. Aliquots were assayed for residual mercaptan[3] at 20-min intervals for 1 h.

The rate of total α-ketoacid liberation in the presence of iodoacetate gives the sum of activities of β- and γ-cystathionase[6]; the activity of the latter can then be subtracted after it has been determined as described above. This assay for β-cystathionase becomes quite inaccurate when the other cleavage enzyme is present in ex-

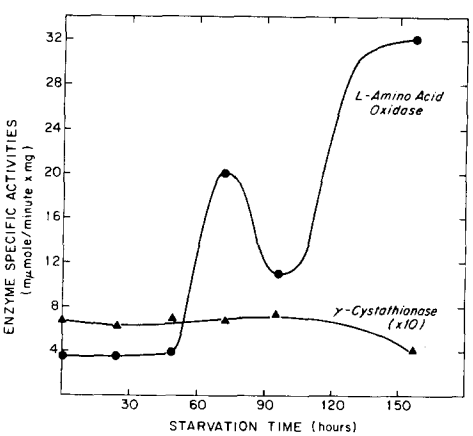
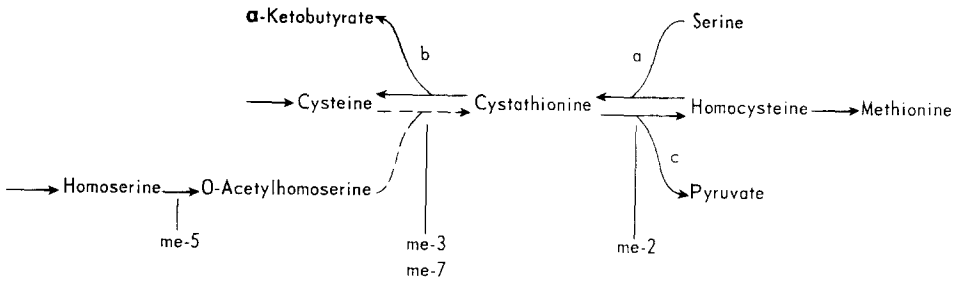


Fig. 1. Starvation and derepression of enzymes in wild-type Neurospora.

* *O*-Acetyl-L-serine[7] was found to be half as effective as L-serine in this reaction. It has not been determined whether this was due to its decomposition to serine.



Scheme I. *Trans*-sulfuration in Neurospora. Reaction (a) is catalyzed by cystathionine β-synthase, (b) by γ-cystathionase, and (c) by β-cystathionase. The enzymes involved in the steps from homoserine to cystathionine have not been fully characterized[2].

cess, but the results were confirmed qualitatively by determining the proportions of pyruvate and α-ketobutyrate liberated from cystathionine[6].

The effect of starvation on γ-cystathionase was investigated by the procedure used by HOROWITZ to derepress tyrosinase and L-amino-acid oxidase[8] (EC 1.4.3.2). Wild-type Neurospora was grown 3 days at 30° in a series of flasks containing complete Vogel's medium. The resulting mycelia were washed with sterile precautions, suspended in buffer[8], and shaken at 30° for periods up to 7 days before being harvested and extracted (Fig. 1). As a control, L-amino-acid oxidase was also assayed, by the procedure of YUGARI AND GILVARG[9]. The reaction cuvettes contained, per 1 ml: 0.57 ml of potassium borate (mole/l), pH 8.0, previously saturated with pure oxygen; 7 μg of catalase; and 5 μmoles of L-histidine. After addition of 0.5–2 mg of extract protein, the rate of ketohistidine formation was determined from the increase in absorbance at 300 mμ at 30°. The molar absorbance of ketohistidine was about 10 000 under these conditions.

RESULTS AND DISCUSSION

When nutrient sulfur was allowed to limit growth, as shown by a 60% decrease in the final cell mass (Table I), the level of γ-cystathionase was found to be increased 30-fold (Table I). The increase occurred between the second and third days, after growth had ceased. Mutant strains[6] me-2 (P162) and me-7 (K79) gave the same results as wild type. Surprisingly, the level of the companion enzyme of reverse *trans*-sulfuration, cystathionine β-synthase, was not correspondingly increased (Table I). *Trans-*

TABLE I

SULFUR DEPRIVATION AND DEREPRESSION OF ENZYMES IN WILD TYPE NEUROSPORA

*Total nutrient sulfur concentration** (μatomo/ml)	*Yields after 3 days growth (from 100 ml of medium)*		*Enzyme specific activities (mμmoles $mg^{-1} \cdot min^{-1}$)*			
	Cell dry weight (mg)	*Extractable protein (mg)*	*γ-Cystathionase*	*Cystathionine β-synthase*	*β-Cystathionase*	*L-amino-acid oxidase*
1.0	500	100	0.5	2	2	3.4
0.1	200	15	15	4	2	3.3

* The same results were obtained whether sulfur was added in the form of sulfate, cysteine, or methionine.

sulfuration in the direction of methionine biosynthesis (β-cystathionase) was also not affected. When methionine constituted the sole source of nutrient sulfur and nitrogen (5 μmoles/ml) the γ-cystathionase was at the same repressed level as in Table I, and the β-cystathionase was reduced to about one fourth.

It appeared that the observed 30-fold derepression of γ-cystathionase might be caused by depletion of a sulfur-containing low molecular weight corepressor, or on the other hand that this enzyme might resemble tyrosinase and L-amino-acid oxidase, which are produced in large amounts in response not only to sulfur deprivation, but also to other types of nutritional deprivation, or to total starvation[8]. In Neurospora, derepression of the observed large magnitude has commonly been found only in the latter group of enzymes.

The effect of starvation was investigated by suspending mycelia in phosphate buffer for varying periods, as described by HOROWITZ[8]. As shown in Fig. 1, conditions giving maximal levels of L-amino-acid oxidase did not cause any increase in γ-cystathionase activity. Conversely, and in confirmation of earlier work[10], L-amino-acid oxidase was not derepressed under the conditions of sulfur deprivation which gave maximal increases of cystathionase (Table I). It thus appears that conditions for derepression of L-amino-acid oxidase are not the same as those for γ-cystathionase, and that sulfur deprivation may derepress the latter through depletion of a low molecular weight sulfur-containing corepressor.

The lack of coordinate control of cystathionine β-synthase should perhaps be a reminder that cystathionine can be considered a storage form of sulfur, as well as a metabolic intermediate. Recently a group of Neurospora enzymes, which seem to be related through their potentiality to function in mobilizing sulfur, have been found to be repressible by methionine, and in some cases independently by a precursor of cysteine[11].

The question concerning the utility of reverse *trans*-sulfuration in Neurospora remains unanswered. In this connection it should not be overlooked that the γ-cystathionase can decompose a wide variety of amino acids besides cystathionine[12]. The current status of the substrate range of this enzyme is presented in Tables II and III. As a result of the derepression described above enzyme can be obtained effectively free of DPNH oxidase after 2 simple purification steps: "step 3" previously described[12]. Kinetic constants can then be determined by measuring the liberation of pyruvate or α-ketobutyrate continuously[12] in the presence of DPNH and lactic dehydrogenase (Table II: *assays removing α-ketoacid*). The same data can be obtained independently for sulfur-containing substrates from the rate of mercaptan liberation measured in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid) (Table II).

As had been expected[12], the previously observed difference in the rates of cystathionine decomposition measured by the 2 assays was found to be a v_{max}'. and not a K_m' effect. Another problem for future work is presented by the observation that, for several substrates, the K_m values appear different from the K_i values that are observed when the same compounds are used to inhibit the reactions of other substrates (Table II). The significance of the high velocity of the elimination of succinate from *O*-succinyl-homoserine has been discussed elsewhere[2,5].

We would like here to call attention particularly to the fact that *S*-methylcysteine is a substrate for γ-cystathionase. The ability of this compound to support the growth of certain methionine auxotrophs of Neurospora, particularly when they

TABLE II

KINETIC CONSTANTS FOR ELIMINATION REACTIONS CATALYZED BY NEUROSPORA γ-CYSTATHIONASE

Substrate	*Determinations by assay removing α-ketoacid*		*Determinations by assay removing mercaptan*			
	K_m	$v_{max.}$	K_m	$v_{max.}$	K_i *against cystathionine*	K_i *against* L-*cystine*
L-Cystathionine	$7 \cdot 10^{-4}$	32	$7 \cdot 10^{-4}$	100		
O-Succinyl-DL-homoserine	$5 \cdot 10^{-3}$	120			$2 \cdot 10^{-2}$	
O-Acetyl-DL-homoserine	$5 \cdot 10^{-2}$	10				
L-Homoserine	$2 \cdot 10^{-2}$	8			$8 \cdot 10^{-3}$	$8 \cdot 10^{-3}$
L-Homocystine			$2 \cdot 10^{-3}$	13		
γ-Elimination: $A—CH_2CH_2CH(NH_2)COOH + H_2O \rightarrow AH + NH_3 + CH_3CH_2COCOOH$						
L-Cystine			$3 \cdot 10^{-5}$	15		
meso-cystine			$1 \cdot 10^{-4}$	50		
Lanthionine (L + *meso*)			$4 \cdot 10^{-4}$	80		
S-Methyl-L-cysteine			$2 \cdot 10^{-2}$	30		
DL-Erythro-β-chloro-α-aminobutyrate	$4 \cdot 10^{-3}$	18			$2 \cdot 10^{-4}$	$2 \cdot 10^{-4}$
L-Serine		0			$1 \cdot 10^{-2}$	$1 \cdot 10^{-2}$
β-Elimination: $A—CH_2CH(NH_2)COOH + H_2O \rightarrow AH + NH_3 + CH_3COCOOH$						
$A—\underset{\displaystyle CH_3}{\overset{\displaystyle |}{C}}HCH(NH_2)COOH + H_2O \rightarrow AH + NH_3 + CH_3CH_2COCOOH$						

are deprived of other sources of sulfur[13], has interested several laboratories[13–15]. *S*-methyl-cysteine is decomposed to methyl mercaptan by γ-cystathionase (Table II), and sulfur deprivation would facilitate the reaction by increasing the enzyme level*. An accompanying paper[16] describes the synthesis of methionine, by a bacterial enzyme, from methyl mercaptan and *O*-succinyl-homoserine. A similar reaction in Neurospora, perhaps utilizing *O*-acetyl-homoserine[2], may underlie the hitherto obscure role of *S*-methyl-cysteine in this organism.

TABLE III

ADDITIONAL β-ELIMINATION REACTIONS CATALYZED BY γ-CYSTATHIONASE

Equivocal, or no, reaction: L-cysteine, L-homocysteine, L-methionine, *S*-carboxyethyl-cysteine, DL-serine, *O*-acetyl-L-serine, DL-threonine, *O*-acetyl-L-threonine, *O*-phosphoryl-L-homoserine, DL-homoserine-lactone.

Substrate	*Concentration (μmole/ml)*	*Relative reaction rate (Cystathionine = 100)*
L-Cystine-L-homocystine mixed disulfide	2	30
L-Cystine	2	21
β-Methyl-cystine isomer A	1	1.1
β-Methyl-cystine isomer B	1	1.4
Djenkolic acid	2	20

* This was brought to our attention by Dr. R. L. METZENBERG.

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