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EFFECT OF REGULATORY MUTATIONS OF SULPHUR METABOLISM ON THE LEVELS OF CYSTEINE- AND HOMOCYSTEINE-SYNTHESIZING ENZYMES IN *NEUROSPORA CRASSA*

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1. Regulation of four enzymes involved in cysteine and homocysteine synthesis, i.e. cysteine synthase (EC 4.2.99.8), homocysteine synthase (EC 4.1.99.10), cystathionine β-synthase (EC 2.1.22) and γ-cystathionase (EC 4.4.1.1) was studied in the wild type and sulphur regulatory mutants of *Neurospora crassa*.

2. Homocysteine synthase and cystathionine β-synthase were found to be regulatory enzymes but only the former is under control of the cys-3-scon system regulating several enzymes of sulphur metabolism, including γ-cystathionase.

3. The results obtained with the mutants strongly suggest that homocysteine synthase plays a physiological role as an enzyme of the alternative pathway of methionine synthesis. Cysteine synthase activity was similar in all strains examined irrespective of growth conditions.

4. The scon<sup>+</sup> strain with derepressed enzymes of sulphur metabolism showed an increased pool of sulphur amino acids, except for methionine. Particularly characteristic for this pool is a high content of hypotaurine, a product of cysteine catabolism.

Fungi are able to utilize sulphate as a sole sulphur source. In a series of enzymatic reactions (Scheme 1) sulphate is reduced to sulphide, a substrate for cysteine synthesis. Methionine is formed from cysteine via cystathionine and homocysteine. The existence of an alternative pathway for homocysteine synthesis directly from O-acetylhomoserine (OAH) and sulphide (Scheme 1, step 4) was described for several fungi. Its physiological role was studied in *Saccharomyces cerevisiae* (Cherest et al., 1969), *Saccharomycopsis lipolytica*.

* This work was supported by the Polish Academy of Sciences within the project 09.7.
(Morzycka & Paszewski, 1979) and Aspergillus nidulans (Paszewski & Grabski, 1974, 1975). In these three organisms, homocysteine synthase is under control of the same regulatory system as the enzymes of the sulphate assimilation pathway. In A. nidulans the same is true for cystathionine β-synthase and γ-cystathionase (Scheme 1, steps 5 and 6), as concluded from the results obtained with the mutants impaired in sulphur amino acids metabolism (Paszewski & Grabski, 1975).

![Scheme 1. Outline of sulphur amino acids biosynthesis in fungi. 1. Cysteine synthase; 2. cystathionine γ-synthase; 3. β-cystathionase; 4. homocysteine synthase; 5. cystathionine β-synthase; 6. γ-cystathionase; APS, adenylyl sulphate; PAPS, phosphoadenylyl sulphate.](image)

In Neurospora crassa two mutations with regulatory effects on sulphur metabolism were described. In the scon<sup>e</sup> mutant, enzymes of the sulphate assimilation pathway as well as arylsulphatase and choline sulphatase are derepressed, even in the presence of methionine, contrary to the wild type (Burton & Metzenberg, 1972). In the cys-3 mutants, on the other hand, there is no induction (or derepression) of any of these enzymes, even under conditions of cysteine deprivation (Marzluf & Metzenberg, 1968). cys-3 mutations are epistatic with respect to scon<sup>e</sup>. In both regulatory mutants, among the enzymes directly involved in sulphur amino acids synthesis only γ-cystathionase was studied in some detail; in scon<sup>e</sup> its activity was high and non-repressible, while in cys-3 it was low and non-inducible.

It was, therefore, of interest to know how the other enzymes involved in cysteine and homocysteine synthesis such as cystathionine β-synthase (step 5), cysteine synthase (step 1) and homocysteine synthase (step 4) are regulated. Homocysteine synthase poses here a special problem as its physiological role in Neurospora is not yet understood. It is also important to know how regulatory mutations of sulphur metabolism influence the sulphur amino acids pool.
MATERIAL AND METHODS

Strains. Wild type strain 74-OR23-1A was used as a control in all experiments. The mutant strains cys-3, sconC, cys-3,sconC and me-2 were kindly supplied by Dr. R. L. Metzenberg.

Media, growth conditions and extract preparation. Minimal medium (MM) of Vogel (Vogel, 1964) was used. It was supplemented with methionine as indicated or devoid of sulphur compounds (minus S) for the sulphur starvation experiments (the amount of sulphur in conidia is sufficient to permit growth for many hours). Portions of 100 ml of liquid medium in 300-ml Erlenmayer flasks were inoculated with a filtered suspension of conidia (absorbance at 420 nm 0.2) and incubated in a rotary shaker at 30 C for 13-15 h, except for “sulphur-free” cultures which were incubated for 24-36 h. Mycelia were collected on cheesecloth, washed with water, blotted on filter paper, mixed with glass powder and ground in a chilled mortar with 0.1 M-potassium phosphate buffer, pH 7.5. The homogenates were centrifuged for 15 min at 15,000 g and the enzyme activities were assayed in the supernatants. Protein was estimated by the method of Lowry et al. (1951).

Enzyme assays. Cysteine synthase (EC 4.2.99.8) and cystathionine β-synthase (EC 2.1.22) were assayed according to Pieniak et al. (1973). Homocysteine synthase (EC 4.1.99.10) was assayed using the reaction mixture described by Paszewski & Grabski (1973); homocysteine formed was then estimated by the method of Kredich & Tomkins (1966). The activities of β-cystathionase (EC 4.4.1.8) and γ-cystathionase (EC 4.4.1.1) were determined as described by Paszewski & Grabski (1974). Arylsulphatase (EC 3.1.6.1) was assayed according to Metzenberg (1968), and ATP-sulphurylase (EC 2.7.7.4) according to de Vito & Dreyfuss (1964). The procedure for determination of 35SO4 uptake was essentially the same as described by Piotrowska et al. (1976). The incubation mixture contained 1 mm-Na235SO4 (18 x 107 c.p.m./mmol) and 35SO4 uptake was followed for 8 min, during which it was found to be linear.

Determination of 35S sulphur amino acid pool. The strains were grown in minimal medium supplemented with Na235SO4 in standard conditions. The mycelia were harvested and 35S-labelled amino acids isolated and separated by thin-layer chromatography on MN cellulose as described previously (Paszewski & Grabski, 1974). The positions of radioactive spots were determined by autoradiography. The main band (90% of radioactivity) containing poorly separated glutathione, cystathionine, cystine, homocystine and mixed disulphides was scraped off from the plates. Amino acids were eluted from cellulose powder with 0.1 M-HCl. The eluate was evaporated and the residue oxidized with a mixture of H2O2 and 80% formic acid (1:9). Amino acids were then separated by high-voltage electrophoresis as described by Bessman et al. (1967), together with internal standards of
identically oxidized L-cysteine, DL-homocysteine, L-glutathione and L-cystathionine. S-Amino acids were localized by staining with ninhydrin. The spots were cut out and counted in a PPO-POPPOP scintillation mixture in a Packard Tricarb Scintillation Spectrophotometer. As both filter paper and ninhydrin cause quenching, only the proportion of individual amino acids in the mixture was calculated. This proportion was, in turn, used to calculate the content of particular $^{35}$S-labelled amino acids of the main chromatographic band, the total radioactivity of which was determined before electrophoretic analysis. $[^{35}$S]$\text{Methionine}$ and $[^{35}$S]$\text{Hypotaurine}$ were estimated directly by scraping the respective spots from the chromatogram and counting the radioactivity.

Reagents. O-Acetylserine and O-acetylhomoserine were synthesized according to Wiebers & Garner (1967). L-Methionine, L-cysteine, DL-homocysteine thiolactone HCl, and L(+)-cystathionine were from Sigma Chemical Comp. (St. Louis, Mo., U.S.A.). Na$_2$SO$_4$ was from the Institute of Nuclear Research (Świerk, Poland).

RESULTS

The results presented in Table 1 indicate that in \textit{N. crassa} the activity of cysteine synthase was similar under all growth conditions tested. The enzyme activity was almost the same in the regulatory mutants and in the wild type.

The activity of cystathionine $\beta$-synthase increased markedly upon the addition of exogenous methionine and slightly under sulphur starvation. No effect of $\text{scon}^c$ and $\text{cys-3}$ mutations could be observed. The slight increase of cystathionine $\beta$-synthase activity upon sulphur starvation was also noted.

\begin{table}
\centering
\caption{Level of cysteine synthase, homocysteine synthase, cystathionine $\beta$-synthase and $\gamma$-cystathionase in wild type and $\text{scon}^c$ and $\text{cys-3}$ mutants in Neurospora \textit{crassa}}
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{Strain} & \textbf{Enzyme activity (nmol of product $\times$ min$^{-1}$ $\times$ mg protein$^{-1}$)} & \textbf{cysteine synthase} & \textbf{homocysteine synthase} & \textbf{cystathionine $\beta$-synthase} & \textbf{$\gamma$-cystathionase} \\
 & & MM & MM + meth & MM & MM + meth & MM & MM + meth & MM & MM + meth \\
\hline
wild type & 1.49 & 2.50 & 1.80 & 17.6 & 20.5 & 40.5 & 0.46 & 1.02 & 0.54 & 0.75 & 1.63 & 6.80 \\
cys-3 & 2.26 & 1.90 & 2.69 & 16.3 & 19.3 & 40.5 & 0.48 & 0.75 & 0.76 & 0.81 & 0.88 & 0.70 \\
\textit{scon}^c & 2.02 & 2.06 & 16.0 & 42.6 & 41.0 & 40.0 & 0.57 & 0.93 & 8.50 & 8.40 & 8.50 & 8.40 \\
\textit{scon}^c$,$\text{cys-3}$ & & & & & & & & & & & & \\
\hline
\end{tabular}
\end{table}

Results based on 4 - 6 experiments. Methionine concentration 4 mm.
previously (Flavin & Slaughter, 1967) and regarded as insignificant. The increase was, however, highly reproducible in our experiments. γ-Cystathionase was derepressed under sulphur starvation and, as observed by Burton & Metzenberg (1972), was under the control of the cys-3 - scon regulatory system.

Homocysteine synthase was non-repressible by exogenous methionine, but showed a two- to three-fold derepression under sulphur starvation (Table 1). In the cys-3 mutant the enzyme activity did not differ from that found in the wild type, but in the strains bearing sconc mutations homocysteine synthase activity was higher and did not respond to the growth conditions tested. The enzyme level in sconc approximated that observed in the wild type under sulphur starvation. The presence of cys-3 mutation in the sconc strain restored the basal level of homocysteine synthase, indicating that the enzyme was under control of the cys-3 - scon system.

The physiological role of homocysteine synthase in N. crassa is obscure. The auxotrophy of mutants impaired in cystathionine γ-synthase or β-cystathionase indicates that, at least under normal conditions, this enzyme does not participate to a significant extent in homocysteine synthesis (Kerr & Flavin, 1970). However, it may be important under specific conditions as it was found in A. nidulans. In this organism strains carrying mutations suppressing lesions in cystathionine γ-synthase and β-cystathionase genes show an elevated level of homocysteine synthase (Paszewski & Grabski, 1975).

By analogy we have looked for mutants with a high homocysteine synthase activity among revertants of the me-2 strain blocked in β-cystathionase (Kerr & Flavin, 1970). Ten revertants, chosen at random, were examined and none showed an increased enzyme activity. The results of β-cystathionase assays were not conclusive as there was a considerable β-cleavage of cystathionine in the crude extracts of the me-2 mutant (about 50% of the wild-type activity). This could be due partly to the leakiness of β-cystathionase in the mutant, but also to the activity of methylcysteine lyase and γ-cystathionase which both have some β-cystathionase activity (Smith & Thompson, 1969; Flavin & Slaughter, 1964). In this situation, sensitivity of our assay was too low to establish that reversion resulted from the restoration of β-cystathionase activity. Two out of ten revertants, me-2.sup26 and me-2.sup1, which were identified in crosses as bearing me-2 suppressors, were studied in some detail. A representative set of data from analysis of the 35S-labelled amino acids pool in the revertants, wild type and sconc strains are given in Table 2. The revertant me-2.sup26 is interesting as it exhibits a markedly lower level of glutathione with the concomitant increase in homocysteine as compared with the other strains. Accumulation of cystathionine suggests that a block in β-cystathionase still persisted in the revertant, and this in turn led to the conclusion that homocysteine was formed by homocysteine synthase. Electrophoretic separa-
tion of the S-containing metabolites revealed the presence of two unidentified sulphur compounds in me-2, sup26, the total radioactivity of which was close to that of glutathione.

Table 2

Content of $^{35}$S-labelled amino acids in the wild type, scon$^c$, me-2, sup26 and me-2, sup1 strains grown in the presence of $^{35}$SO$_4$

Mycelia were grown in minimal medium supplemented with Na$_2^{35}$SO$_4$ (4.6 x 10$^9$ c.p.m./mmol) at 29°C for 14 h (wild type and scon$^c$) or for 19 h (me-2, sup26 and me-2, sup1). Mycelia were harvested and content of $^{35}$S-labelled amino acids was estimated as described in Methods; data on only major radioactive compounds are given.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration ($10^{-4}$ x c.p.m./g dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wild type</td>
</tr>
<tr>
<td>Cysteine$^a$</td>
<td>162.9</td>
</tr>
<tr>
<td>Homocysteine$^a$</td>
<td>78.2</td>
</tr>
<tr>
<td>Glutathione$^a$</td>
<td>1153.9</td>
</tr>
<tr>
<td>Hypotaurine</td>
<td>traces</td>
</tr>
<tr>
<td>Cystathionine$^b$</td>
<td>156.6</td>
</tr>
<tr>
<td>Methionine</td>
<td>122.8</td>
</tr>
<tr>
<td>Total</td>
<td>2535.0</td>
</tr>
</tbody>
</table>

$^a$ Oxidized and estimated as sulphonic acids.

$^b$ Estimated as cystathionine sulphone.

Since homocysteine synthase activity in this revertant was not elevated, we checked whether the increase in the homocysteine pool and in the total pool of $^{35}$S-labelled compounds did not result from derepression of the sulphur assimilation pathway. $^{35}$SO$_4$ uptake and ATP-sulphurylase activity were examined in the me-2, sup26 and wild type strains (not shown), but no difference was found. The same holds for the activities of arylsulphatase and cysteine synthase. In spite of high homocysteine pool, me-2, sup26 grew at the rate by 70% lower than the wild type strain (as estimated by dry weight determination). Growth of this revertant was stimulated by methionine. Thus homocysteine evidently is not a growth-limiting factor. It is tempting to think that the phenotype of this strain may be related to a low glutathione pool, but the primary cause of this low pool is not clear. The me-2, sup1 strain grew very slowly and, for an unknown reason, the pool of sulphur amino acids was lower than that of the wild type. It seems likely that a suppressor mutation restores $\beta$-cystathionase activity.

Scon$^c$ accumulated more cysteine, homocysteine and glutathione, but not cystathionine and methionine, as compared with the wild type (Table 2). It should be noted, however, that scon$^c$ accumulated also a considerable amount of hypotaurine, a product of cysteine catabolism. The large total pool of $^{35}$S-labelled compounds in this mutant is not surprising in view of its non-repressible sulphur assimilation pathway.
DISCUSSION

The presented results show that, in N. crassa, homocysteine synthase, in contrast to cysteine synthase, is regulated by the cys-3 - scon system, controlling a number of other enzymes of sulphur metabolism (see review by Marzluf, 1978). Kerr (1971) described the enzyme as constitutive, contrary to the earlier reports on its repressible character (Wiebers & Garner, 1967). Our results indicate that homocysteine synthase of exponential cultures of N. crassa is already maximally repressed. The enzyme was derepressed in the scon\(^c\) but not in the scon\(^c\), cys-3 double mutant. The derepression is only about two-fold but is similar to that observed in A. nidulans. There is evidence that this enzyme is involved in the synthesis of methionine by the mutants requiring this amino acid, when grown on S-methylcysteine (Wiebers & Garner, 1964). This compound is enzymatically cleaved to mercapto-methyl (Smith & Thompson, 1969) which, in turn, is a substrate for methionine synthesis in the reaction catalysed by homocysteine synthase:

\[O\text{-Acetylhomoserine} + \text{CH}_3\text{SH} \rightarrow \text{methionine} + \text{acetate}.\]

However, the methyl group of methylcysteine comes from methionine, this reaction cannot be therefore regarded as de novo methionine synthesis.

The results obtained with the me-2, sup26 strain indicate that homocysteine synthase may be involved in de novo synthesis of homocysteine in Neurospora as observed previously in Aspergillus nidulans mutants blocked in the synthesis of cysteine from O-acetylserine (Pieniążek et al.; 1973, Paszewski & Grabski, 1975). No similar mutants have been identified in Neurospora.

In Aspergillus mutant, suAmeth, which shares many properties with scon\(^c\), homocysteine synthase, cystathionine \(\beta\)-synthase and \(\gamma\)-cystathionase - the three enzymes constituting the alternative pathway of cysteine synthesis - are simultaneously and proportionally derepressed. In scon\(^c\) there is a slight derepression of homocysteine synthase coinciding with considerable derepression of \(\gamma\)-cystathionase. This may account for the fact that suAmeth accumulates large amounts of cystathionine, while scon\(^c\) metabolizes this compound efficiently to cysteine and further to hypotaurine. suAmeth suppresses lesions in cystathionine \(\gamma\)-synthase and \(\beta\)-cystathionase genes. Whether scon\(^c\) mutations suppress similar lesions in Neurospora remains an open question, difficult to answer because, when scon\(^c\) is crossed with another strain, the trait is transmitted to the progeny with a very low frequency (Burton & Metzenberg, 1972).

It is worth noting that in the scon\(^c\) and suAmeth strains methionine content is not increased above the level found in the wild type. This could mean that both in Neurospora and Aspergillus the rate of methionine synthesis is limited by the second precursor, that is methyltetrahydrofolate, or the activity of methionine synthase (methyltetrahydrofolate: homocysteine methyltransferase, EC 2.1.1.14).
REFERENCES


**Wpływ mutacji regulatorowych w metabolizmie siarki na aktywność enzymów syntetyzujących cysteинę i homocysteинę u Neurospora Crassa**

**Streszczenie**

1. Badano regulację czterech enzymów biorących udział w syntezie cysteiny i homocysteiny, t.j. syntazy cysteiny, syntazy homocysteiny, β-syntazy cystationiny i γ-cystationazy, w szczepie dzikim i mutanach regulacyjnych systemu siarkowego u *Neurospora crassa*.

2. Syntaza homocysteiny i β-syntaza cystationiny podlegają regulacji, ale tylko pierwsza pozostaje pod kontrolą systemu regulacyjnego *cys-3*-scon, o którym wiadomo, że kontroluje szereg enzymów metabolizmu siarkowego, z γ-cystationazą włącznie.

3. Wyniki otrzymane dla niektórych mutantów silnie sugerują, że fizjologiczną rolą syntazy homocysteiny jest udział w alternatywnej drodze syntez myoniny. Aktywność syntazy cysteiny była podobna we wszystkich badanych szczepach i nie zależała od warunków wzrostu.

4. Szczep *scon*, o zdereprymowanym poziomie enzymów metabolizmu siarkowego, wykazuje podwyższoną pulę aminokwasów siarkowych z wyjątkiem metioniny. Szczególnie charakterystyczna jest zawartość w puli hipotauryny --- produktu katabolizmu cysteiny.

Received 2 April, 1980.