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# Comparative studies on *O*-acetylhomoserine sulfhydrylase: physiological role and characterization of the *Aspergillus nidulans* enzyme*

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***O*-acetylhomoserine sulfhydrylase (OAH SHLase) from *Aspergillus nidulans* is an oligomeric protein with a broad substrate specificity with regard to sulfhydryl compounds. As its *Saccharomyces cerevisiae* counterpart the enzyme also reacts with *O*-acetylserine and is inhibited by carbonyl reagents but not by antiserum raised against the yeast enzyme. In contrast to *Saccharomyces cerevisiae* the enzyme is not essential for *Aspergillus nidulans* as indicated by the completely prototrophic phenotype of OAH SHLase-negative mutants. Its major physiological role in *Aspergillus nidulans* seems to be recycling of the thiomethyl group of methylthioadenosine but it is also a constituent of the alternative pathway of cysteine synthesis.**

There are two pathways for synthesis of homocysteine and cysteine in fungi (Fig. 1) but their physiological role varies from species to species (for review see [1]). For instance, in filamentous fungi like *Aspergillus nidulans* and *Neurospora crassa* cysteine is mainly synthesized from *O*-acetylserine (OAS)[1] and it serves as a precursor of homocysteine synthesized *via* cystathionine. It was shown in *A. nidulans* that the alternative pathway of cysteine synthesis involving *O*-acetylhomoserine sulfhydrylase (OAH SHLase), cystathionine β-synthase and cystathionine γ-lyase (Fig.1 steps 11, 12 and 13) is active when the main pathway is impaired [2, 3]. Consequently both pathways must be blocked in the cysteine-requiring mutant. A similar situation was found in *Cephalosporium acremonium* [4]. In contrast, single mutants defective in OAH SHLase in *Saccharomyces cerevisiae* are auxotrophs which grow either on methionine or cysteine, indicating that the enzyme is essential for this yeast. The yeast enzyme has been purified and characterized by Yamagata and coworkers [5 - 8] and the gene coding for the enzyme has been cloned and sequenced [9]. It was of interest to determine if differences in physiological role of OAH SHLase between *A. nidulans* and *S. cerevisiae* are reflected in the differences in the enzyme properties. For this purpose we have purified and partly characterized the enzyme from *A. nidulans* [10]. In this work we present additional biochemical and genetic data bearing on physiological role of OAH in *A. nidulans* and show further differences with the homologous *S. cerevisiae* enzyme.

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[1] Abbreviations: DMDSe, dimethyldiselenide; $CH_3SeH$, methylselenide; OAH SHLase, *O*-acetylhomoserine sulfhydrylase; OAS, *O*-acetylserine

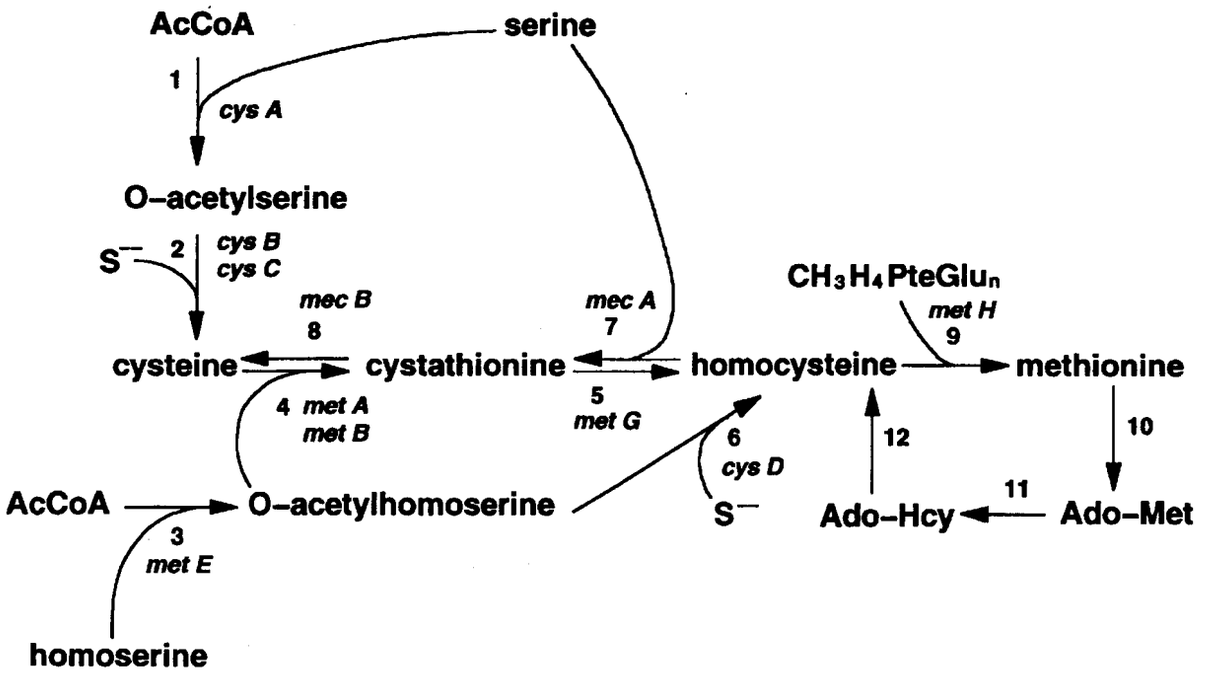


*Fig. 1. An outline of sulfur-containing amino acid metabolic pathways in fungi.*
Enzymes: 1, serine transacetylase; 2, *O*-acetylserine sulfhydrylase; 3, homoserine transacetylase; 4, cystathionine γ-synthase; 5, cystathionine β-lyase; 6, *O*-acetylhomoserine sulfhydrylase; 7, cystathionine β-synthase; 8, cystathionine γ-lyase; 9, methionine synthase; 10, *S*-adenosylmethionine synthase; 11, various methyltransferases; 12, *S*-adenosylhomocysteine hydrolase.

## MATERIALS AND METHODS

***Biological material.*** The following strains of *Aspergillus nidulans* were used: *sG8, paba-A2,yA1; cysB102,pyroA4,yA1; cysB10,biA1,phen-A2; cysB102,cysD11; cysD11,riboB5,yA1.* The wild type strains with respect to sulfur metabolism used as reference were *pyroA4,yA1* and *prolA2,pabaA2,biA1.*

*paba, pyro, cys, bi, phen* and *ribo* denote blocks in the synthesis of paraaminobenzoic acid, pyridoxine, cysteine, biotine, phenylalanine and riboflavine, respectively; *y* denotes yellow conidia.

***Media, culture conditions and extract preparation.*** Liquid minimal medium and culture conditions were as described previously [2]. Mycelia were collected on surgical gauze, washed with water and blotted on filter paper. Mycelial pads were mixed with an equal volume of powdered glass and ground in a chilled mortar with 0.1 M potassium phosphate buffer, pH 7.5. The homogenates were centrifuged at 4°C at 15000 × *g* for 10 min and supernatants (4 - 8 mg protein/ml) were used for enzyme assays.

***Isolation of OAH SHLase deficient mutants.*** Mutants defective in OAH SHLase were isolated in *cysB* strain as resistant to dimethyldiselenide (DMDSe) by the modified procedure of Treichler *et al.* [4]. In cells the reagent is reduced to methylselenide ($CH_3SeH$), a substrate for OAH SHLase. In the reaction with OAH very toxic selenomethionine is formed. Therefore mutants defective in OAH SHLase are resistant to DMDSe.

The *cysB102* strain was used for mutagenesis since mutations affecting *cysD* gene in this strain lead to auxotrophy due to blocking of both pathways of cysteine synthesis (Fig. 1). Conidia of this strain, suspended in 0.9% NaCl ($2 \times 10^7$ cells/ml), were mutagenized with 254 nm UV at 1 - 10 per cent survival and plated on minimal medium supplemented with 0.2 mM L-methionine. A drop of 1000-fold diluted DMDSe in ethanol was applied on the inner side of the cover of a Petri dish with plated conidia in the amount corresponding to 1 - 2 μM concentration. As DMDSe is volatile at room temperature the dishes were sealed with

plastic packing tape and incubated at 37°C. The DMDSe-resistant colonies appeared within 7 - 14 days, and were retested for drug resistance, cysteine or methionine auxotrophy and enzyme activity.

***Purification of O-acetylhomoserine sulfhydrylase.*** Large-scale purification of OAH SHLase to homogeneity from *A. nidulans* has been described previously [10]. In this work we adapted a simplified, small-scale purification procedure: the extract from 20 g of blotted mycelium in 50 mM Tris/HCl buffer, pH 7.8, containing 80 μM pyridoxal 5'-phosphate and 1 mM EDTA was prepared by grinding in a mortar. The homogenate (5 mg protein/ml) was heated at 65°C for 10 min and cleared by centrifugation. The supernatant was applied onto a DEAE-cellulose column (2 × 10 cm), washed with 40 ml of the extraction buffer followed by 80 ml of 60 mM NaCl in the same buffer. The enzyme was eluted with 60 ml of 100 mM NaCl in the same buffer. Fractions of 8 ml, containing highest enzyme activity were pooled, concentrated with Amicon and kept in ice or stored at –20°C.

***Enzyme assays.*** Protein was estimated by the method of Bradford [11]. OAH SHLase was assayed in the reaction mixture described previously [12] using 1 mM final concentration of $Na_2S$. Homocysteine formed was estimated by the method of Kredich & Tomkins [13]. One unit of the enzyme was defined as the amount producing 1 μmole of the product per minute under the assay conditions used.

Reactivity of the enzyme towards various sulfhydryl compounds was studied by substituting them for sulfide in the standard reaction mixture. Formation of thioethers was followed by monitoring the disappearance of thiol groups by the method of Kredich & Tomkins [13]. The same sulfhydryl compounds were added to the growth medium. The appearance of the resulting thioethers in the reaction mixture and in mycelial extracts was demonstrated by thin-layer chromatography on cellulose plates (20 × 20 cm) with n-butanol:acetic acid:water (12:3:5) as solvent. The thioethers were identified by staining the plates with chloroplatinic reagent [15].

***Immunochemical method.*** The enzyme was incubated at 37°C for 10 min in 1 ml of 0.05 M Tris/HCl buffer, pH 7.8, containing 1 mM EDTA, 0.2 mM PLP and appropriate amount of the antiserum raised against OAH/OAS SHLase from *S. cerevisiae*. The reactions were started by the addition of OAH and sulfide to the reaction mixtures and incubated for 10 min.

***Other methods.*** Molecular mass of the enzyme was determined by HPLC gel filtration using a G3000SW column equilibrated with 0.05 M Tris/HCl buffer, pH 7.5, containing 1 mM EDTA, 0.2 mM PLP, 0.1 M NaCl and 20% glycerol. Ferritin (540 kDa), liver glutamate dehydrogenase (330 kDa), catalase (240 kDa), lactic dehydrogenase (140 kDa) and hemoglobin (64.5 kDa) were used as molecular mass standards. Molecular mass of OAH SHLase was also determined by gel filtration through AcA34 in 50 mM Tris/HCl buffer using catalase (240 kDa), albumin (68 kDa) and ovalbumin (45 kDa) as standards and by sucrose gradient centrifugation (5 - 35%) at 40000 r.p.m. for 45 h.

***Reagents.*** Dimethyldiselenide was purchased from Janssen Biochemika. *O*-Acetylserine and *O*-acetylhomoserine were synthesized by the method of Wiebers and Garner [14]. L-Methionine, L-cysteine, DL-homocysteine and pyridoxal 5'-phosphate were from Sigma. DEAE-cellulose DE52 was from Whatman, Sephadex G-150, and Blue Dextran 2000 were from Pharmacia, AcA34 was from LKB. The rest of chemicals were of the highest purity available.

## RESULTS

Table 1 summarizes a 70-fold purification of OAH SHLase. High recovery of the enzyme which was also observed in a large-scale purification [10] may suggest that some inhibitors were present in the crude extract and were removed during purification. This partly purified preparation gave three bands on SDS polyacrylamide gel electrophoresis (not shown) and was stable for at least one week at –20°C when frozen. It could be further stabilized at least for 6 weeks by addition of glycerol (20%). The enzyme is adsorbed on phenyl-Sepharose and octyl-Sepharose which indicates its hydrophobic character. However, the use of these adsorbents did not lead to further purification of the enzyme.

Molecular mass determined by gel filtration through AcA34 was 267000 ± 19000 Da whereas determined by HPLC gel filtration through

Table 1
*Purification of O-acetylhomoserine sulfhydrylase. For details see Materials and Methods*

| Steps | Volume (ml) | Total protein (mg) | Specific activity (units/mg protein) | Total activity (units) |
|---|---|---|---|---|
| 1. Extract | 84 | 391 | 0.063 | 24.5 |
| 2. Heat treatment | 74.5 | 184 | 0.139 | 25.5 |
| 3. DEAE-cellulose chromatography | 26.4 | 5.7 | 4.440 | 25.5 |

G3000SW was 263000 Da (Fig. 2). However, when molecular mass was determined by sucrose gradient centrifugation the enzyme sedimented more slowly than catalase (240000 Da). These results may imply that the enzyme has a rather loose structure which gives a large molecular mass in gel filtration.

Inhibition of the enzyme by carbonyl reagents was investigated (Table 2). Among six reagents tested, hydroxylamine-hydrochloride and phenylhydrazine-hydrochloride were most effective; 2-mercaptoethylamine inhibited moderately. This pattern of inhibition by the reagents is similar to many PLP-requiring enzymes, as shown for *Schizosaccharomyces pombe* OAH sulfhydrylase [17], indicating that the enzyme catalyzes the sulfhydrylase reaction with PLP as the coenzyme. Since a strong binding of PLP to OAH SHLase was suggested in the previous paper [10], the effect of PLP on the enzyme protection from denaturation was investigated. The results presented in Fig. 3 demonstrate that PLP added to the reaction mixture did not increase protection of the enzyme from denaturation by urea, guanidine hydrochloride (0 - 0.36 M) and heating (35 - 70°C). These results imply that the enzyme was in the form of holo-enzyme during the experiments.

Various sulfhydryl compounds were found to be substitutes for sulfide in the reaction catalyzed by OAH SHLase. Except for cysteine, the

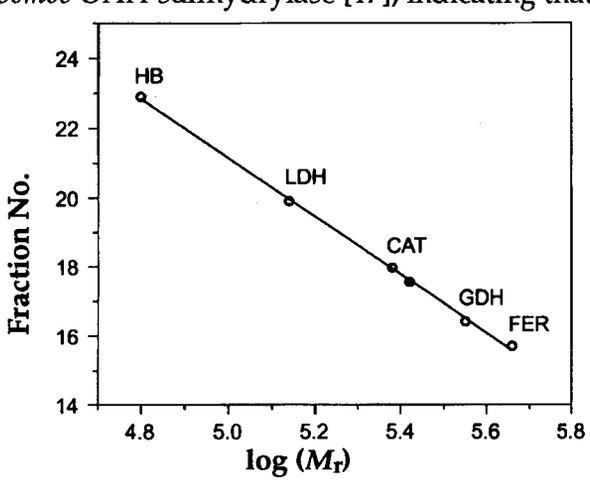


*Fig. 2. Determination of the relative molecular mass of the enzyme by HPLC on a G3000SW column.*
The enzyme (0.02 units) and standard proteins (0.04 - 0.06 ml each of 2 mg/ml solutions) were applied separately to a G300SW column (Tosoh, 0.75 x 30 cm) and eluted at 26°C at a flow rate of 0.3 ml/min. The eluate was fractionated by 0.51 ml. Other conditions are described in the text. Symbols are as follows: HB, bovine hemoglobin ($M_r$ 64,500); LDH, rabbit muscle lactate dehydrogenase [EC 1.1.1.27] ($M_r$ 140,000); CAT, bovine liver catalase [EC 1.11.1.6] ($M_r$ 248,000); GDH, bovine liver glutamate dehydrogenase [EC 1.4.1.3] ($M_r$ 330,000); FER, bovine liver ferritin ($M_r$ 540,000).

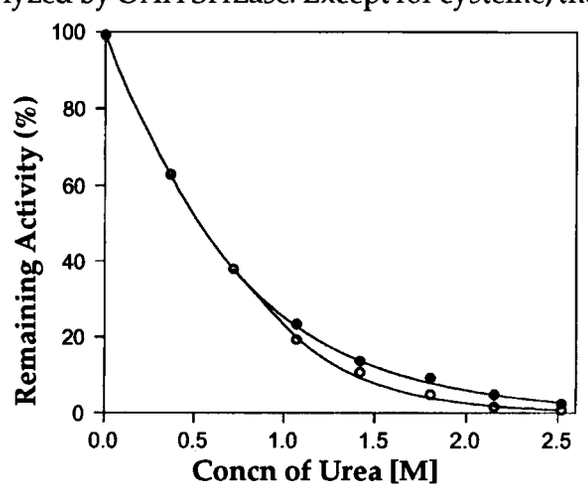


*Fig. 3. Denaturation of the enzyme with urea.*
7 milliunits of the enzyme were dissolved in 0.5 ml of 0.1 M Tris/HCl buffer (pH 7.8) containing 1 mM EDTA, 10% glycerol and urea at various concentrations as indicated. The mixtures were incubated at 37°C for 15 min in the presence ( ● ) and absence ( ○ ) of 0.2 mM PLP. After the incubation, PLP was added at a concentration of 0.2 mM to the enzyme solutions incubated without PLP. The sulfhydrylase reaction was started by the addition of OAH (5 mM) and $H_2S$ (1 mM) and continued for 10 min. Values shown in the figure were obtained from duplicated determinations of the activity.

Table 2

*Inhibition of O-acetylhomoserine sulfhydrylase by carbonyl reagents*

The enzyme (0.06 units) was preincubated at 30°C for 10 min in 0.1 ml of Tris/HCl buffer, pH 7.8, containing 1 mM EDTA, 0.2 mM dithiothreitol and 25% (v/v) glycerol with the carbonyl reagents indicated at the concentration of 1 mM. The enzyme reaction was carried out for 10 min with 5 mM OAH as the substrate

| Reagents | Activity (μmoles homocysteine/reaction) | Ratio |
|---|---|---|
| None | 1.76 | (100) |
| Hydroxylamine-HCl | 0.27 | 15 |
| L-Penicillamine | 1.59 | 90 |
| Semicarbazide-HCl | 1.75 | 99 |
| Phenylhydrazine-HCl | 0.53 | 30 |
| KCN | 1.71 | 97 |
| 2-Mercaptoethylamine | 1.16 | 66 |

Table 3

*Reactivity of sulfhydryl substrates with O-acetylhomoserine catalyzed by O-acetylhomoserine sulfhydrylase in vitro*

Sulfur substrates were added to a final concentration of 0.05 mM. Potassium phosphate buffer concentration was 10 mM and O-acetylhomoserine concentration was 2 mM. Reaction was carried out for 60 min. The disappearance of sulfhydryl groups was followed by the method of Kredich & Tomkins [13]

| Substrate | Reaction efficiency (%) |
|---|---|
| Sulfide | 100 |
| Mercaptoethanol | 97 |
| Thioglycerol | 81 |
| Thioglycolic acid | 65 |
| Cysteine | 0 |

Table 4

*Dependence of strain sensitivity to DMDSe on activity of O-acetylhomoserine sulfhydrylase*

| Strain | Specific activity of OAH SHLase (milliunits/mg protein) | Growth inhibiting concentration of DMDSe (μmole/litr) |
|---|---|---|
| *cysB* | 243 | 0.1 |
| Wild type | 78 | 0.4 |
| *metE* | ND* | 2.0 |
| *cysD* | 15 | 2.0 |
| *cysBcysD* | 25 | 2.0 |

*ND, not determined

other three compounds tested reacted readily (Table 3). The identical thioethers were formed *in vitro* and *in vivo* in the *cysB* strains by OAH SHLase (Fig. 4). We have not observed formation of the respective thioethers in the strains carrying *cysD* mutation (not shown). Thioglycolic acid is probably not transported to the cells so there was no accumulation of the corresponding thioether in the mycelium.

Differences between OAH SHLase from *A. nidulans* and *S. cerevisiae* were demonstrated using antiserum raised against the *S. cerevisiae*

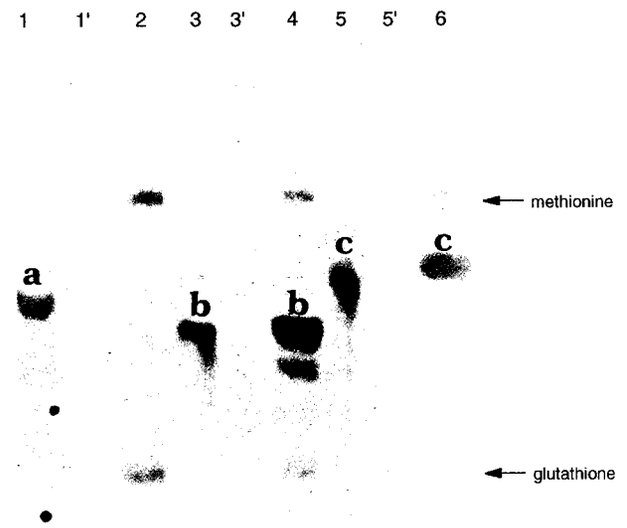


*Fig. 4. Formation of thioethers from different mercaptans in crude extracts and mycelium of cysB strain.* Amount of material applied was adjusted to minimize the background of other sulfur-containing compunds so that thioethers of interest (arrows) are better visible. The results are qualitative. The photograph presents the negative of the original stained plate. Lanes 1, 3, 5 – products of *in vitro* reaction of OAH with thioglycolic acid (a), thioglycerol (b) and mercaptoethanol (c), respectively (1', 3', 5' – controls without OAH). Lanes 2, 4, 6 – extracts of mycelia grown in the presence of thioglycolic acid (no accumulation of thioether corresponding to that found in *in vitro* reaction), thioglycerol and mercaptoethanol.

enzyme since no inhibition was observed in the reaction catalyzed by the *A. nidulans* enzyme for the same amounts of antiserum which caused 50% inhibition of the reaction catalyzed by *S. cerevisiae* enzyme.

In order to determine the physiological role of OAH SHLase in *A. nidulans* we isolated mutants defective in this activity. The positive selection protocol for isolation of such mutants was based on the observation that the sensitivity of a strain towards dimethyldiselenide correlates with the level of the enzyme activity: the higher the activity, the higher the sensitivity to the reagent (Table 4). A number of OAH SHLase negative mutants (*cysD*) along with mutants impaired in acetylation of homoserine (*metE*, see Fig. 1) and some mutants of unknown nature were isolated (Table 5). All mutants classified as impaired in OAH sulfhydrylase exhibit some residual activity of the enzyme (from 7 to 32 per cent of the parental strain). This residual activity does not seem to provide a physiological pathway of homocysteine synthesis as the double mutants *cysD,cysB* are tight auxotrophs. When *cysD* mutations obtained in the present work were outcrossed from *cysB* mutation present in the parental strain it appeared that none of them causes auxotrophy.

Table 5

*Growth characteristics of dimethyldiselenide resistant mutants isolated in cysB strain*

| Mutant type | Growth on minimal medium supplemented with: | | | | Number of mutants obtained | Mutant genotype inferred |
|---|---|---|---|---|---|---|
| | none | methionine | cysteine | *O*-acetylhomoserine | | |
| 1 | – | + | + | – | 22 | *cysD* |
| 2 | – | + | – | + | 12 | *metE* |
| 3 | + | + | + | + | 46 | not known |

## DISCUSSION

The results presented here indicate that O-acetylhomoserine sulfhydrylase of *A. nidulans* exhibits a broad specificity towards sulfhydryl substrates including mercaptoethanol, thioglycerol, thioglycolic acid and methylselenide. Therefore, besides its role in the recycling of methylthio groups from methylthioadenosine [16] the enzyme may be involved in removing the excess of other mercaptans. It does not react, however, with cysteine. This is an important feature of the enzyme as cysteine, along with OAH, is a substrate for cystathionine γ-synthase, the enzyme of the main pathway of methionine synthesis (Fig.1). Therefore, when cysteine is available and cystathionine γ-synthase is active, most, if not all OAH is used for cystathionine and not for direct homocysteine synthesis. Auxotrophy of *metG* mutants and the accumulation of cystathionine in them [3] supports this conclusion.

The reactivity of the enzyme with methylselenide with formation of the very toxic selenomethionine enabled us to isolate a large number of OAH SHLase negative mutants. Interestingly, all of them showed a considerable residual OAH SHLase activity which means that the previously isolated *cysD11* mutant [2] was not an exceptionally leaky one. It seems likely that the fungus shows an additional enzyme with OAH SHLase activity which is unstable as its activity was lost both in the large-scale [10] and small-scale purification procedures. The use of the double mutants *cysD11,metA17* and *cysD11,mecA1* ruled out a possibility that the residual activity can be attributed to cystathionine γ-synthase or cystathionine β-synthase – in all of them residual OAH SHLase activity was found. It is worth noting that two OAH SHLases have been found in *S. pombe* (Brzywczy & Paszewski, unpublished) one of which reacts only with OAH but not with OAS. This monofunctional OAH SHLase had been purified earlier from *S. pombe* by Yamagata [17]. It seems possible that the residual activity observed in *A. nidulans cysD* mutants is due to a similar enzyme.

The *Aspergillus* sulfhydrylase also shows activity with O-acetylserine (OAS) [10] so it resembles the *S. cerevisiae* enzyme and thus can be called OAH/OAS sulfhydrylase. However, the prototrophy of OAH SHLase – defective mutants described here indicates that the enzyme is not essential for *A. nidulans*. This is in marked contrast to the situation observed in *S. cerevisiae*. In fact the latter organism seems rather unique in this respect as OAH SHLase-negative mutants isolated by the same method in a few other yeast genera were found to be prototrophs as is *A. nidulans* (Brzywczy & Paszewski, unpublished). The enzyme thus appears to play different physiological roles in various fungi.

The results described here confirm those described earlier [10] in that OAH SHLase from *A. nidulans* differs in many aspects from the homologous enzyme of *S. cerevisiae*. Molecular mass of the *Aspergillus* enzyme is about 250000 - 260000 Da with a subunit weight of approx. 43000. This suggests a hexameric structure while the *S. cerevisiae* enzyme is a tetramer. The *Aspergillus* enzyme is more heat resistant and binds PLP much more strongly than its yeast counterpart [7]. In fact it was impossible to establish the dissociation constant for the *Aspergillus* enzyme due to a very low stability of the apoenzyme [10]. The fact that antiserum raised against the *S. cerevisiae* enzyme does not inhibit the *Aspergillus* enzyme provides further evidence of marked differences between these two enzymes. Molecular characterization of the *Aspergillus* enzyme will provide additional evidence whether it is homologous with its yeast counterpart.

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