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Cystathionine Metabolism in Methionine Auxotrophic and Wild-Type Strains of *Saccharomyces cerevisiae*

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The role of cystathionine in methionine biosynthesis in wild-type and auxotrophic strains of *Saccharomyces cerevisiae* was studied. Homocysteine and cysteine-requiring mutants were selected for detailed study. Exogenously supplied cystathionine, although actively transported by all strains tested, could not satisfy the organic sulfur requirements of the mutants. Cell-free extracts of the wild-type, homocysteine, and cysteine auxotrophs were shown to cleave cystathionine. Pyruvic acid and homocysteine were identified as the products of this cleavage. A mutant containing an enzyme which could cleave cystathionine to homocysteine in cell-free experiments was unable to use cystathionine as a methionine precursor in the intact organisms. The significance of this finding is discussed.

In most organisms, the classical biosynthetic pathway for methionine includes a step for the conversion of the sulfur-containing amino acid cysteine to its four carbon homologue, homocysteine. It has been postulated that this conversion involves the enzymatic synthesis and cleavage of the seven carbon thioether, cystathionine.

It was concluded from early isotopic competition studies with *Escherichia coli* that cystathionine was not involved in methionine formation in that organism (3, 16). These early studies were reviewed by Balish and Shapiro, whose recent experiments affirmed that cystathionine is a methionine precursor in *E. coli* and *Aerobacter aerogenes* (1). The accumulation of cystathionine by methionine auxotrophs has been demonstrated in *A. aerogenes* and *Neurospora crassa* (11, 12).

The role of cystathionine in methionine biosynthesis in *N. crassa* was studied extensively by Wiebers and Garner (22–24). They recently concluded that cystathionine is not an obligate intermediate in methionine formation; in fact, cysteine and methionine are synthesized by separate, direct biosynthetic pathways (25).

Although several hundred methionine auxotrophs of *Saccharomyces cerevisiae* have been isolated and characterized in our laboratory, none was found to grow when supplied cystathionine. Many clones, however, responded to either cysteine or homocysteine. This paper reports the results of our studies on the metabolism of cystathionine by wild-type and methionine-requiring mutants of yeast.

MATERIALS AND METHODS

Modified Wickerham's synthetic medium (15) without methionine was used as the basic medium for testing the growth requirements of various methionine auxotrophs. Appropriate additions were made to the medium to give a final concentration of 1 mM of the additive. The mutants were grown for 24 hr in yeast-complete medium (18), washed three times in sterile distilled water, resuspended in water, and streaked onto the surface of the prepoured agar. The plates were observed for growth at 24, 48, and 72 hr. We considered using one or more of the auxotrophs in a microbiological assay of the cystathionine enzymatic cleavage products. Serine, homoserine, alanine, α-amino-*n*-butyric acid, pyruvic acid, α-ketobutyric acid, cysteine, homocysteine, cystathionine, and methionine were tested for their ability to sustain growth of the mutants.

^{35}S-cystathionine was isolated from *N. crassa* according to the methods of Wiebers and Garner (22, 24; *personal communications*). ^{35}S-cysteine-HCl was obtained from Calbiochem (Los Angeles, Calif.), and $Na_2{}^{35}SO_4$ was obtained from Abbott Laboratories (North Chicago, Ill.).

Radioactivity was assayed by use of a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) by the procedure of Pigg et al. (15). Amino acids were assayed according to the procedure of Moore and Stein (13).

In the investigation of amino acid accumulation, the procedure of Sorsoli et al. was used (17). In one series of experiments, the cells were collected onto membrane filters (Carl Schleicher and Schuell Co.; type B6) and were washed with iced minimal medium. The filters were then dried and placed in scintillation vials. The accumulated radioactivity was assayed with

the liquid scintillation spectrometer. To recover transported ^{35}S-cystathionine from the organisms, the cells were exposed to labeled cystathionine for 2 hr. An extract was made from ethyl alcohol, and the amino acids were chromatographically separated (14). To locate the radioactive compounds, the migration path was cut into squares. The papers were added to scintillation vials, and the radioactivity was determined.

Cell numbers were measured nephelometrically with a model 9 nephlo-colorimeter (Coleman Instruments Corp., Maywood, Ill.). One nephelos unit was equivalent to 1.8×10^4 cells per ml of culture. Direct microscopic cell counts and samples of aqueous cell suspensions dried to constant weight were used for preparing calibration curves.

The strains of *Saccharomyces* used in the cell-free experiments were grown aerobically in yeast-complete medium (18) for 24 to 48 hr at 30 C. The organisms were then harvested by centrifugation and resuspended in synthetic minimal medium overnight. The cells were recollected, washed, and suspended in a 25% volume of 0.1 M sodium citrate, *p*H 7.5. The viscous cell suspensions were disrupted by subjection to 10,000 lb. of pressure in an Eaton press (7). An equal volume of citrate buffer was added to the disrupted cells to extract the soluble protein and to thaw the mixture. Cells and debris were removed by centrifugation in a Servall RC-2 refrigerated centrifuge at 0 C at approximately $20,000 \times g$ for 30 min. The cell-free extract was frozen in 5-ml samples. We observed that the preparation lost activity upon thawing and refreezing.

Small molecular weight constituents were eliminated from the crude cell extracts by gel filtration through a column of Sephadex G-50. The protein was eluted with 0.1 M phosphate buffer (*p*H 7.5).

To produce cleavage of cystathionine, the complete reaction mixture was composed of 1 ml of the cell extract (10 to 15 mg of protein), 10 μg of pyridoxal phosphate, 1.6 μg of magnesium sulfate, and 50 μmoles of DL-allo-cystathionine in a final volume of 3 ml. The reaction was incubated for 30 min at 37 C and was terminated by addition of 0.5 ml of freshly prepared 25% trichloroacetic acid.

Enzymatic activity (8) was followed by use of 5,5′-dithiobis-(2-nitrobenzoic acid). The yellow color was easily discernible; it developed instantaneously and showed no fading during the experiment.

The extraction sequence of Friedman and Haugen (9) was used to determine the keto acids by the formation of the 2,4-dinitrophenyl-hydrazone (5). The keto acid phenylhydrazones were chromatographically separated (4). In addition, we recorded (by use of a Cary model 11 spectrophotometer) the absorption spectra of the colored solution which developed when strong base was added to the phenylhydrazones of the cleavage products of cystathionine. The 2,4-dinitrophenylhydrazones of authentic pyruvic acid and ketobutyric acid were prepared and treated in the same manner.

Sulfhydryl compounds were identified (10) as adducts of *N*-ethylmaleimide (NEM). The complete reaction mixture was the same as described above, except that 100 μmoles of NEM and 2 μc of ^{35}S-cystathionine were also included. Following an incubation period of 1 hr, the reaction was terminated by the addition of trichloroacetic acid. The mixture was filtered and the components of the filtrate were chromatographically separated (14). Chloroplatinate reagent (21) was used to locate the sulfur-containing compounds, and this process was followed by use of 0.5% ninhydrin in *n*-butyl alcohol. We made strips [1 or 1.25 inches (2.5 or 3.2 cm) wide] of the filter paper by cutting in the direction of the solvent migration, so that the excised area contained all of the material originally applied to the chromatogram. The strips were assayed for radioactive areas by use of a Vanguard-TMC chromatogram scanner; the results were compared to the ninhydrin- and sulfur-positive reference spots of the NEM derivatives of cysteine and homocysteine.

In addition to the chromatographic technique used for identification of the sulfhydryl components, a microbiological assay was employed. Following an incubation period of 30 min at 37 C, the reaction was terminated by use of trichloroacetic acid. The protein precipitate was extracted with ether. The precipitate was filtered, brought to 12 ml by the addition of 0.1 M phosphate buffer (*p*H 7.5), and filter sterilized. A 2.5-ml amount of the sterile material was added to four tubes, each containing 5 ml of double-strength modified Wickerham's medium without methionine. Each growth mixture was brought to a final volume of 10 ml by the addition of sterile phosphate buffer, *p*H 6.5. Following the same procedure, four additional tubes containing medium were filled with a mixture of 25 μmoles of L-cysteine, 25 μmoles of L-homocysteine, 25 μmoles of cystathionine, phosphate buffer, and the product of a control reaction mixture without substrate. Cultures (24 hr) of yeast mutant strains me-3 and me-4 (grown in yeast-complete medium) were harvested, washed, and resuspended in sterile distilled water. Each organism was inoculated into two tubes of each type of medium. The tubes were incubated at 30 C with agitation. Growth was determined nephelometrically at 12, 24, and 48 hr.

RESULTS

The growth requirements of selected methionine auxotrophs are shown in Table 1. Mutants in group me-4 required cysteine, homocysteine, or methionine for growth and were designated cysteine mutants. On the other hand, group me-3 mutants required homocysteine or methionine for growth and were designated homocysteine mutants. Neither of these mutant groups could grow when inoculated onto a medium containing cystathionine as a sole organic sulfur source.

The rapid uptake of randomly ^{14}C-labeled algal hydrolysate and methionine in wild-type yeast has been reported. The accumulation process was shown to be under the control of a single gene

and was relatively nonspecific (17). Figure 1 shows that ^{35}S-cysteine, ^{35}S-cystathionine, and ^{14}C-methionine were actively transported by wild-type cells. The experiment revealed that cystathionine was transported by yeast cells. A direct comparison of cystathionine uptake in wild-type, me-3, and me-4 clones is shown in Fig. 2.

The possibility remained that an enzyme cleaved the thioether before its active transport, and only the radioactive sulfur was accumulated. To test this possibility, we isolated the ^{35}S-cystathionine accumulated by the organisms. A compound migrating with an R_F of 0.16 to 0.24 and identifiable with authentic radioactive cystathionine was extractable from these cells.

TABLE 1. *Growth requirements for selected methionine auxotrophs of Saccharomyces cerevisiae*[a]

Addition to growth medium	Auxotrophic group		
	Me-4	Me-3	Me-1
None	−	−	−
L-Cysteine	+	−	−
Cystathionine	−	−	−
L-Homocysteine thiolactone	+	+	−
L-Methionine	+	+	+

[a] None of the following compounds was able to sustain growth of the organisms: DL-serine, DL-homoserine, DL-alanine, α-amino-*n*-butyric acid, pyruvic acid, α-ketobutyric acid.

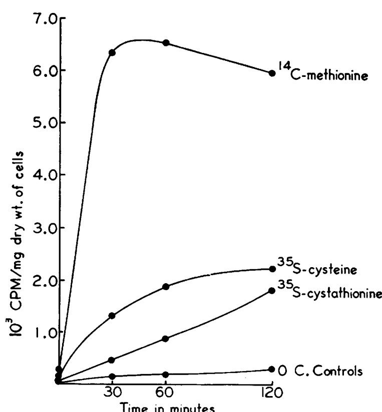

FIG. 1. *Methionine, cysteine, and cystathionine accumulation in wild-type Saccharomyces cerevisiae. The procedure used to measure labeled amino acid uptake was identical to that previously reported (17). The amino acids were at a final concentration of 1 mM and contained 0.25μc of either ^{14}C- or ^{35}S-labeled substrate per ml of reaction mixture. The 0 C controls were held in an ice bath during the reaction period and washing procedures.*

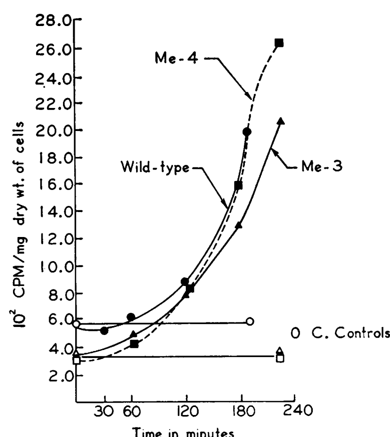

FIG. 2. *Cystathionine accumulation in wild-type and methionine auxotrophic strains of yeast. The reaction conditions were the same as those in Fig. 1, except that the cells were recovered on membrane filters, as described in Materials and Methods. The ^{35}S content was normalized to allow for isotopic decay. Symbols; ▲, me-3; ■, me-4; and ●, wild-type; △, me-3 at 0 C; □, me-4 at 0 C; ○, wild-type at 0 C.*

It was obvious that rapid degradation of cystathionine was occurring in these cultures. The enzymatic cleavage products of cystathionine were analyzed by use of cell-free extracts of the organisms. The R_F values of the chromatographed keto acid 2,4-dinitrophenyl-hydrazones (derived from the enzymatic assays) are listed in Table 2. Slight variations in R_F values are attributed to differences in sodium carbonate concentration in the samples. However, chromatography of the unknown phenylhydrazone with that of pyruvate resulted in a single spot. α-Ketobutyric acid phenylhydrazone was added to a control mixture, and two distinct migrating materials were detected. These materials corresponded to the predicted R_F values for the α-ketobutyrate and pyruvate conjugates. Within the limits of this procedure, no α-ketobutyrate was produced enzymatically from cystathionine. Pyruvate was the only keto acid detected in these experiments.

We determined the absorption spectra of the NaOH-developed color of the phenylhydrazones of the keto acids formed by enzymatic cleavage of cystathionine. These spectra were compared to those of authentic α-ketobutyric acid and pyruvic acid. The derivative of pyruvate, as well as the phenylhydrazones from the product of the enzymatic reaction mixtures prepared from the wild-type clone and mutant me-3, showed identical spectra with an absorbancy maximum at 435 mμ. Neither an absorption peak nor a shoulder was observed at 385 mμ, where the α-ketobutyrate derivative demonstrated maximal absorption.

TABLE 2. R_F *Values of keto acid 2,4-dinitrophenyl-hydrazones*[a]

Phenylhydrazone	R_F		
	Me-3	Me-4	WT
Unknown	0.39	0.37	0.38
Pyruvic acid	0.41	0.40	0.42
α-Ketobutyric acid	0.71	0.63	0.60
Unknown + pyruvic acid	0.37	0.37	0.36
Unknown + α-ketobutyric acid	0.38	0.37	0.37
	0.66	0.67	0.58

[a] Phenylhydrazones of reference compounds and enzymatic cleavage products were chromatographically separated as described in Materials and Methods.

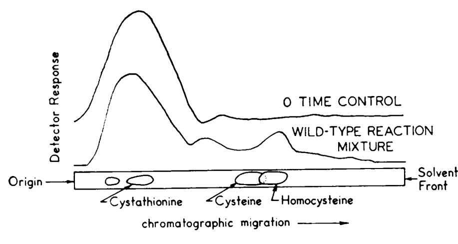

FIG. 3. *Cystathionine cleavage by a wild-type clone of yeast. The enzymatic reaction mixture and the chromatographic conditions were described in Materials and Methods. Only the chromatogram for the incubated reaction mixture is shown. The zero-time control contained only cystathionine, as is indicated by the detector response of the radiochromatogram scanner.*

These data substantiate the chromatographic evidence that pyruvic acid is the keto acid formed from cystathionine by the enzymes of wild-type and homocysteine-cysteine mutants.

Although spots produced by the paper chromatographic separation of the NEM derivates oı homocysteine and cysteine overlapped, these spots were discernibly different (Fig. 3). When the NEM derivatives of the enzymatic cleavage of ^{35}S-cystathionine by wild-type cells were chromatographed and scanned, the response of the detector to the radioactivity on the paper spot which, as expected, was the major peak; we also observed a second unknown peak and a third discrete peak which corresponded to the homocysteine reference spot. Although the cysteine and homocysteine reference spots overlapped, the radioactivity was associated with the homocysteine spot. The radioactivity did not extend far enough to include all of the area which would be associated with cysteine. The area on the strip corresponding to the homocysteine derivative was found to be chloroplatinate and ninhydrin positive and contained ^{35}S. The control tracing, to which trichloroacetic acid was added at zero time, showed only a single radioactive peak. The area on the strip was chloroplatinate and ninhydrin positive and corresponded to the cystathionine reference spot.

The enzymatic mixture extracted from me-3, a homocysteine-requiring mutant, cleaved cystathionine in the same manner (Fig. 4). The degradative products of cystathionine formed in this reaction were identical to those produced by the wild-type organism. Homocysteine was one of these products. The ^{35}S-containing component migrating between cystathionine and cysteine was tentatively identified as homocysteine thiolactone.

As a further check on the above studies, a microbiological assay of the products of cystathionine cleavage was attempted by use of me-3, the homocysteine-requiring mutant. If homocysteine is produced by the enzymatic extracts, it should be able to satisfy the organic sulfur requirement for this organism (cysteine was not able to do so). The results of the assay may be seen in Table 3. The mutant responded to some compound in the reaction mixture, as shown by an increase in growth. Homocysteine appeared to be the growth-promoting compound, since the organism was previously shown to be unresponsive to the other cleavage products of cystathionine (Table 1). The growth response of me-4, a cysteine-requiring mutant, was significantly above the control values but was not as extensive as the growth response of the other mutant.

DISCUSSION

Cystathionine did not satisfy the organic sulfur requirement for mutant clones of yeast blocked in the methionine biosynthetic pathway. We thought that this occurred because cystathionine was unable to penetrate the yeast cell membrane. Our experimental results, however, indicated that

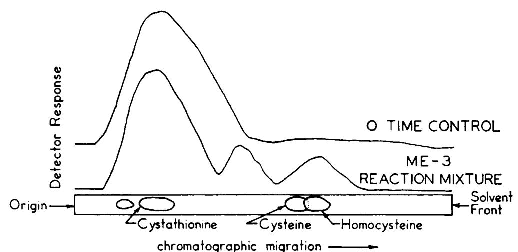

FIG. 4. *Cystathionine cleavage by extracts of yeast mutant me-3. The conditions were identical to those in Fig. 3.*

TABLE 3. *Microbiological assay of enzymatic cleavage products of cystathionine*[a]

Growth medium addition	Incubation of Me-3 (hr)			Incubation of Me-4 (hr)		
	12	24	48	12	24	48
Buffer	11	10	11	32	35	55
Cysteine	10	8	11	38	51	93
Homocysteine thiolactone	23	67	100	49	100	100
Cystathionine	10	8	16	28	33	57
Substrateless reaction products	13	14	14	20	18	31
Reaction products	48	61	61	52	65	93

[a] The growth indicated beneath the time designation is expressed as nephelos units and is the average of duplicate determinations. Growth medium was Wickerhams without methionine. The enzymatic reaction mixtures are described in Materials and Methods.

exogenously supplied cystathionine was transported intracellularly in wild-type and methionine auxotrophic clones of this organism. Our data also showed that both the wild-type *Saccharomyces* and the homocysteine-requiring groups contained an enzymatic mechanism for producing pyruvate and homocysteine from cystathionine. It is difficult to understand why the auxotrophs me-3 and me-4 were unable to utilize exogenously supplied cystathionine, since the enzymatic mechanism for homocysteine formation from cystathionine exists in extracts of these organisms.

The possibility exists that the degradation of cystathionine to homocysteine in vitro was due to a reaction of no biosynthetic importance. Evidence indicates that the enzyme involved was cysteine desulfhydrase (6). This enzyme is believed to produce thiocystine (T. W. Szczepkowski and J. L. Wood, Federation Proc. **24**:219, 1965), as well as pyruvate, H_2S, and ammonia (2), from cysteine.

Since enzymes may be oriented in such a manner to facilitate transfer of substrate, it is possible that the enzyme which catalyzed the reaction also produced a compound which was compartmentalized in the whole cell. Such compartmentalization may make the homocysteine formed unavailable for methionine biosynthesis but still subject to degradative reactions. It is possible that, in whole cells, homocysteine is desulfhydrated to become a source of homoserine or desulfhydrated and deaminated to become an additional source of α-ketobutyrate. Compartmentalization and metabolic isolation have been demonstrated in yeast (19, 20).

An inhibitor which prevents the enzymatic cleavage of cystathionine to homocysteine may exist in the whole cell, and this inhibitor could be labile under the conditions of extract preparation. If such a specific inhibitor does exist, its structure would be genetically determined, and cystathionine-utilizing mutants lacking the inhibitor should be obtainable. None has ever been observed in this organism, although an extensive search for such mutant clones was made in our laboratory.

Thus, whatever the explanation, the paradox remains. Exogenously supplied cystathionine cannot be used as a homocysteine precursor by organisms which have the mechanism to cleave the thioether to homocysteine. Our data do not permit an evaluation of the possible involvement of endogenously generated cystathionine as a methionine precursor. In another ascomycete, however, it seems clear that cystathionine is not an obligate intermediate in methionine biosynthesis (25).

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