JOURNAL OF BACTERIOLOGY, Oct. 1972, p. 299-303
Vol. 112, No. 1

Cystathionine Synthesis in Yeast: an Alternative Pathway for Homocysteine Biosynthesis

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Received for publication 3 April 1972

Cystathionine synthesis from *O*-acetylhomoserine and cysteine has been demonstrated in yeast extracts for the first time. The activity is less than that of *O*-acetylhomoserine sulfhydrylase, but it is higher than that reported for homoserine *O*-transacetylase and therefore should not be growth limiting. Cystathionine synthase seems to share the regulatory properties of the sulfhydrylase, and both activities are missing from the methionine auxotroph *Saccharomyces cerevisiae* EY9, suggesting that both reactions are catalyzed by the same enzyme. However, cystathionine synthase activity was lost during purification of the sulfhydrylase, suggesting that the two reactions may be catalyzed by separate enzymes. Since previous studies have shown that yeast extracts can catalyze the cleavage of cystathionine to homocysteine, our results show the existence of two complete alternate pathways for homocysteine biosynthesis in yeast. Which of these is the major physiological pathway remains to be determined.

Homocysteine is synthesized in *Neurospora* (8), and in *Escherichia coli* (12) and *Salmonella* (3), by reactions 1 and 2 (the bacterial substrate being the succinyl, rather than the acetyl, ester of homoserine).

O-Acetylhomoserine + cysteine →
cystathionine + acetate (1)

Cystathionine + H_2O →
homocysteine + NH_3 + pyruvate (2)

These organisms are also able to synthesize homocysteine directly from sulfide by reaction 3, first discovered in *Salmonella* (4).

O-Acetylhomoserine + sulfide →
homocysteine + acetate (3)

Various kinds of evidence indicate that reaction 3 does not function effectively in the cell (7), among them being the fact that mutants lacking only β-cystathionase (EC 4.2.1.15) (catalyzing reaction 2) require methionine for growth. In *Salmonella*, reactions 1 and 3 are both catalyzed by the same enzyme (4), which has been isolated in pure form (6). The acetylhomoserine sulfhydrylase of *Neurospora* (reaction 3) has been extensively purified and does not catalyze reaction 1 (7); the latter is catalyzed by a complex of two easily separated proteins (M. A. Savin and M. Flavin, *unpublished results*).

Yeast extracts catalyze reaction 2, but reaction 1 has been detected only at minute rates (9). Cherest et al. (2) have found that sulfhydrylase activity is high in wild-type extracts and is missing from the methionine auxotroph EY9 (*met-8*), and they have concluded that homocysteine is synthsized primarily by reaction 3. We were prompted to reexamine the possible alternate role of reaction 1 in yeast by the recent finding that certain folate derivatives are allosteric activators of cystathionine γ-synthase in *Neurospora* (13).

MATERIALS AND METHODS

Microbial strains and growth media. *Saccharomyces cerevisiae* AN33 (α thr_1 arg_1) and EY9 (α met_8) were a gift of R. K. Mortimer. *Saccharomyces carlsbergensis* was obtained from the American Type Culture Collection (ATCC 9373). Sources and growth media for the strains of *Salmonella typhimurium* (3) and *Neurospora crassa* (9) used in these experiments have been previously described. *Neurospora tetrasperma* was obtained from the Fungal Genetics Stock

Center at Dartmouth College (FGSC 1270) and grown as previously described (9). Yeasts were grown either on nutrient medium (1% Difco Bacto-Peptone, 1% Difco yeast extraction, 2% glucose) or on the minimal salts medium of de Robichon-Szulmajster and Magee (11), supplemented where indicated with 2×10^{-3} M L-methionine.

Enzyme preparations. Fifty-milliliter starter cultures of yeasts were used to inoculate 500 ml of medium in Fernbach flasks. Cells were grown at 30 C on a rotary shaker to mid-exponential phage, harvested by centrifugation at 14,000 × *g*, washed with water, and then suspended in 2 volumes of the following buffer: potassium phosphate, *p*H 7.5, 0.5 M; ethylenediaminetetraacetic acid, 0.01 M; pyridoxal phosphate, 5×10^{-5} M; and dithiothreitol, 0.01 M. The cells were broken in an Aminco french pressure cell at 8,000 psi, and then subjected to 120 sec of ultrasonic treatment at 2 C in 30-sec bursts. The extracts were centrifuged at 14,000 × *g* for 20 min and the pellet was discarded. The resultant crude extract was subjected to gel filtration on Sephadex G-25 to remove all low-molecular-weight species (9) prior to assay. *Neurospora* (9) and *Salmonella* (5) extracts were prepared as previously described.

Materials. The preparation and sources of *O* acetylhomoserine (10) and tris(hydroxymethyl)aminomethane-^{35}S-sulfide (7) have been described. N^5-methyltetrahydrofolate monoglutamate and triglutamate were the generous gift of Warwick Sakami, and the N^5-methyltetrahydrofolate heptaglutamate was the gift of Bernard T. Kaufman. ^{35}S-Cystathionine was prepared enzymatically from ^{35}S-cysteine and *O*-succinylhomoserine by using purified *Salmonella* cystathionine γ-synthase (6) and was isolated by high-voltage paper electrophoresis (9). *O*-acetylhomoserine sulfhydrylase activity was assayed by using radioactive sulfide as described by Kerr (7). The rate of cystathionine synthesis was measured by using the procedure developed for *Neurospora* by Kerr and Flavin (9), except that reactions were carried out at *p*H 8.2 in potassium pyrophosphate buffer. In this assay, extracts are incubated with ^{35}S-cysteine and *O*-acetylhomoserine for 30 min at 30 C, and the reaction products are isolated by high-voltage paper electrophoresis at *p*H 1.7 for 4 hr. The procedure separates ^{35}S-cystathionine from the radioactive substrate and from ^{35}S-homocysteine. In several experiments, the identity of the cystathionine spot on electrophoresis was confirmed by thin-layer and paper chromatography in several solvent systems.

Purification of O-acetylhomoserine sulfhydrylase. Fifteen liters of minimal salts medium in a carboy was innoculated with a 500-ml culture of *S. carlsbergensis*. After overnight growth, cells were harvested by centrifugation in a Sharples centrifuge and extracted as described above. The extract was diluted to a protein concentration of 20 mg/ml with the same buffer used for extraction. After adjusting the *p*H to 7.1, the extract was rapidly brought to 60 C in a boiling water bath, held at that temperature for exactly 5 min, and then rapidly cooled to 15 C in an ice bath. The heated extract was centrifuged, the pellet was discarded, and the supernatant fraction was diluted with extraction buffer (*p*H 7.1) to a protein concentration of 10 mg/ml. 1.1% (w/v) protamine sulfate was added with constant stirring to bring the final protamine sulfate concentration to 0.1% (w/v). After 10 min of stirring, the mixture was centrifuged at 14,000 × *g* for 20 min and the pellet was discarded. The supernatant fluid was brought to 80% saturation with solid ammonium sulfate (535 gm/liter), keeping the *p*H at about 6.1 with NH_3. After 30 min of stirring, the material was centrifuged at 14,000 × *g* and the pellet was dissolved in sufficient extraction buffer (*p*H 7.1) to bring the protein concentration to 10 mg/ml. After overnight dialysis against the same buffer, the solution was brought to 40% saturation with solid ammonium sulfate (230 g/liter) while keeping the *p*H at 7.1 and was centrifuged after 30 min, and the pellet was discarded. The supernatant was brought to 55% saturation with an additional 95 g of solid ammonium sulfate/liter, was stirred for 30 min, and centrifuged, and the pellet was dissolved in a small amount of extraction buffer. A 1.2- by 20-cm column of diethylaminoethylcellulose (Whatman DE52) was equilibrated with the same buffer at *p*H 7.32. The sample was dialyzed overnight to remove residual ammonium sulfate. After adjusting the *p*H of the sample to 7.32 with NH_3, it was applied to the column and eluted with a linear gradient of KCl from 0 to 0.5 M in the same buffer. Fractions showing peak sulfhydrylase activity were combined and concentrated by precipitation with 80% ammonium sulfate. The peak of sulfhydrylase activity appeared near the beginning of the gradient.

RESULTS AND DISCUSSION

The new extraction procedure described here has yielded yeast extracts which synthesize cystathionine from acetylhomoserine and ^{35}S-cysteine at a rate at least 10 times greater than previously observed (9). Protoplast lysates give similar results. Although the rate is only one-fifth of that in *Neurospora* extracts (Table 1), it exceeds the rates reported for the synthesis of acetylhomoserine in yeast (2, 10), and is probably sufficient to support growth. With these reaction mixtures, *Neurospora* extracts also yield small amounts of labeled homocysteine, which can be attributed to the presence of β-cystathionase (reaction 2); yeast extracts yield proportionately more homocysteine (see below). A second difference is that the rate of reaction 3 is much higher in yeast than in *Neurospora* (Table 1). The rates of reactions 1 and 3 were both reduced when methionine was present in the growth medium, and both reactions were absent from the yeast methionine auxotroph EY9 (Table 1). These results suggested that yeast resembles *Salmonella*, where reactions 1 and 3 are catalyzed by the same enzyme (4). However, when the sulfhydrylase was purified (Table 2), the abil-

ity to catalyze reaction 1 was lost, as in *Neurospora* (7). Yamagata (14) has also recently reported that sulfhydrylase partially purified from baker's yeast does not catalyze reaction 1.

This study of yeast was prompted by the discovery that N^5-methyl-tetrahydrofolate polyglutamates are allosteric activators of cystathionine γ-synthase in *Neurospora* (13); the activity of fresh, gel-filtered extracts of *N. crassa* was stimulated by and, in some other *Neurospora* species, almost completely dependent upon, the addition of the activator (Table 3). However, in yeast extracts, reaction 1 was not stimulated by the folate derivatives but was somewhat inhibited (Table 3), perphaps nonspecifically since the mono-, tri-, and heptaglutamates had similar effects. The folate derivatives had no significant effect on reaction 3 in either yeast or *Neurospora*.

Cherest et al. (2) reported that the sulfhydrylase in yeast is inhibited by methionine with a K_i of 1.9×10^{-3} M. Cystathionine synthesis in yeast was also inhibited by comparable levels of methionine. *Neurospora* cystathionine γ-synthase is subject to allosteric inhibition by *S*-adenosyl-methionine; the reaction

TABLE 1. *Rates of catalysis of cystathionine and homocysteine synthesis by crude extracts of several strains of yeast and by cystathionine synthase-deficient mutants of Neurospora crassa and Salmonella typhimurium*

Organism	Growth medium	Relative rates of synthesis (nmole/min/mg)		
		From ^{35}S-cysteine and *O*-acetylhomoserine[a]		From ^{35}S-sulfide and *O*-acetylhomoserine[a]
		Cystathionine	Homocysteine	Homocysteine
Saccharomyces carlsbergensis	Minimal	3.0	1.9	165
S. carlsbergensis	Methionine	1.2	1.0	27
Saccharomyces cerevisiae AN33	Complex	2.0	1.5	70
S. cerevisiae EY9 (*met$_8$*)	Complex	0	0	0
Neurospora crassa Wild-type EM 5297	Minimal	15	≤1.5	8
N. crassa 36104A (*me-3*)	Methionine	0	0	8
N. crassa 4894a (*me-7*)	Methionine	0	0	8
Salmonella typhimurium Wild-type (Lt-2)	Minimal	24	0	21
S. typhimurium Wild-type (Lt-2)	Methionine	21	0	18
S. typhimurium met-B15	Methionine	0	0	0

[a] In the *Salmonella* strains *O*-succinylhomoserine was used.

TABLE 3. *Effects of N^5-methyltetrahydrofolate derivatives on the rate of cystathionine synthesis in extracts of fungi and bacteria*

Organism	Folate	Concentration (mM)	Cystathionine synthesis (nmole/min/mg)	Percent of control rate (without added folate)
Saccharomyces carlsbergensis	None		3.67	100
	CH_3-FH_4G_1[a]	0.015	1.51	41
	CH_3-FH_4G_3[b]	0.015	1.57	43
	CH_3-FH_4G_7[c]	0.015	1.44	39
Neurospora crassa	None		3.06	100
	CH_3-FH_4G_7[c]	0.015	8.30	272
Neurospora tetrasperma	None		0.17	100
	CH_3-FH_4G_7[c]	0.015	2.76	1620
Salmonella typhimurium	None		24.0	100
	CH_3-FH_4G_3[b]	0.015	24.0	100

[a] N^5-methyltetrahydrofolate monoglutamate.
[b] N^5-methyltetrahydrofolate triglutamate.
[c] N^5-methyltetrahydrofolate heptaglutamate.

TABLE 2. *Purification of O-acetylhomoserine sulfhydrylase activity from Saccharomyces carlsbergensis and rates of cystathionine synthesis in fractions*

Step	Treatment	Protein (mg)	Activity (nmole/min/mg)		Total units sulfhydrylase (nmole/min)	Recovery (%)
			Sulfhydrylase	Cystathionine synthesis		
1	Crude extract	10,000	55	1.61	550,000	100
2	Heat-protamine sulfate	1,110	340	0	377,000	69
3	40 to 55% $(NH_4)_2SO_4$	244	1,200	0	294,000	53
4	Diethylaminoethyl-cellulose	38	2,100	0	80,000	15

was somewhat inhibited in yeast extracts by adenosylmethionine, but only at higher concentrations than would indicate a regulatory function. One other attempt was made to unmask higher cystathionine synthase activity in yeast by using cells "permeabilized" by nystatin (1), but these preparations catalyzed reaction 1 at about the same rate as extracts and were not stimulated by folate derivatives.

The results so far indicate that the yeast sulfhydrylase is separable from cystathionine synthase activity, as in *Neurospora*, but that both activities are absent from the same mutant and share the same regulatory properties, unlike *Neurospora*. The question arises whether one product (homocysteine or cystathionine) is formed secondarily from the other, so that the same enzyme is needed for both reactions but a second enzyme is also needed for one of them. To determine whether the relatively large amounts of homocysteine formed in incubations with cysteine and acetylhomoserine (Table 1) originated secondarily from cystathionine by reaction 2, yeast extract was incubated with 1 mM ^{35}S-cystathionine and the radioactive products were identified by high-voltage paper electrophoresis. No labeled homocysteine could be detected. The second possibility, sequential formation of cystathionine from homocysteine, implies that the labeled cysteine is contaminated with sulfide; this is likely, since the purified sulfhydrylase (Table 2) continued to form homocysteine from ^{35}S-cysteine and acetylhomoserine. In this case homocysteine would be formed first by reaction 3, and cystathionine would be formed from serine and homocysteine; the enzyme responsible for the latter reaction, cystathionine β-synthase, is present in yeast (3). This seemed unlikely since there was no evident pool of serine in the reaction mixtures. It was further ruled out by the results of Table 4: added serine did not increase cystathionine formation, and a pool of unlabeled homocysteine decreased cystathionine labeling slightly, but not enough to have been a precursor. It appears, then, that cystathionine is formed directly from cysteine and acetylhomoserine by yeast.

TABLE 4. *Effects of unlabeled pools of* L-*serine and* L-*homocysteine on the rate of incorporation of* ^{35}S *into cystathionine from* ^{35}S-*cysteine in a crude extract of Saccharomyces cerevisiae AN33*

Addition	Concentration (μmole/ml)	^{35}S-cystathionine formed (μmole/30 min/ml)
None		0.21
L-Serine	2.0	0.18
L-Homocysteine	2.0	0.14

The absence of both activities from EY9 might be explained if EY9 produced an inhibitor of both activities, if EY9 were a regulatory mutant, or if in fact both activities were catalyzed by one enzyme, and the disappearance of one activity were an artifact of purification. The first possibility is ruled out because addition of extracts of EY9 did not inhibit reaction 1 or 3 in wild-type extracts. The last possibility has been discussed elsewhere in relation to *Neurospora* (7).

The results show the presence of two alternate pathways for homocysteine biosynthesis in yeast, either by trans-sulfuration, reactions 1 and 2, or by direct sulfhydrylation, reaction 3. Which of these is the major physiological pathway remains to be determined.

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