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Cystathionine as a Precursor of Methionine in *Escherichia coli* and *Aerobacter aerogenes*

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ABSTRACT

BALISH, EDWARD (Argonne National Laboratory, Argonne, Ill.), AND STANLEY K. SHAPIRO. Cystathionine as a precursor of methionine in *Escherichai coli* and *Aerobacter aerogenes*. J. Bacteriol. 92:1331–1336. 1966.—Cystathionine has been shown to be a precursor of methionine biosynthesis in *Escherichia coli* and *Aerobacter aerogenes*. A double enzyme assay was developed to show the formation of homocysteine from cystathionine. The results obtained support the concept that cystathionine serves as a precursor of methionine via the intermediate formation of homocysteine. The latter compound is methylated by the homocysteine methyltransferase of these microorganisms. Sulfhydryl and keto acid assays were used to demonstrate cystathionase activity. Methionine represses both homocysteine methyltransferase formation and cystathionase formation. However, the presence of methionine in reaction mixtures resulted in product inhibition of homocysteine methyltransferase activity, but not of cystathionase activity.

The thioether, cystathionine, has been implicated as an intermediate in the biosynthesis of methionine in microorganisms. Cystathionine has been found to accumulate in methionine mutants of *Aerobacter aerogenes* (7) and *Neurospora crassa* (8). It has also been demonstrated that cystathionine can satisfy the methionine requirement of some mutant strains of *Escherichia coli* (9, 21, 25; J. S. Gots and W. Y. Koh, Bacteriol. Proc., p. 134, 1950), *Neurospora* (8), and *Salmonella* (2). Enzymes have been described in cell-free extracts of *Neurospora* (2, 3, 5), yeasts (2), *Salmonella* (2), and *E. coli* (2, 14, 25) which cleave cystathionine to homocysteine, pyruvate, and ammonia. The biosynthesis of methionine by methylation of the homocysteine derived from cystathionine has been demonstrated with cell-free extracts of *E. coli* PA 15 by Rowbury and Woods (14). On the other hand, Roberts et al. (13) found that the addition of unlabeled cystathionine to cultures of *E. coli* growing in the presence of S^{35}-sulfate did not decrease the level of incorporation of label into methionine, indicating that cystathionine is not an intermediate in methionine biosynthesis. It has also been demonstrated that *N. crassa* can form homocysteine enzymatically from homoserine and H_2S (24), which implies that cystathionine is not an obligate precursor of homocysteine.

The following report demonstrates the formation of methionine in reaction mixtures with cystathionine as the source of the methyl acceptor for the adenoslymethionine:homocysteine methyltransferase or the methylmethionine:homocysteine methyltransferase of various strains of *E. coli* and *A. aerogenes*.

MATERIALS AND METHODS

Organisms. E. coli Texas and *E. coli* Texas M, a methionine auxotroph, were originally described by McRorie et al. (11). *E. coli* K-12 (ATCC 10798), *E. coli* B (ATCC 11303), and two other methionine auxotrophs, *E. coli* K-12 W-6 (1) and *E. coli* 113-3 (ATCC 11105), as well as various strains of *A. aerogenes* (16), were also employed in these studies. *Saccharomyces cerevisiae* was used to prepare yeast methyltransferase (18).

Compounds. Commercial L-methionine-$C^{14}H_3$ was used to prepare the corresponding labeled sulfonium compounds. Adenosylmethionine was isolated from yeast cells by the procedure of Shapiro and Ehninger (19). Methylmethionine was synthesized according to the method of Toennies and Kolb (23). L-Homocysteine was prepared by the exposure of commercial L-homocysteine thiolactone to 0.3 M NaOH for 7 min. An equal amount of 0.3 M KH_2PO_4 was added for neutralization. All other compounds were of commercial origin. The DL form of cystathionine was used.

Media. A minimal medium (16) and a medium with

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1.5% peptonized milk (Difco) with 0.75% K_2HPO_4 (*p*H 7.0) were employed in these studies.

Preparation of cell-free extracts. Unless otherwise stated, the cells were cultured in minimal medium with 0.5 μmole/ml of methionine where required. A 30% suspension of washed, log-phase cells (in 0.1 M phosphate buffer, *p*H 7.0) was exposed to sonic oscillation for 15 min in a Raytheon 10-kc oscillator. The disrupted cells were then centrifuged for 15 min at 15,000 × *g*, and the supernatant material was stored in a deep freeze (−10 C) until used in the reaction mixtures. Protein concentrations of all cell-free extracts were determined according to the procedure of Lowry et al. (10).

Bacterial enzyme assay. The enzymatic synthesis of methionine was measured by the tracer assay of Shapiro and Yphantis (20). Assays were performed with 0.1 ml of a cell-free extract, 4 μmoles of homocysteine, 4 μmoles of methylmethionine-$C^{14}H_3$ or adenosylmethionine-$C^{14}H_3$, and 0.05 μmole of Zn^{++} in 0.1 M phosphate buffer (*p*H 7.0) in a total volume of 0.5 ml. DL-Cystathionine, 8 μmoles/0.5 ml of reaction mixture, was substituted for L-homocysteine in some reaction mixtures. After incubation for 2 hr at 37 C, the reaction mixture was stopped by rapid cooling to 0 C. A 0.1-ml sample of the reaction mixture was placed on a short ion-exchange column [0.5 × 2 cm (Dowex 50-X4), 100 to 200 mesh, Li^+]. The radioactive substrate was retained by the column, whereas the labeled product, methionine, was eluted quantitatively with 1.0 ml of water. Each column eluate was collected in a vial, and 20 ml of a solution of 0.4% 2,5-diphenyloxazole in equal volumes of toluene and ethyl alcohol was added. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. In all cases, the specific activity of the methyl donor was adjusted to permit the detection of 10 mμmoles of methionine per reaction mixture with an accuracy of ±5%. Paper and thin-layer chromatography and high-voltage paper electrophoresis were employed to demonstrate that methionine was the only radioactive product formed in the reaction mixtures.

Double enzyme assay. Homocysteine produced by the enzymatic degradation of cystathionine was detected by use of a double enzyme assay. DL-Cystathionine (8 μmoles) and the bacterial enzyme (2 to 5 mg of protein) were incubated for 2 hr at 37 C in 0.05 M phosphate buffer (*p*H 7.0) in a total volume of 1.2 ml. After the initial incubation period, the bacterial enzyme was inactivated by heating at 70 C for 10 min, and the denatured bacterial protein was removed by centrifugation. A sample of 0.5 ml of the supernatant fluid was added to tubes containing 4 μmoles of methylmethionine-$C^{14}H_3$ and purified yeast methyltransferase (2 to 5 mg of protein) in 0.5 ml of 0.05 M phosphate buffer (*p*H 7.0). A similar quantity of methylmethionine-$C^{14}H_3$ was also added to the remaining 0.7 ml of the heat-inactivated reaction mixture as a control. Further control tubes consisted of yeast enzyme plus cystathionine, and cystathionine alone. After 2 hr of incubation at 37 C, the reaction mixtures were analyzed for methionine-$C^{14}H_3$ by the ion-exchange procedure outlined previously.

Sulfhydryl and keto acid assays. Sulfhydryl production from cystathionine was estimated by use of an aromatic disulfide, 5,5′-dithiobis-(2-nitrobenzoic acid), and then measuring the amount of colored aryl mercaptan formed (4). Keto acids were estimated by use of the methods of Friedeman and Haugen (6).

Chromatography and high-voltage electrophoresis. Whatman no. 1 paper and Eastman Chromagram sheets were employed for ascending chromatography of Dowex 50 eluates and reaction mixtures which had been deproteinized by adding 0.01 ml of glacial acetic acid per reaction mixture. Solvent systems of butanol-acetic acid-water (60:15:25, v/v) and ethyl alcohol-acetic acid-water (64:1:35, v/v) were employed to identify methionine. Amino acids were detected with a spray of 0.25% ninhydrin in acetone containing 1% acetic acid. Pyruvate was identified by the method of Metzler and Snell (12).

High-voltage paper electrophoresis was carried out on Whatman 3-mm paper. A running time of 1 hr at 2,500 v and 120 amp with a buffer consisting of 3% formic acid and 12% acetic acid was used to separate methionine, adenosylmethionine, methylmethionine, adenosylhomocysteine, and cystathionine.

Radioactivity was detected on developed chromatograms with an automatic scanning and recording device (W. Eisler, W. Chorney, and W. E. Kisieleski, Abstr. Am. Chem. Soc. Meeting, p. 12C, 1958).

RESULTS

Methionine biosynthesis with cystathionine as the methyl acceptor. The fact that homocysteine is readily methylated to form methionine in the methylmethionine (or adenosylmethionine), homocysteine methyltransferase system of *E. coli* Texas and *E. coli* Texas M suggested that this might be a very sensitive means to ascertain whether cystathionine was the immediate precursor of homocysteine in this system. To test this hypothesis, cystathionine was substituted for homocysteine in the reaction mixtures, and the

TABLE 1. *Methionine biosynthesis by cell-free extracts of Escherichia coli*[a]

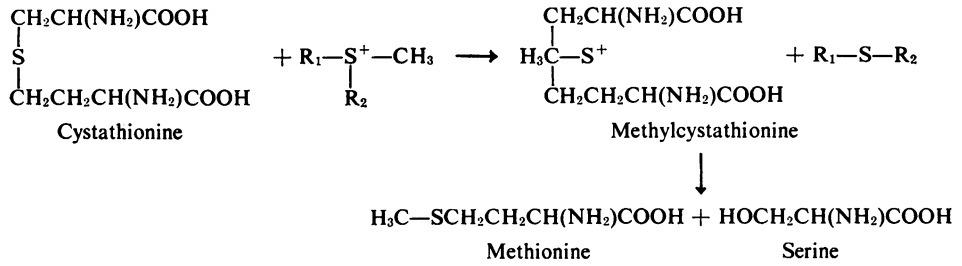
Enzyme source	Methyl donor	Methyl acceptor[b]	
		Homocysteine	Cystathionine
E. coli Texas	Adenosylmethionine	45	31
	Methylmethionine	295	272
E. coli Texas M	Adenosylmethionine	65	36
	Methylmethionine	390	354

[a] Cells were cultured in the peptonized milk medium.

[b] Results are expressed as millimicromoles of methionine per milligram of protein per hour.

results of a typical experiment are shown in Table 1. It is evident that cystathionine is capable of acting as a source of the methyl acceptor for the biosynthesis of methionine. Paper chromatography, thin-layer chromatography, and high-voltage electrophoresis were employed to show that methionine is the only radioactive compound formed in the reaction mixtures with cystathionine. No methionine is formed when either cystathionine or homocysteine is omitted from the reaction mixture. It is also of interest to note that methylmethionine is a more efficient methyl donor than adenosylmethionine.

The fact that cystathionine can substitute for homocysteine in the methyltransferase reaction does not necessarily mean that cystathionine is split to homocysteine prior to methylation. The reaction could occur by a direct methylation of cystathionine with the intermediate formation of methylcystathionine as is shown below. The latter compound was previously proposed by Toennies (22).



No role for homocysteine is postulated in this mechanism.

To determine whether homocysteine is an intermediate in the pathway of methionine biosynthesis from cystathionine, the double enzyme assay described above was employed to detect homocysteine (Table 2). In the double enzyme assay, it may be seen that a substantial amount of radioactive methionine was formed after the addition of purified yeast enzyme (18) and radioactive methylmethionine. In no case was radioactive methionine detected when yeast enzyme was omitted. Since the yeast homocysteine methyltransferase does not utilize cystathionine as a substrate, it seems reasonable to assume that homocysteine was formed in the incubation mixtures containing cystathionine and the various bacterial enzymes. It is also evident (Table 2) that cell-free extracts of all of the bacterial species, except *E. coli* B, were able to form methionine from cystathionine, as shown by the results of the bacterial enzyme assay. However, *E. coli* B can form homocysteine from cystathionine, as indicated by the results obtained from the bacterial and yeast enzyme assay (Table 2).

More direct evidence for the formation of homocysteine from cystathionine is shown in Table 3. It can be seen that all of the bacterial cell-free extracts produced sulfhydryl and keto acid when incubated with cystathionine. Incubation mixtures lacking either cystathionine or enzyme showed no detectable sulfhydryl or keto acid formation. Enzyme from *S. cerevisiae* did not form any sulfhydryl or keto acid when incubated with cystathionine. The values for keto acid production were consistently higher than were the values for sulfhydryl groups. This suggests some loss of sulfhydryl groups during the 2-hr incubation at 37 C. Similar losses of sulfhydryl occurred even when dithiothreitol (10^{-4} M) was added to the reaction mixtures at the start of the experiment.

It was also evident that much more sulfhydryl and keto acid were produced (Table 3) than was indicated by the amount of methionine formed from cystathionine (Table 2). However, no attempts were made to achieve completion of the latter reaction. Since yeast methyltransferase utilizes D- or L-homocysteine, the split of DL-cystathionine to D- or L-homocysteine should not

TABLE 2. *Evidence for homocysteine as an intermediate in methionine biosynthesis from cystathionine and methylmethionine*[a]

Enzyme source	Bacterial enzyme assay[b]	Bacterial and yeast enzyme assay[c]
Escherichia coli Texas	265	135
E. coli Texas M	350	115
E. coli K-12	188	141
E. coli K-12 W-6	97	95
E. coli B	—	25
E. coli 113-3	116	91
Aerobacter aerogenes	232	104
Saccharomyces cerevisiae	—	—

[a] Results are expressed as millimicromoles of methionine per milligram of protein per hour.

[b] Assay for methionine biosynthesis from cystathionine.

[c] Assay for methionine biosynthesis from cystathionine via homocysteine.

TABLE 3. *Sulfhydryl group and keto acid production from cystathionine by cell-free extracts of various microorganisms*[a]

Enzyme source	Sulfhydryl group	Keto acid
Escherichia coli Texas	720	828
E. coli Texas M	980	1,000
E. coli K-12	728	840
E. coli K-12 W-6	408	540
E. coli B	260	362
E. coli 113-3	705	740
Aerobacter aerogenes	800	975
Saccharomyces cerevisiae	—	—

[a] Results are expressed as millimicromoles per milligram of protein per hour.

have affected methionine production as measured by the double enzyme assay. The discrepancy between sulfhydryl and keto acid formation as compared with methionine production could also be explained by an enzymatic split of cystathionine to cysteine and butyrate. The latter reaction has been demonstrated in yeast (2) and liver (15). Although no capacity to split cystathionine was indicated with the purified yeast enzyme employed in these studies, it is possible that the bacterial enzymes used could carry out a similar split. Chromatography of deproteinized reaction mixtures showed that homocysteine and pyruvate, identified as the 2,4-dinitrophenylhydrazine derivative, were the main products of the reaction. However, this does not rule out the possibility that quantities of cysteine and butyrate, too small to be detected by chromatography, were present.

Effect of methionine on methionine biosynthesis. Rowbury and Woods (14) reported that enzyme extracts prepared from *E. coli* which had been grown in the presence of methionine had far less cystathionase activity than those grown without methionine. To determine if methionine repressed cystathionase activity in *E. coli* Texas and *E. coli* Texas M, 0.5 μmole of methionine per ml of medium was added to the peptonized milk and the minimal medium. Cell-free extracts prepared from cells grown in the presence of methionine showed a decrease in methionine biosynthesis when cystathionine was the source of the methyl acceptor. The greatest repression of enzyme activity occurred with cell-free extracts prepared from cells grown in the minimal medium supplemented with methionine (Table 4). Further experiments were carried out to determine whether the effect was due to repression of cystathionase. Table 5 shows that this enzyme was repressed, as indicated by the decrease in activity of the extracts prepared from cells grown in the presence of methionine.

TABLE 4. *Methionine biosynthesis from cystathionine and methylmethionine by cell-free extracts prepared from cells grown in the presence and absence of methionine*[a]

Enzyme source	Culture medium			
	Peptonized milk	Peptonized milk + methionine[b]	Minimal medium	Minimal medium + methionine[b]
Escherichia coli Texas	280	225	340	185
E. coli Texas M	370	265	—	221

[a] Results are expressed as millimicromoles of methionine per milligram of protein per hour.

[b] A 0.5-μmole amount of methionine per ml of culture medium was used.

TABLE 5. *Influence of methionine in the growth medium on cystathionase activity*

Enzyme source	Methionine concn	Sulfhydryl formed[a]	Keto acid formed[a]
	mμmoles/ml		
Escherichia coli Texas	0	980	1,005
	100	915	1,000
	500	552	640
	1,000	500	650
E. coli Texas M	100	1,000	1,100
	500	565	645
	1,000	447	600

[a] Results are expressed as millimicromoles per milligram of protein per hour.

Methionine also inhibited the biosynthesis of methionine, in vitro, with cell-free extracts of *E. coli* Texas and *E. coli* Texas M. Figure 1 shows that methionine acted as an inhibitor of methionine biosynthesis when cystathionine was the source of the methyl acceptor. It should be noted that methionine also inhibits homocysteine methyltransferase activity in *S. cerevisiae* (17). However, up to 10 μmoles of methionine in a reaction mixture failed to inhibit the capacity of cell-free extracts of *E. coli* Texas and Texas M to form sulfhydryl from cystathionine. Therefore, the inhibition observed in extracts of *E. coli* Texas and *E. coli* Texas M is probably due to inhibition of the homocysteine methyltransferase, rather than to inhibition of the cystathionase.

Enzyme stability. A 70 to 75% decrease in methionine production from cystathionine oc-

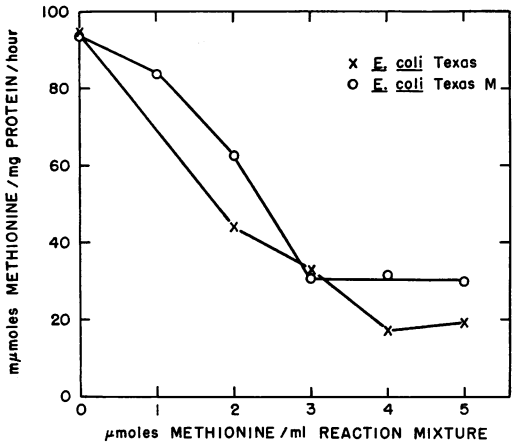


FIG. 1. *Product inhibition of methionine biosynthesis from cystathionine. Standard assay mixtures were prepared with various amounts of methionine.*

curred after the cell-free extracts of *E. coli* Texas and *E. coli* Texas M had been stored at −10 C for 1 month (Table 6). On the other hand, very little loss of methionine-forming capacity was evident when the same enzyme extracts were used with homocysteine as the methyl acceptor. This indicates that the decrease in methionine biosynthesis was due to a loss of cystathionase activity (Table 7). The formation of both sulfhydryl and keto acid were greatly reduced after the enzymes were stored at −10 C for 1 month.

In an effort to stabilize enzyme activity, dithiothreitol was added to the cell-free extracts, but similar losses in activity were observed in these preparations. However, if dithiothreitol (10^{-3} M) was added to reaction mixtures containing fresh enzyme preparations, it resulted in a 35% stimulation of methionine production from cystathionine. It should be pointed out that sulfhydryl reagents are known to stimulate homocysteine methyltransferase activity with extracts of *S. cerevisiae* (17). No increase in the production of sulfhydryl from cystathionine was evident when dithiothreitol was added to protect the sulfhydryl group of homocysteine. As a result, the increased formation of methionine from cystathionine in the presence of dithiothreitol is probably due to a stimulation of the homocysteine methyltransferase enzyme and not due to a stimulation of homocysteine production from cystathionine.

DISCUSSION

The implication of cystathionine as an intermediate compound in the biosynthesis of methionine has resulted from several types of investigation. The data in this report give further impetus

TABLE 6. *Effect of storage at −10 C on methionine biosynthesis from methylmethionine and two methyl acceptors*[a]

Enzyme source	Methyl acceptor			
	Homocysteine[b]		Cystathionine[b]	
	A	B	A	B
Escherichia coli Texas.....	319	302	280	90
E. coli Texas M.....	444	420	370	95

[a] Results are expressed as millimicromoles of methionine per milligram of protein per hour.
[b] A, fresh enzyme preparation; B, activity after 30 days of storage at −10 C.

TABLE 7. *Effect of storage at −10 C on the capacity of cell-free extracts to produce sulfhydryl and keto acid from cystathionine*[a]

Enzyme source	Sulfhydryl formed[b]		Keto acid formed[b]	
	A	B	A	B
Escherichia coli Texas...	715	248	835	297
E. coli Texas M.....	995	304	1,025	323

[a] Results are expressed as millimicromoles per milligram of protein per hour.
[b] A, fresh enzyme preparation; B, activity after 30 days of storage at −10 C.

to the role of cystathionine in methionine biosynthesis. Cystathionine can serve as the source of a methyl acceptor (homocysteine) resulting in the production of methionine by cell-free extracts of *E. coli* Texas, Texas M, K-12, K-12 W-6, and 113-3. *A. aerogenes* also has the enzymatic capacity to form methionine from cystathionine via homocysteine. Rowbury and Woods (14) described the formation of methionine from cystathionine with cell-free extracts of *E. coli* PA 15, and they concluded that cystathionine was first split to homocysteine, which was then methylated to form methionine. These investigators also stated that the final step in the conversion of homocysteine to methionine required serine and a source of the folic acid cofactor system (tetrahydropteroylglutamate and cobalamin). The methylation of homocysteine in the current study involves a direct transfer of a methyl group from adenosylmethionine or methylmethionine to homocysteine. This gives rise to the possibility that different systems exist for the terminal step in methionine biosynthesis.

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