Cleavage of *S*-Ribosyl-L-Homocysteine by Extracts from *Escherichia coli*

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ABSTRACT

DUERRE, JOHN A. (University of North Dakota, Grand Forks), AND CHRIS H. MILLER. Cleavage of *S*-ribosyl-L-homocysteine by extracts from *Escherichia coli*. *J. Bacteriol.* 91:1210-1217. 1966.—Cell-free extracts prepared from *Escherichia coli* catalyze the cleavage of the thioether linkage of *S*-ribosylhomocysteine. One of the products has been identified as homocysteine by paper chromatography of the *N*-ethyl-maleimide derivative and can be measured directly in reaction mixtures by sulfhydryl reagents. The other compound has been tentatively identified as ribose by column chromatography. Free sulfhydryl groups were also detected with use of *S*-adenosylhomocysteine as substrate; however, enzymatic cleavage of the glycosidic linkage of this compound appears to be required prior to cleavage of the thioether linkage. Homocysteine formed from either substrate could be converted readily to methionine, provided the necessary cofactors were added. Some of the properties of the ribosylhomocysteinase are discussed.

It had been reported earlier (6, 7) that various microorganisms, particularly *Escherichia coli*, incorporate substantial amounts of tritiated *S*-ribosylhomocysteine during growth. Inasmuch as the bulk of the tritium from ribosyl-L-homocysteine-H³ appeared in the protein residues, it was suggested that ribosylhomocysteine was split, and the homocysteine moiety was incorporated into protein methionine after methylation. Shapiro (23) came to similar conclusions by use of methionine-requiring mutants of *Aerobacter aerogenes*. In these investigations, it was shown that *S*-ribosylhomocysteine completely replaced the requirement for methionine, provided the methyl donor *S*-methylmethionine was present.

An alternate explanation to the incorporation of homocysteine from *S*-adenosylhomocysteine or its degradation product, *S*-ribosylhomocysteine (4), would be remethylation of either of these compounds to regenerate *S*-adenosylmethionine as follows:

The incorporation of the homocysteine moiety into methionine would then involve the transadenosylation reaction proposed by Pfeffer and Shapiro (18). However, the findings by Rosenthal, Smith, and Buchanan (20), that *S*-adenosylhomocysteine could not serve as methyl acceptor with a transmethylase enzyme from *E. coli*, fails to support such a mechanism. None of these studies identified the necessary enzymes or reaction steps leading to methionine biosynthesis from *S*-ribosylhomocysteine.

This paper reports a cell-free system from *E. coli* which can synthesize methionine with the homocysteine moiety of *S*-ribosylhomocysteine and the hydroxymethyl group of serine. One of the required enzymes catalyzes the cleavage of the ribose-sulfur linkage, yielding free homocysteine and ribose.

MATERIALS AND METHODS

Analytical methods. Amino nitrogen was measured quantitatively by the method of Yemm and Cocking (27), and pentose was measured by the orcinol reaction (3). Protein concentration was determined by the method of Lowry et al. (16). Tetrahydrofolic acid was prepared by the method of Blakley (1). Homocysteine was freshly prepared by treatment of L-homocysteine thiolactone with alkali (9). *S*-Ribosylhomocysteine sulfoxide and homocysteine sulfoxide were prepared by treatment of the corresponding thiols with H₂O₂ (5, 25).

Compounds. Coenzymes, homocysteine thiolactone, methionine, serine, and adenosine were obtained from Calbiochem. *S*-Adenosyl-L-homocysteine and *S*-adenosyl-L-H³-homocysteine were prepared as outlined previously (5). *S*-Adenosylmethionine was prepared by the method of Schlenk and DePalma (22). *S*-Ribosyl-L-homocysteine was prepared enzymatically from crystalline *S*-adenosylhomocysteine as described previously (4). However, it has been found that HCl used as an eluting solvent can be removed successfully by flash evaporation, provided the temperature is maintained below 30 C. The material prepared in this way was found to contain an excessive amount of ninhydrin-positive material in relation to the amount of orcinol-positive material present. Samples were subjected to chromatographic analysis with Dowex 50 H⁺ resin (Fig. 1). The compound was found to be contaminated with a ninhydrin-positive, sulfur-negative, orcinol-negative material with an elution pattern similar to ammonia. This contamination may have resulted from the incomplete removal of ammonium sulfate used in the fractionation of *S*-adenosylhomocysteine nucleosidase (4).

Enzyme. *E. coli* strain W was cultured in 20-liter carboys containing 15 liters of M-9 medium as described previously (4). The cells were harvested and washed twice with 0.05 M tris(hydroxymethyl)-aminomethane (Tris) buffer (pH 7.5). Washed cells were suspended in the same buffer, 1.0 ml for each

400 mg of wet cells, and were disrupted in a nitrogen-cooled pressure cell at 20 to 25,000 psi. Remaining whole cells and debris were removed by centrifugation at 10,000 $\times g$ for 15 min. The supernatant fluid, containing approximately 20 mg of protein per ml, was applied to a column of Sephadex G-25, 2.0 $\times$ 35 cm, previously equilibrated with 0.05 M Tris buffer (pH 7.5), and elution was carried out with the same buffer. Such a column was suitable for removal of small molecular weight material from 30 to 35 ml of cell-free extract.

Enzyme assays and identification of reaction products. The reaction mixture, containing 3.0 μ moles of *S*-ribosylhomocysteine, 100 μ moles of Tris buffer (pH 8.0), and cell-free extract in a total volume of 1.0 ml, was incubated under an atmosphere of nitrogen at 37 C for 4 hr. To stop the reaction, the tubes were placed in a boiling-water bath for 3.0 min and cooled in ice; the precipitate was removed by centrifugation at 15,000 $\times g$ for 10 min. The reaction mixture was then examined for the possible presence of free homocysteine and ribose by thin-layer chromatography.

Thin-layer chromatography. The Serva Thin Layer Chromatography System (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.) was employed with use of Serva TLC cellulose. A slurry, made up of seven parts of water to one of cellulose, was poured onto glass plates measuring 4 $\times$ 7 inches. A layer 500 μ in thickness was left on the plates, by use of the Serva applicator, and the plates were left overnight to dry.

FIG. 1. Chromatographic separation of ammonia and homocysteine from *S*-ribosylhomocysteine. Dowex 50-H⁺ resin, 200 to 400 mesh, was used. The column was 0.7 cm² $\times$ 20 cm; the flow rate, 1.3 ml/min. The column was eluted by a pH gradient formed by mixing 0.2 M sodium citrate (pH 8.3) with an equal volume of 0.2 M citrate buffer (pH 2.9). Both solutions contained 2.0 ml of thiodiglycol and 1.0 g of Brij-35 per liter. Reducing compounds were measured by the orcinol method, whereas amino nitrogen and ammonia were measured by the quantitative ninhydrin tests. (A) Elution pattern of 3.0 μ moles of ribose, 3.0 μ moles of NH₄Cl, and 3.0 μ moles of L-homocysteine. (B) Elution pattern of 3.0 μ moles of *S*-ribosylhomocysteine (crude product).

Samples of deproteinized reaction mixtures, *S*-ribosylhomocysteine, *S*-ribosylhomocysteine sulfoxide, and homocysteine (0.02 to 0.1 μ mole) were placed on spots about 3.0 cm from the bottom of the plate. Ethyl alcohol-water-acetic acid (65:34:1) and propanol-water (80:20) were used as solvents. Homocysteine has a tendency to oxidize during chromatography. This difficulty was overcome by coupling homocysteine with *N*-ethylmaleimide (12). To 0.3 ml of the reaction mixture was added an equal volume of hot 0.375 M *N*-ethylmaleimide in water. The resulting solution was spotted with repeated application (5 to 10 times) of 2.0 μ liters. Amino acids were located by spraying with 0.5% ninhydrin in acetone. The reducing compounds were detected with ammoniacal silver nitrate (2) and sulfur-containing compounds with the iodoplatinate reagent (25).

Direct assays for homocysteine. It was found that the *N*-ethylmaleimide derivative of homocysteine gave a pink color upon the addition of alkali, whereas *S*-ribosylhomocysteine did not. The assay method was carried out as follows: 0.3 ml of deproteinized reaction mixture was added to an equal volume of hot 0.375 M *N*-ethylmaleimide, and the tubes were allowed to stand at least 10 min; then 0.22 ml of 1.0 N NaOH was added. Since the color tends to fade, all tubes were read 30 sec after the addition of alkali in a microcuvette at 520 m μ with a Beckman DU spectrophotometer. Free homocysteine added to the reaction mixture gave a linear response over a range of 30.0 to 360 μ moles (Fig. 2). The linearity of the assay was not affected when homocysteine had been incubated with *E. coli* extract (2.0 mg) for 4.0 hr, nor did any measurable color result from deproteinized solutions without added homocysteine. Neither *S*-ribosylhomo-

cysteine, *S*-ribosylhomocysteine sulfoxide, nor homocysteine sulfoxide gave positive reactions. This test was compared with the Folin reagent (10), by which free sulfhydryl compounds are measured. Considerable difficulty was encountered with this reagent, since a precipitate formed upon the addition of the sodium bicarbonate. This precipitate was removed by centrifugation prior to reading at 725 m μ . It was also found that the deproteinized zero-time cultures gave considerable color. This necessitated the running of a zero-time control for every reaction tube.

RESULTS

Identification of reaction products. Homocysteine was tentatively identified as a reaction product by thin-layer chromatography. A chromatogram illustrating the main points is shown in Fig. 3. In this experiment, samples deproteinized at zero-time were compared with samples taken after 4 hr at 37 C. Samples of *S*-ribosylhomocysteine sulfoxide (RH-S), *S*-ribosylhomocysteine (RH), and homocysteine (H) are shown in the first three spots. Homocysteine incubated with extract for 4 hr (H-4) is also

FIG. 2. Standard curve for determination of free homocysteine with *N*-ethylmaleimide. Known quantities of L-homocysteine (X—X); L-homocysteine incubated with *Escherichia coli* extract (2.0 mg) for 4 hr at 37 C under an atmosphere of nitrogen (●—●); *E. coli* extract alone (■—■). Protein was precipitated as described under Materials and Methods.

FIG. 3. Separation of homocysteine-*N*-ethylmaleimide derivative from *S*-ribosylhomocysteine by thin-layer chromatography. The solvent system used was 80% aqueous propanol. The chromatogram was sprayed with 0.5% ninhydrin in acetone and developed by heating at 110 C. Abbreviations used are ribosylhomocysteine (RH), ribosylhomocysteine sulfoxide (RHS), and homocysteine (H). Numbers after abbreviations denote time of incubation.

shown. Homocysteine-*N*-ethylmaleimide derivative gave a ninhydrin-positive spot with an R_F value of 0.41. Regardless of the length of time homocysteine is left in contact with *N*-ethylmaleimide, a very faint ninhydrin-positive spot appears ahead of the main spot. Both spots gave a positive test for sulfur when treated with iodo-platinate reagent. Although not shown in the chromatogram, free homocysteine spreads out along the path of developing solvent. The leading materials may be free homocysteine. Both homocysteine sulfoxide and *S*-ribosylhomocysteine sulfoxide (RH-S) gave R_F values of 0.08 with use of this solvent system. In the sample containing *S*-ribosylhomocysteine which had been incubated for 4 hr (RH-4), a ninhydrin-positive, sulfur-positive spot with the same R_F value as homocysteine was observed. In the zero-time control (RH-O), no spot was noted. This indicated that free homocysteine was formed during the course of the incubation. Similar chromatograms were sprayed with ammoniacal silver nitrate. No definite indication of reducing compounds with R_F values similar to ribose could be seen. However, the amount of carbohydrate necessary for detection was considerably more than necessary for the detection of homocysteine with iodo-platinate or ninhydrin.

Because of the inability to detect free ribose in the reaction mixture by paper chromatography, experiments were undertaken to determine if any ribose could be detected by column chromatography. The techniques used were similar to those described for the separation of carbohydrates by Khym and Zill (14). Ribose was eluted from Dowex 1 in a very sharp band after passage of about 100 ml of 0.02 M borate through the column. Neither *S*-ribosylhomocysteine nor homocysteine could be removed from Dowex-1 with borate, whereas both were eluted as a single peak with 0.1 N HCl. When *S*-ribosylhomocysteine was incubated with Sephadex-treated cell-free extract, a peak corresponding to ribose could be detected in the reaction mixture (Fig. 4). No such peak was observable in a zero-time control or from extracts incubated for 4 hr without *S*-ribosylhomocysteine. It was found that the amount of orcinol-positive material disappearing from the *S*-ribosylhomocysteine peak was greater than the amount of orcinol-positive material recovered as ribose.

Attempts were made to measure homocysteine directly in the reaction mixtures. It was found that the *N*-ethylmaleimide derivative of homocysteine gave a pink color upon the addition of alkali. This test was compared with the Folin reagent (10), by which free sulfhydryl compounds can be measured. The *N*-ethylmaleimide reagent

FIG. 4. Chromatographic separation of ribose from *S*-ribosylhomocysteine. Dowex 1 resin (borate form); 200 to 400 mesh; 12 cm $\times$ 0.7 cm²; flow rate, 1.0 ml/min. Cross-hatched areas denote orcinol-positive material calculated as ribose equivalents.

was less sensitive than the Folin reagent; but it was routinely used because of closer agreement between duplicates, less interference from other sulfhydryl compounds (presumably proteins), and more reproducible results. Neither test afforded reproducible quantitative measurements which could be used for kinetic studies. However, within the limits of the assays, both were in agreement as to the amount of homocysteine formed from *S*-ribosylhomocysteine (Table 1). The concentration of *S*-ribosylhomocysteine for optimal homocysteine formation was about 0.002 M. It can be seen from Table 1 that *S*-adenosylhomocysteine also served as substrate; however, at a much reduced rate.

Properties of the enzyme. The enzyme was unstable in solutions of low ionic strength, and all activity was lost on dialysis. The greatest protection of the enzyme was achieved by maintaining the concentration of protein above 15 mg/ml in 0.05 M Tris buffer (pH 7.5). Enzyme stored in that way was found to retain about 75% of its activity after 48 hr at 2 C, and 50 to 60% after 1 week. If cell-free extracts were prepared in phosphate buffer, a marked reduction in activity was noted after standing overnight at 2 C. If phosphate buffer was substituted for Tris in the assay, a reduction in activity of about 50% was observed. Passage of extract through Sephadex G-25 appeared to enhance the activity slightly (10 to 20%) over the crude extract. This may have been due to the excessively high readings obtained with zero-time controls with use of the crude extract. No cofactor or metal ion requirements were noted when using Sephadex-treated extracts. No observable difference in activity was noted with hydrogen or nitrogen used in the gas

TABLE 1. Formation of free homocysteine from *S*-adenosylhomocysteine and *S*-ribosylhomocysteine

Substrate	Amt	Gas phase	Free homocysteine as determined by	
			<i>N</i> -ethylmaleimide*	Folin reagent*
	$\mu\text{moles/ml}$		μmoles	μmoles
None.....		N ₂	—†	15
Ribosylhomocysteine.....	0.5	N ₂	45	55
Ribosylhomocysteine.....	1.0	N ₂	108	91
Ribosylhomocysteine.....	2.0	N ₂	216	198
Ribosylhomocysteine.....	3.0	N ₂	254	208
Ribosylhomocysteine.....	3.0	H ₂	208	220
Ribosylhomocysteine.....	3.0	Air	58	74
Homocysteine.....	3.0	N ₂	3,100	2,800
Ribose.....	3.0	N ₂	—†	10
<i>S</i> -Adenosylhomocysteine.....	3.0	N ₂	121	154

* The system contained 100 μmoles of Tris buffer (pH 8.0), varying concentrations of substrate, and 4.3 mg of Sephadex-treated *Escherichia coli* extract per ml. Anaerobic conditions were obtained by passing either nitrogen or hydrogen gas through the tubes for 10 min, at which time the tubes were sealed off and cell-free extract was added by syringe. All tubes were incubated at 37 C for 1 hr. All samples, except tubes containing homocysteine, were read against a blank containing cell-free extract and corresponding substrate deproteinized at zero-time.

† Not measurable.

phase (Table 1). With oxygen in the gas phase, only about 25% as much free homocysteine was measurable as under anaerobic conditions (Table 1). This does not necessarily mean that the thioether linkage of *S*-ribosylhomocysteine was not cleaved in the presence of air. If any of the deproteinized reaction mixtures were allowed to stand in air for a few hours, a marked reduction in measurable free homocysteine was noted.

Figure 5 presents a study of the amount of homocysteine formed after various times of incubation with *S*-ribosylhomocysteine and *S*-adenosylhomocysteine as substrates. It can be seen from the curve that the formation of homocysteine from *S*-ribosylhomocysteine remained essentially linear for the first 2 hr. Thereafter, the activity tended to fall off slightly. Such a decrease in activity was also noted when the concentration of the enzyme was reduced by one half. The formation of free sulfhydryl groups from *S*-adenosylhomocysteine was also measurable; the rate increased over the first 2 hr, then leveled off. In accord with earlier observations, the Sephadex-treated extract was found to hydrolyze the glycosidic linkage of *S*-adenosylhomocysteine, although at a much reduced rate, since the reaction was carried out at pH 8.0. The pH optimum for the *S*-adenosylhomocysteine nucleosidase was reported to be between 6.0 and 7.0 (4). Under the conditions used, 90% of the *S*-adenosylhomocysteine had been degraded to *S*-ribosylhomocysteine after 3 hr as measured by Benedict's Reagent (4). The optimal pH for cleavage of the thioether linkage of *S*-ribosyl-

FIG. 5. Time course of formation of free sulfhydryl groups from *S*-ribosylhomocysteine and *S*-adenosylhomocysteine. Each tube contained 100 μmoles of Tris buffer (pH 8.0), 2.0 mg of Sephadex-treated cell-free extract, and 2.0 μmoles of *S*-ribosylhomocysteine or *S*-adenosylhomocysteine. Tubes were incubated at 37 C under an atmosphere of nitrogen. The rate of homocysteine formation was assayed by *N*-ethylmaleimide method described under Materials and Methods.

homocysteine was 8.0 (Fig. 6); no measurable free homocysteine was found below 7.2.

S-Adenosylhomocysteine and *S*-ribosylhomocysteine as substrates in methionine biosynthesis. In view of the above findings, it was decided to test the ability of *S*-adenosylhomocysteine and *S*-ribosylhomocysteine to serve as substrates in the

FIG. 6. Dependency of enzymatic reaction on pH. Each tube contained 2.0 μ moles of S-ribosylhomocysteine, 100 μ moles of Tris buffer, and 2.5 mg of Sephadex-treated cell-free extract per ml. Tubes were incubated at 37 C for 1 hr under an atmosphere of nitrogen. The pH value of reaction mixture was measured at end of the incubation. Homocysteine was determined by N-ethylmaleimide method outlined under Materials and Methods.

formation of methionine. It was found that methionine biosynthesis was obtained with Sephadex-treated *E. coli* extracts under the conditions described by Hatch, Larrabee, Cathou, and Buchanan (13). Serine was used as a donor of 1-carbon units, and L-homocysteine was used as methyl acceptor (Table 2). S-Adenosylmethionine was added at a level of 0.1 μ mole/ml in place of adenosine triphosphate (15). S-Ribosylhomocysteine and S-adenosylhomocysteine were also utilized as methyl acceptors, although at much reduced rates (Table 2). The participation of reduced nicotinamide adenine dinucleotide (NADH₂), Folate-H₄, serine, and S-adenosylmethionine in the formation of methionine from S-ribosylhomocysteine is apparent. No requirement for reduced flavin adenine dinucleotide (FADH₂) was noted; however, if the sole function of this compound is to maintain a reduced environment as suggested by Rosenthal et al. (20), then the anaerobic condition obtained with nitrogen might have satisfied this requirement.

DISCUSSION

Experiments reported in a previous communication showed that the glycosidic linkage of S-

TABLE 2. Cofactor requirements for methionine biosynthesis from S-ribosylhomocysteine

System*	Methionine found† mμmoles/ml
Complete.....	25
Minus RH.....	3
Minus S-AM.....	3
Minus Folate-H ₄	8
Minus FADH ₂	25
Minus NADH ₂	16
Minus serine.....	12
Minus RH + S-AH (10 μmoles)...	15
Minus RH + homocysteine (10 μmoles).....	55
RH alone.....	4

* The complete system contained (per milliliter) 10 μ moles of S-ribosylhomocysteine (RH), 0.1 μ mole of S-adenosylmethionine (S-AM), 0.2 μ mole of FADH₂, 0.25 μ mole of pyridoxal phosphate, 2.0 μ moles of NADH₂, 10 μ moles of Mg⁺⁺, 0.5 μ mole of Folate-H₄, 10 μ moles of L-serine, and 0.6 mg of Sephadex-treated *Escherichia coli* extract. Incubation was at 37 C for 3 hr under H₂.

† The reaction mixtures were deproteinized with 5% trichloroacetic acid, the acid was extracted with ether, and methionine was assayed with *Leuconostoc mesenteroides* P-60 (ATCC 8042).

adenosylhomocysteine is split, leading to the formation of adenine and S-ribosylhomocysteine (4). This enzyme was found in extracts prepared from a number of gram-negative bacteria (4) but could not be detected in extracts prepared from mammalian liver or yeast (Duerre, unpublished data). The formation of free homocysteine from S-adenosylhomocysteine or its degradation product, S-ribosylhomocysteine, had not been detected during the course of these studies. However, in vivo studies with radioactive S-ribosylhomocysteine indicated that the homocysteine portion of the molecule was incorporated into protein methionine (6, 7). The present results with cell-free extracts from *E. coli* indicate that the thioether linkage of S-ribosylhomocysteine is cleaved enzymatically to yield free homocysteine, and that the homocysteine was utilized in the formation of methionine if the necessary cofactors were present. Since free ribose was not recovered quantitatively from the reaction mixture by chromatography on Dowex-1, the possibility exists that ribose per se was not a product of the reaction, or that ribose was degraded by other enzymes in the reaction mixture. Reaction-rate studies presented here suggest that cleavage of the glycosidic bond of S-adenosylhomocysteine is necessary prior to cleavage of the

thioether linkage. Thus, the reaction sequence leading to the incorporation of the homocysteine moiety of *S*-adenosylhomocysteine into protein methionine of *E. coli* is tentatively proposed as follows.

SCHEME 1. Proposed reaction sequence.

The enzyme has been tentatively designated as Ribosylhomocysteinase. The mechanism proposed in Scheme I appears to be important for regeneration of homocysteine from *S*-adenosylhomocysteine. The homocysteine formed could be transmethylated via *S*-adenosylmethionine (20, 24) or 5'-methyltetrahydrofolate (11, 13, 15, 20) to yield methionine which could then be incorporated into protein or utilized in the formation of *S*-adenosylmethionine (17).

Such a mechanism for recycling of homocysteine might not be the only role that *S*-adenosylhomocysteine plays in methionine or *S*-adenosylmethionine biosynthesis, since experiments with other biological systems implicate *S*-adenosylhomocysteine as a methyl acceptor to regenerate *S*-adenosylmethionine without prior degradation (8, 19, 21). An alternative would be that *S*-adenosylhomocysteine in protein-bound form plays some catalytic role in the formation of methionine (11, 26).

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