Direct Homocysteine Biosynthesis from *O*-Succinylhomoserine in *Escherichia coli*: an Alternate Pathway That Bypasses Cystathionine

MARCIA SIMON† AND JEN-SHIANG HONG‡*

Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154

Received 12 August 1982/Accepted 4 October 1982

Mutations were found which enable *Escherichia coli* K-12 to form homocysteine in the absence of cystathionase. The formation of homocysteine in the mutant strains required cystathionine γ -synthetase, the *metB* gene product, but bypassed the normal intermediate cystathionine. It is concluded that cystathionine γ -synthetase catalyzes the formation of homocysteine directly from *O*-succinylhomoserine and an as-yet-unidentified sulfur donor. The mutation apparently causes the formation of this sulfur donor and has been named *metQ*. The expression of the *metQ* gene is under catabolite repression.

The biosynthesis of the methionine precursor homocysteine in *Escherichia coli* normally occurs by β -elimination from cystathionine catalyzed by cystathionase. Cystathionine is formed by the transsulfuration of *O*-succinylhomoserine by cysteine catalyzed by cystathionine γ -synthetase. Mutants deficient in cystathionase (*metC*) or cystathionine γ -synthetase (*metB*) are methionine (or homocysteine) auxotrophs.

In the course of investigating the reversion properties of *metC* mutations generated by the energy-uncoupled *ecf* mutants (8), we discovered that homocysteine is formed directly from *O*-succinylhomoserine and an as-yet-unidentified sulfur donor in *Met*⁺ revertants of *metC* deletion mutants. This paper describes the isolation and characterization of the mutations that permit homocysteine synthesis from *O*-succinylhomoserine, bypassing cystathionine.

All strains used in this work were derived from *E. coli* K-12 and are listed in Table 1. Minimal media contained minimal salt medium E (14) with a 0.5% carbon source. Cystathionase activity was assayed by the methods of Flavin (5) and Delavier-Klutchko and Flavin (3) at 26°C spectrophotometrically by coupling the hydrolysis of cystathionine to reaction with the Ellman reagent (4). Protein concentration was determined by the method of Lowry et al. (10), using bovine serum albumin as the standard. Transport assays were done as described elsewhere (8).

Cystathionase was purified from strain JSH211 bearing the colicin E1-*E. coli* hybrid plasmid pLC4-13 (2; M. Simon, Ph.D. thesis, Brandeis University, Waltham, Mass., 1981) by a procedure developed in this laboratory (M. Simon, unpublished data). The purity of the enzyme was greater than 95% as judged by sodium dodecyl sulfate-gel electrophoresis.

Antibodies against the purified cystathionase were prepared as follows. An adult female New Zealand albino rabbit was given a footpad inoculation of 2 mg of purified cystathionase in complete Freund adjuvant. Two booster injections of 2 mg were given at 1-week intervals, and the rabbit was bled 1 month later and once again two weeks after that. The antisera obtained were used without further purification.

Extracts were prepared as follows. Cells were harvested after overnight growth at 37°C by centrifugation and washed once with 50 mM K⁺ phosphate (pH 7.4), containing 0.1 mM each of pyridoxal 5-phosphate and β -mercaptoethanol (standard buffer). The pellet was suspended in 2 ml of standard buffer and placed in a methanol ice bath. The suspension was sonicated for 5 $\times$ 5 s with a Bronson Sonifier at maximum setting. Membrane particles were removed by centrifugation for 1 h at 17,000 rpm in an SS34 rotor. The supernatant was assayed and stored at -20°C. In no case were membranes seen to have cystathionase activity.

From the finding that approximately half of the revertants of *ecf* mutants were *metC* (8) and the fact that *ecf* and *metC* are distinct genes (Simon, Ph.D. thesis), we suspected that the *metC ecf*⁺ revertants might be deletion mutants, with the deletion encompassing both the *ecf* and

† Present address: Department of Physiology, Harvard Medical School, Boston, MA 02115.

‡ Present address: Department of Cell Physiology, Boston Biomedical Research Institute, Boston, MA 02114.

metC genes. To test this possibility, we carried out an analysis of the reversion of these revertants to methionine prototrophy. We found that spontaneous revertants able to grow in the absence of added methionine occurred with a frequency of 2×10^{-7} on media containing succinate, fumarate, or malate as a sole carbon source, but not on glucose.

An examination of the cystathionase activity was then carried out to determine whether it was restored in these methionine prototrophs. To facilitate this determination, the methionine prototrophs were isolated from *metC* revertants of the *ecf* mutant (JSH216) bearing a *metJ* mutation. This *metJ* mutation derepresses the synthesis of methionine biosynthetic enzymes, including cystathionase (12). Extracts prepared from five independent *Met*⁺ revertants of JSH216 grown in minimal medium containing 0.5% succinate had no detectable cystathionase activity (less than 0.01 U/mg of cell protein). Wild-type *MetC*⁺ strains carrying a *metJ* mutation, such as JSH212, had 0.6 to 1.0 U/mg of cell protein.

An immunochemical test (Ouchterlony double diffusion) was also done to determine whether cystathionase appeared in the *Met*⁺ prototrophs. No cross-reactivity with antisera prepared against purified cystathionase was observed in the extracts prepared from five independent revertants.

The absence of both cystathionase activity and immunological cross-reactivity in these revertants thus indicates that the *Met*⁺ prototrophs do not result from mutations going from *metC* to *MetC*⁺, but rather from mutations in a hitherto unknown gene. We tentatively call these mutations *metQ* mutations.

metQ mutations were also readily derived from two other *metC* strains unrelated to *ecf* mutations: one was the *metC* deletion in strain S942 generated by the improper excision of phage from a chromosome site near the *metC* gene and which was obtained from K. Shimada (13), and the other in the strain JSH211 is a point mutation (*metC69*) derived from the Taylor strain AT2699.

The expression of the *metQ* mutations fulfilled conditions characteristic of catabolite repression. First, *metQ* strains from JSH210, JSH211, and S942, isolated on succinate, did not grow on glucose or glucose-6-phosphate, but grew well on malate, fumarate, melibiose, glycerol, and lactose. The doubling time of JSH210 *metQ* at 37°C was 1.0 h on 1% Na⁺ succinate, whereas it was 10 h on 0.5% glucose. In the presence of methionine, the doubling time was 1.0 h irrespective of the carbon source, indicating that the limiting factor for growth on glucose was methionine. Second, cyclic AMP was capable of overcoming the methionine requirement for growth

on glucose or glucose-6-phosphate as tested in crystal tests.

If the *metQ* mutation results in methionine synthesis in the absence of cystathionase activity and if all other methionine biosynthetic enzymes are required for the synthesis, then the introduction of a *metA*, *-B*, or *-E* mutation into the *metQ* strains should reverse these methionine prototrophs to *met* auxotrophs. This was found to be the case. *metQ* strains carrying these mutations, MS421 (*metC metQ metA*), MS405 (*metC metQ metB*), and MS406 (*metC metQ metE*), were all incapable of growth on

TABLE 1. Bacterial strains

Strain	Relevant genetic markers	Comments
CGSC1911	<i>thi metA argH</i> (or <i>E</i>)	Obtained from B. Bachmann
CGSC4404	<i>F</i> ⁻ <i>metB</i>	Obtained from B. Bachmann
CGSC4529	<i>F</i> ⁻ <i>metC69</i>	Obtained from B. Bachmann
CGSC4212	<i>F</i> ⁻ <i>metE</i>	Obtained from B. Bachmann
JSH210 <i>metQ</i>	<i>F</i> ⁻ <i>thi metC69 metQ</i>	Spontaneous revertant of JSH210
JSH210	<i>F</i> ⁻ <i>thi metC69</i>	(9)
JSH211	<i>F</i> ⁻ <i>thi metC69 metJ</i>	(7)
JSH212	<i>F</i> ⁻ <i>thi metJ</i>	(7)
JSH216	<i>F</i> ⁻ <i>thi metJ metC</i>	<i>metC</i> revertant of JSH215 [<i>thi metJ ecf</i> (Ts)]
MAL300	<i>F</i> ⁻ <i>thi ecf</i> (Ts)	(9)
MAL303	<i>F</i> ⁻ <i>thi ilv metC</i>	(9)
MAL303 <i>metQ</i>	<i>F</i> ⁻ <i>thi ilv metC metQ</i>	Spontaneous revertant of MAL303
MS405	<i>F</i> ⁻ <i>thi metC metQ metB</i>	<i>metB</i> derived from CGSC4404
MS406	<i>F</i> ⁻ <i>thi metC metQ metE</i>	<i>metE</i> derived from CGSC4212
MS408	<i>F</i> ⁻ <i>thi metB</i>	<i>metB</i> derived from CGSC4404
S942	<i>F</i> ⁻ <i>thi ΔmetC</i>	Gift of K. Shimada
MS421	<i>F</i> ⁻ <i>thi metC metQ metA</i>	<i>metA</i> derived from CGSC1911
MS422	<i>F</i> ⁻ <i>thi ecf</i> (Ts) <i>metQ</i>	From JSH210 <i>metQ</i> by transduction with P1 (MAL300) selecting for <i>met</i> ⁺ on glucose at 26°C

FIG. 1. Inability of *metC metQ* strain to form homocysteine from cystathionine. Strains MS405 (*metC metQ metB*) and MS408 (*metB*) were grown on minimal succinate plus methionine and thiamine at 37°C overnight and washed once with minimal salt medium E (14). Samples (0.1 ml) were mixed with 2 ml of soft agar (0.6% in 0.5% NaCl solution) and overlaid onto minimal succinate plates containing thiamine. Approximately 2 mg each of L-cystathionine and methionine was placed on the surface of the solidified agar, and after 24 h of incubation at 37°C, photography was taken. L-cystathionine was on 1; L-methionine was on 2.

succinate or glucose plates in the absence of methionine. However, all grew normally in the presence of methionine. These results indicate that the *metA*, *-B*, and *-E* genes are required for methionine synthesis in *metQ* strains.

To determine whether the requirement for the *metB* gene can be attributed to the gene product, cystathionine synthetase, or the reaction product, cystathionine, a crystal test was performed on a succinate plate with the *metC metQ metB* strain MS405; the *metB* strain MS408 served as a positive control. Cystathionine conferred growth on MS408 but not on MS405 (Fig. 1). Since cystathionine was transported into MS405 about two times better than into MS408 (data not shown) it is apparent that the requirement for the *metB* gene is due to cystathionine synthetase, not to cystathionine. This indicates that the *metQ* mutation does not result in the induction of a new cystathionase and that the *metB* gene product catalyzes the formation of homocysteine directly, bypassing the normal cystathionine route.

The formation of homocysteine from *O*-succinylhomoserine, bypassing cystathionine, that we report here indicates that the sulfur donor cannot be cysteine and must be something else.

This sulfur donor may be a novel one induced by the *metQ* mutation or a known one whose concentration is normally inadequate to effect homocysteine synthesis, but is elevated as a result of the *metQ* mutation. Inorganic sulfide could be such a sulfur donor, since Flavin and Slaughter (6) have shown that cystathionine γ -synthetase can catalyze homocysteine formation in vitro by the direct condensation of sulfide with *O*-succinylhomoserine. This possibility was therefore tested with sodium sulfide by a radial streaking technique (11). The results of such tests showed that *metC* strains JSH210 and JSH211 were unable to grow on succinate in the presence of sulfide without methionine. Similar tests were also done with cysteine and cystine, and the results were also negative, indicating that sulfide, cysteine, and cystine are probably not the sulfur donor required for the observed formation of homocysteine in the absence of β -cystathionase.

The work presented here demonstrates that *E. coli* K-12 can be mutated such that it is capable of homocysteine biosynthesis in the absence of β -cystathionase, the enzyme normally required for its synthesis. The alternate pathway for homocysteine biosynthesis in the *metQ* strains apparently requires *O*-succinylhomoserine as a reactant and is catalyzed by cystathionine γ -synthetase since *metA* and *metB* mutations abolish homocysteine formation.

It is unlikely that a new cystathionase is induced in the *metQ* mutants. No *metC*-like cystathionase activity could be detected in extracts of *metQ* revertants of JSH211. Further, exogenous cystathionine did not allow *metB metC metQ* strains to grow. If the function of the cystathionine γ -synthetase were to supply cystathionine as a substrate for a new cystathionase, then the cystathionine transported into the cell should obviate the need for the *metB* enzyme; this was not observed.

The requirement for cystathionine γ -synthetase, but not for cystathionine, suggests a novel in vivo function for this enzyme. Flavin and Slaughter (6) have shown that cystathionine γ -synthetase can function in vitro as a sulfhydrylase, utilizing sulfide and *O*-succinylhomoserine to form homocysteine. Experiments with sulfide suggest, however, that such sulphydration probably plays no physiological role since exogenously supplied sulfide failed to confer the *metQ* phenotype on *metC* strains.

It is unknown what the *metQ* mutations induce. It is possible that these mutations induce the formation of a novel sulfur donor that can condense with *O*-succinylhomoserine to form homocysteine directly or result in increasing the concentration of a known sulfur donor capable of direct condensation with *O*-succinylhomoserine.

ine to form homocysteine. This donor normally would exist at a concentration inadequate to effect homocysteine formation in the absence of *metQ* mutations. It is also possible that the *metQ* mutations may result in altering cystathionine γ -synthetase specificity such that a direct condensation of sulfide and *O*-succinylhomoserine is favored over the normal reaction of *O*-succinylhomoserine with cysteine forming cystathionine. Such an alteration may, for instance, allow the synthetase to act as a cystine desulfhydrase. The formation of thiocysteine and its subsequent elimination of sulfide would supply a high local concentration of sulfide for direct condensation with *O*-succinylhomoserine. Cystine desulfhydrase activity has previously been shown by Cavallini et al. (1) for the β -cystathionase. The finding that *metQ* mutations are not located in the *metB* gene indicates, however, that if this is the case, the alteration must occur either post-translationally or by the production of an effector of that enzyme.

We thank Barbara Bachmann for providing bacterial strains.

This work was supported by grant GM22576 from the National Institute of General Medical Sciences.

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