METHIONINE BIOSYNTHESIS IN ENTEROBACTERIACEAE: BIOCHEMICAL, REGULATORY, AND EVOLUTIONARY ASPECTS

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I. INTRODUCTION

Methionine was discovered in 1922 by Mueller[1] in the course of efforts to describe the growth factors for a hemolytic *Streptococcus*. Along with threonine, methionine is the only amino acid to have been isolated during the elucidation of a nutritional requirement.[2] Apart from its role as a building block of proteins, methionine in its acylated form plays an essential role as the initiator amino acid of protein synthesis and is a precursor of S-adenosylmethionine, the universal methyl donor, and of the polyamine spermidine.

The methyl group of methionine is derived from the β-carbon of serine; the remainder of its carbon atoms comes from aspartic acid, and the sulfur atom comes from cysteine. It is impossible to dissociate the study of methionine biosynthesis from that of the other amino acids which derive all or part of their carbon atoms from aspartic acid. The corresponding pathway is summarized in Figure 1.

One can note three branch points leading, respectively, from aspartate semialdehyde to diaminopimelate and lysine, from homoserine to methionine, and from threonine to isoleucine. The first enzyme of each branch is subject to feedback inhibition by its respective end product.

The known regulatory mechanisms of methionine synthesis involve, in addition to the feedback inhibition of the first reaction of the specific branch, repression of the synthesis of the methionine biosynthetic enzymes when cells are grown in the presence of methionine. Another pathway converges at the homocysteine level of the specific methionine branch and provides the methyl group involved in the homocysteine → methionine transformation (Figure 2).

This review deals with the biochemical and genetic aspects of methionine biosynthesis, of its regulation in *Enterobacteriaceae*, and with evolutionary considerations derived from our studies.

II. ENZYMES INVOLVED IN METHIONINE BIOSYNTHESIS AND ITS REGULATION

A. Synthesis of Homoserine: The Common Pathway

1. Aspartate Kinases-Homoserine Dehydrogenases I and II and Aspartokinase III

(ATP: L-aspartate phosphotransferase, E.C. 2.7.2.4; L-homoserine: $NADP^+$ oxidoreductase, E.C. 1.1.1.3.)

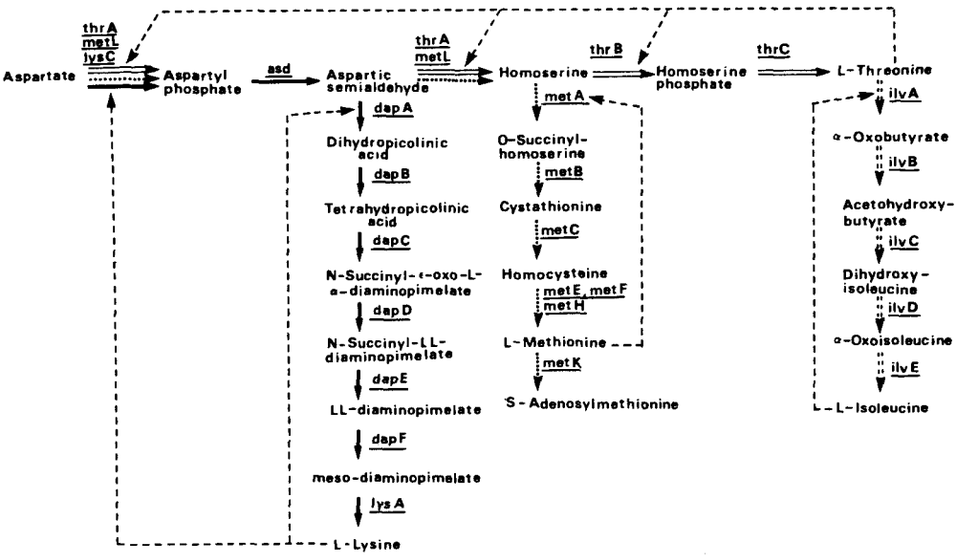


FIGURE 1. The pathway leading to lysine, threonine, isoleucine, methionine, and S-adenosylmethionine in *E. coli* and *S. typhimurium*. Details of the specific methionine pathway are given in Figure 2. Relevant enzymes corresponding to the mentioned genes are given in the text and Table 1. Long dashed arrows represent regulation at the level of enzymatic activity. Expression of the genes is regulated specifically by the different end products of the pathway (⇒,·· ·· ··> , →, and :: :: ::> regulation by threonine and isoleucine, methionine, lysine, and isoleucine, respectively).

$$\text{L-aspartate} + \text{ATP} \rightleftarrows \text{L-}\beta\text{-aspartylphosphate} + \text{ADP}$$

$$\text{L-aspartate semialdehyde} + \text{NADPH} + \text{H}^+ \rightleftarrows \text{L-homoserine} + \text{NADP}^+$$

The synthesis of L-β-aspartylphosphate is catalyzed by three distinct aspartokinases, all of which catalyze the same reaction but differ in the mode of regulation of their synthesis and activity. Aspartokinase I is inhibited by threonine and its synthesis is repressed by threonine *plus* isoleucine; the synthesis of aspartokinase II is repressed by methionine; and lysine inhibits the activity and represses the synthesis of aspartokinase III. The reduction of L-aspartate semialdehyde to homoserine is likewise catalyzed by two distinct homoserine dehydrogenases. Homoserine dehydrogenase I activity is inhibited by threonine and its synthesis is repressed by threonine *plus* isoleucine, whereas methionine represses the synthesis of homoserine dehydrogenase II.

In the mid-1960s, it became apparent that the aspartokinase I and homoserine dehydrogenase I reactions were carried out by a bifunctional protein. Similarly, the aspartokinase II and homoserine dehydrogenase II reactions are carried out by another bifunctional protein.[3,4]

The existence of three isofunctional aspartokinases and two isofunctional homoserine dehydrogenases allows an efficient and fine regulation of amino acid biosynthesis. A given end product in excess will inhibit the activity of the relevant enzyme and repress its synthesis, thereby causing a decrease in production of the common intermediate. When the flow reaches the next branch point, this common intermediate, which is already present in lower amounts, is not directed to the end metabolite in excess since there is a new enzyme subject to regulation at this point. Instead, it is directed toward the amino acid(s) which is needed for the growth of the organism. The whole biosynthetic machinery can easily be brought to a stop in a medium containing an excess of all of the end products.

Several attempts have been made to answer questions regarding the similarity of the three

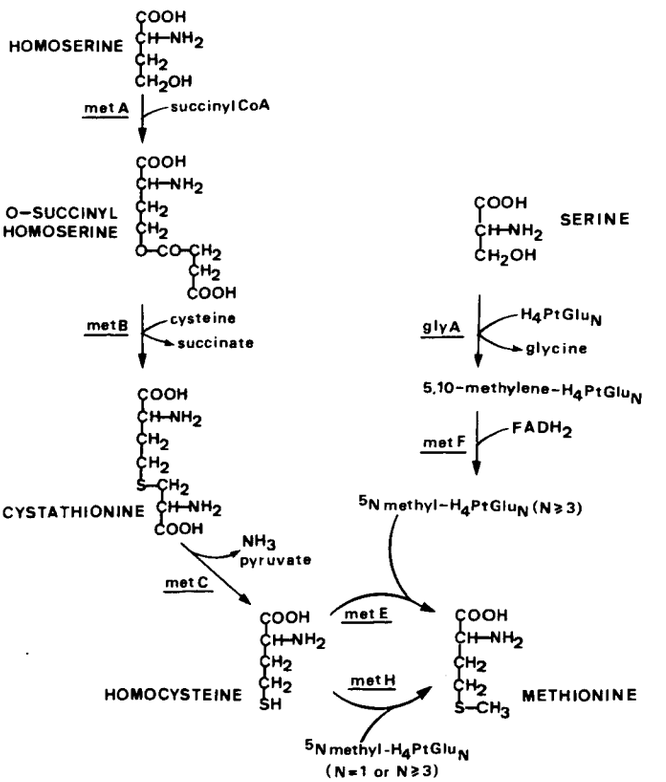


FIGURE 2. Biosynthesis of methionine from homoserine and serine in *E. coli* and *S. typhimurium*. Relevant enzymes corresponding to the mentioned genes are given in the text and Table 1. ^{5}N-methyl-H_4PtGlu_N stands for tetrahydropteroyl glutamate. The product of the *metH* gene can use the mono- or polyglutamate forms (N = 1 or N ≥ 3), whereas the product of the *metE* gene can only utilize the polyglutamate form of ^{5}N-methyl-H_4PtGlu (N ≥ 3) as substrate.

proteins and their evolutionary origin.[3,4] Aspartokinase I-homoserine dehydrogenase I and aspartokinase II-homoserine dehydrogenase II possess binding sites for the same substrates and catalyze identical reactions. In addition, the type I enzyme binds threonine, its allosteric inhibitor. Although the turnover numbers are different, the apparent affinity constants for substrates of the two proteins are quite similar, as are the subunit molecular weights of the two proteins. However, aspartokinase I-homoserine dehydrogenase I is a tetramer (M_r of the subunit: 89,020), whereas aspartokinase II-homoserine dehydrogenase II is a dimer (M_r of the subunit: 88,726). In both cases, modification of cysteinyl groups leads to the loss of kinase activity with almost total retention of the dehydrogenase activity.[5,6]

The results of limited proteolysis of aspartokinase I-homoserine dehydrogenase I and the study of nonsense mutations of the corresponding gene show that the protein is composed of two functional domains: one with kinase activity (N terminal) and the other with dehydrogenase activity (C terminal).[7] A more elaborate analysis has led to the formulation of a triglobular model for the native enzyme.[8] Limited proteolysis of aspartokinase I-homoserine dehydrogenase I from *Escherichia coli* by type VI protease from *Streptomyces griseus* yields five proteolytic fragments: three are dimeric and two are monomeric. One of the monomeric fragments (27 kdaltons) exhibits residual aspartokinase activity, while the second (33 kdaltons) possesses residual homoserine dehydrogenase activity. The smallest of the dimeric

species (2 × 25 kdaltons) is inactive; the two other dimers exhibit either only homoserine dehydrogenase activity (2 × 59 kdaltons) or both activities (hybrid fragment, 89 + 59 kdaltons). The properties of isolated fragments are consistent with the existence of three compact regions of defined size in the aspartokinase I-homoserine dehydrogenase I chain: an N-terminal fragment (27 kdaltons) with aspartokinase activity, a C-terminal fragment (33 kdaltons) with dehydrogenase activity, and a central, enzymatically inactive domain which links the two other domains.[8]

The relative proportions of the three isofunctional enzymes vary in different Enterobacteriaceae. Aspartokinase II-homoserine dehydrogenase II is undetectable in *E. coli* K12. In order to detect it and to study its properties, it was necessary to construct a derepressed strain (MetJ$^-$) devoid of aspartokinase I-homoserine dehydrogenase I activity.[4] Limited proteolysis of aspartokinase II-homoserine dehydrogenase II has shown that it, like aspartokinase I-homoserine dehydrogenase I, is composed of three globular domains.[9] The N-terminal domain is endowed with kinase activity, while the C-terminal domain has dehydrogenase activity. The two parts of the polypeptide chain are separated by a central, enzymatically inactive domain. Thus, the polypeptide chains of the two multifunctional proteins are homologous not only in their sequence (see Section V), but also in their triglobular domain structure.[9]

Antibodies raised against native aspartokinase I-homoserine dehydrogenase I or aspartokinase II-homoserine dehydrogenase II can recognize only the homologous antigen, irrespective of its native or denatured state.[10,11] On the contrary, antibodies raised against denatured proteins recognize both the homo- and heterologous denatured antigens.[10] The existence of a specific cross reaction between the two denatured aspartokinases-homoserine dehydrogenases suggests that they share structural similarities. The regions of similarity are probably buried inside the native proteins and become exposed only upon denaturation. Serological cross-reactivity of two denatured proteins indicates homologies between their amino acid sequences and suggests that the two bifunctional enzymes are derived from a common ancestor.[10]

Aspartokinase I-homoserine dehydrogenase I and aspartokinase III have also been compared using antibodies directed against native and denatured proteins. Cross reaction was detected only when denatured species were used as both immunogens and as antigens, suggesting that these two proteins are evolutionarily related.[12]

The primary structure of aspartokinase II-homoserine dehydrogenase II, deduced from the DNA sequence of the corresponding gene, is given in Section III.[13] Its comparison with the amino acid sequences of aspartokinase I-homoserine dehydrogenase I and aspartokinase III is presented and discussed in Section V dealing with evolutionary considerations.[14,15] The properties of aspartokinase III have been reviewed recently.[16]

2. *Aspartate Semialdehyde Dehydrogenase*

(L-aspartate-β-semialdehyde: NADP$^+$ oxidoreductase, phosphorylating, E.C. 1.20.2.11.)

$$\text{L-β-aspartylphosphate} + \text{NADPH} + \text{H}^+ \rightleftarrows \text{L-aspartate semialdehyde} + \text{NADP}^+ + \text{Pi}$$

Aspartate semialdehyde dehydrogenase catalyzes the reversible, substrate-dependent reduction of NADP$^+$ in the presence of phosphate or arsenate. The reaction is formally similar to that catalyzed by glyceraldehyde 3-phosphate dehydrogenase. Aspartate semialdehyde dehydrogenase has been obtained at 90% purity from *E. coli* K12 grown under conditions of lysine limitation.[17] The procedure for preparing the enzyme was subsequently simplified and improved through the use of a genetically derepressed strain.[18] The enzyme is a dimer of identical subunits, with the N-terminal sequence Met-Lys-Asn-Val-Gly-. Each subunit contains three cysteine residues: two are reactive in the native enzyme and one is partially protected by the substrate. Formation of an acyl-enzyme intermediate has been detected.

The substrate and nucleotide-binding sites of asparate semialdehyde dehydrogenase have been explored with affinity labels. Thus, 2-amino-4-oxo-5-chloropentanoate inactivates the enzyme with pseudo-first-order kinetics and with half-site reactivity.[19] Aspartate semialdehyde protects against the inactivation. A single group is labeled at the active site.[19] Amino acid sequencing of a peptide obtained by peptic digestion of the labeled enzyme yields the sequence Phe-Val-Gly-Gly-Asn-modified residue ↔ Thr-Val-Ser. Biellmann et al.[19] suggested that the side chain of a histidine residue was modified; however, DNA sequencing indicates that a cysteine is the modified residue.[20] Aspartate semialdehyde dehydrogenase has also been alkylated with the coenzyme analog chloroacetylpyridine-ADP, which irreversibly inactivates the enzyme with pseudo-first-order kinetics.[21] NADP and NADPH protect against alkylation, whereas the aldehyde does not. The stoichiometry for total inactivation is again 1 mol of analog per mole of dimer.[21] The DNA sequence of the gene coding for aspartate semialdehyde dehydrogenase and the deduced amino acid sequence that are given in the following section indicate M_r = 39,972 for the protein subunit.

B. Synthesis of Homocysteine

1. Homoserine Succinyltransferase

(Succinyl-CoA: L-homoserine *O*-succinyltransferase, E.C. 2.3.1.46.)

$$\text{Succinyl CoA} + \text{L-homoserine} \rightarrow \text{succinyl-L-homoserine} + \text{CoA}$$

Homoserine succinyltransferase, the product of the *metA* gene, catalyzes the first specific step of methionine synthesis by transforming homoserine into *O*-succinylhomoserine in the presence of succinyl-CoA. *Salmonella typhimurium* mutants blocked in the synthesis of the succinyltransferase will not grow on medium supplemented with *O*-succinylhomoserine. This might be due to an inability to take up the succinylated derivative. However, such mutants can be identified nutritionally, for although they do not produce *O*-acetylhomoserine, they can slowly utilize it for the synthesis of cystathionine. Homoserine succinyltransferase from *E. coli* has been purified 30-fold.[22] It is the only enzyme specific for the methionine branch of the pathway which is subjected to an allosteric control. Its activity is allosterically inhibited in a synergistic way by methionine and *S*-adenosylmethionine.[23] The enzyme is inhibited by α-methylmethionine, which prevents the growth of wild-type bacteria.[24] Resistant mutants carry mutations in the *metA* gene. They overproduce methionine and their enzyme is insensitive to methionine and *S*-adenosylmethionine.[25] The *metA* gene has been cloned: expression in minicells yields a polypeptide of M_r = 40,000.[26] Since gel filtration of the partly purified native enzyme yields a M_r = 84,000, homoserine succinyltransferase appears to be a dimer.[27]

Homoserine succinyltransferases of many Enterobacteriaceae are unusually temperature sensitive. Elevated growth temperatures result in methionine limitation or even starvation. This has been shown to be specifically due to the inactivation of homoserine succinyltransferase.[28] The temperature sensitivity of this enzyme presumably evolved to limit growth at elevated temperatures. Elevated temperatures might cause unbalanced growth and hence cell death, whereas inactivation of homoserine succinyltransferase blocks a wide range of metabolic functions, thereby stopping growth while preserving the viability of the organisms, which can recover rapidly if the temperature falls.

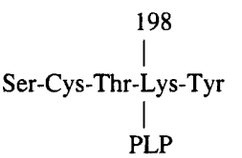
The second and third specific steps of methionine biosynthesis involve the transfer of sulfur from cysteine to homoserine.

2. Cystathionine-γ-Synthase

(*O*-succinyl-L-homoserine succinate lyase, adding cysteine, E.C. 4.2.99.9.)

$$O\text{-succinyl-L-homoserine} + \text{L-cysteine} \rightleftarrows \text{L-cystathionine} + \text{succinate}$$

The formation of cystathionine from *O*-succinylhomoserine in the presence of cysteine is catalyzed by cystathionine-γ-synthase which is encoded by the *metB* gene. The enzyme has been obtained in the pure state from *S. typhimurium* and from *E. coli*.[29,30] In both cases, it is composed of four subunits, each of M_r = 40,000. The *S. typhimurium* enzyme has been shown to contain four pyridoxal phosphate molecules. The *E. coli* protein contains 386 amino acid residues (M_r = 41,503), as deduced from the DNA sequence of the corresponding *metB* gene (see Section III).[31] Its deduced N-terminal sequence agrees with the experimentally determined N-terminal protein sequence.[30] The absorption spectrum of the pure protein shows two maxima — at 278 and 420 to 425 nm — characteristic of the lysine-PLP aldimine structure. Most of the substrates of cystathionine-β-lyase (see Section II.B.3) are also substrates for cystathionine-γ-synthase, but with an elevated K_m and a much lower V_{max}. Upon reduction by tritiated borohydride, the 420-nm band is displaced toward 325 nm, and the enzyme is inactivated. A peptide isolated from the reduced, tritiated protein by tryptic digestion has the following structure:[172]

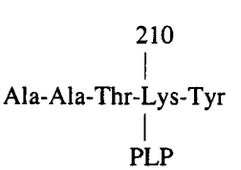


3. Cystathionine- β-Lyase

(Cystathionine-L-homocysteine-lyase, deaminating, E.C. 4.4.1.8.)

$$\text{L-cystathionine} + H_2O \rightleftarrows \text{L-homocysteine} + \text{pyruvate} + NH_3$$

Cystathionine-β-lyase, a product of the *metC* gene, cleaves cystathionine to homocysteine, pyruvate, and ammonia. The *E. coli* enzyme was purified from a strain harboring a multicopy number plasmid carrying the *metC* gene.[32] The protein was reported to be composed of six identical subunits of M_r = 45,000, each binding one molecule of pyridoxal phosphate.[32] However, a more recent study[173] led to the conclusion that the native enzyme is a tetramer. Its pH optimum, substrate specificity, and kinetic parameters have been reported. The dissociation constant of the enzyme for L-cystathionine is four times higher than that determined by Dwivedi et al.[32] The enzyme is specific for β-elimination reactions and does not act on homocysteine and *O*-succinylhomoserine. Different substrates and inhibitors have been tested.[172] 3,3,3-Trifluoroalanine, in particular, binds covalently to and irreversibly inhibits the enzyme. Reduction of the holoenzyme by tritiated borohydride displaces the adsorption band from 420 to 325 nm, which is characteristic of phosphopyridoxyllysine, and leads to the total loss of catalytic activity. The chymotryptic peptide containing the tritiated PLP has been identified as



The enzyme from *S. typhimurium* is less well characterized.[33]

The *E. coli metC* gene has been sequenced; the deduced sequence of the protein (395 residues, M_r = 43,032), supported by the experimentally determined sequence of the first 10 residues, shows strong homology with that of cystathionine-γ-synthase, pointing to a common ancestor for the 2 proteins (see Section V).[34]

C. Methyltaion of Homocysteine

The last two steps in methionine biosynthesis involve the methylation of homocysteine. Two branches converge at this level: one deriving from aspartate involving the synthesis of homocysteine (previously discussed) and the other allowing the formation and the transfer of the methyl group (Figure 2). The source of the methyl group is the β-carbon atom of serine. In the reaction catalyzed by glycine hydroxymethyltransferase, the hydroxymethyl group of serine is transferred to tetrahydropteroylglutamate (H_4PtGlu_N) (in this case, $N \geq 1$). The product, $^5N,^{10}N$-methylene H_4PtGlu, is the precursor of the methyl donor in the synthesis of thymine, hydroxymethylcytosine, purines, and methionine. $^5N,^{10}N$-methylene H_4PtGlu is reduced to ^5N-methyl H_4PtGlu ($N \geq 1$) by the enzyme $^5N,^{10}N$-methylenetetrahydrofolate reductase coded by *metF*. This reaction yields the specific methyl group donor for methionine synthesis. The very last step in methionine synthesis is homocysteine methylation itself.

1. Glycine Hydroxymethyltransferase

(5-10, Methylenetetrahydrofolate: glycine hydroxymethyltransferase, E.C. 2.1.2.1.)

$$\text{5-10,Methylenetetrahydrofolate} + \text{glycine} \rightleftarrows \text{Tetrahydrofolate} + \text{L-serine}$$

The conversion serine ⇄ glycine is catalyzed by this enzyme, which is commonly referred to as serine hydroxymethyltransferase:[35]

$$\text{Serine} \rightleftarrows \text{glycine} + \text{formaldehyde}$$

The mechanism of this reaction, which can be considered a model for the transfer of one-carbon residues, is worthy of attention. It has been shown that the C_1 fragment obtained from serine is a derivative of formaldehyde, that this derivative is a tetrahydrofolate compound, and that pyridoxal phosphate is also necessary for the reaction. It is thought that pyridoxal phosphate participates in this reaction through the formation of a Schiff base between the amino group of the amino acid and the formyl group of pyridoxal phosphate. The resulting system of conjugated double bonds extends from the β-carbon of the amino acid to the nitrogen of the pyridine ring, thus labilizing the hydroxymethyl group of serine and facilitating its cleavage.

The *S. typhimurium* and *E. coli glyA* genes coding for glycine hydroxymethyltransferase have been cloned.[36,37] The *E. coli* gene has been sequenced. It corresponds to a polypeptide of 417 residues (M_r = 45,265). Two regions of the sequence show considerable homology with the pyridoxal phosphate-binding site and with the active site of the rabbit liver enzyme, respectively.

2. 5,10-Methylenetetrahydrofolate Reductase

(5-10,Methylenetetrahydrofolate reductase (FADH2); 5-methylenetetrahydrofolate (FAD) oxidoreductase, E.C. 1.7.99.5.)

This enzyme catalyzes the reaction:

$$\text{5-10,Methylenetetrahydrofolate} + FADH_2 \rightleftarrows \text{5-methyltetrahydrofolate} + FAD$$

The enzyme of *E. coli* has been purified 100-fold from a strain grown under derepression conditions and some of its properties have been studied.[38] (The reaction catalyzed by this enzyme is essentially irreversible, which explains the accumulation of ^5N-methyltetrahydrofolate in vitamin B_{12}-starved cells that do not have the B_{12}-independent transmethylase.) It definitely does not require pyridine nucleotide, the latter being part of another enzyme

required to generate $FADH_2$. The corresponding gene (*metF*) has been cloned from *E. coli* and sequenced.[39] A simplified in vitro DNA-directed system has identified the N-terminal dipeptide sequence of the enzyme.[40] The polypeptide chain is 296 residues long and has M_r = 33,065.

3. Vitamin B_{12}-Dependent and -Independent Homocysteine Methylases

(5-Methyltetrahydrofolate-L-homocysteine *S*-methyltransferase, E.C. 2.1.1.13 and 5-methyltetrahydropteroyltri-L-glutamate:L-homocysteine *S*-methyltransferase E.C. 2.1.1.14.)

Two enzymes can catalyze homocysteine methylation (Figure 2): a transmethylase (product of the *metH* gene) whose activity depends on the presence of vitamin B_{12} and which can use the mono- or polyglutamate forms of ^{5}N-methyl-H_4PtGlu as methyl donor, and a vitamin B_{12}-independent transmethylase (product of *metE*) which can only utilize the polyglutamate forms of ^{5}N-methyl-H_4PtGlu ($N \geq 3$) as substrate.[41,42] *E. coli* and *S. typhimurium* are exceptional in that they possess both enzymes, whereas most other organisms have only one. The use of one or the other transmethylase depends on the availability of vitamin B_{12} in the medium. Strains mutated in *metE* require either methionine or vitamin B_{12} for growth.[43] Both transmethylases have been purified.[44,45]

The mechanism of the vitamin B_{12}-dependent pathway is as follows: the 5-methyltetrahydrofolate homocysteine transmethylase contains a vitamin B_{12} derivative as the prosthetic group. This derivative can have either a hydroxyl group or a methyl group coordinated in the sixth position of the cobalt atom. Catalytic amounts of *S*-adenosylmethionine are required for the functioning of this system. (*S*-adenosylmethionine acts catalytically and provides the first methyl group transferred to homocysteine. Thus, *S*-adenosylmethionine primes the enzyme by methylating the B_{12} prosthetic group. After this methyl group is transferred to homocysteine, subsequent methyl groups come from ^{5}N-methyltetrahydrofolate.[46,47]) We do not understand why the B_{12}-dependent enzyme can use either of the two forms of tetrahydrofolate derivative, whereas the other transmethylase uses only the polyglutamate derivative. One should not overlook the fact that six of the methyl groups of vitamin B_{12} are derived from methionine.[48]

(The B_{12}-independent transmethylase does not involve a methylated enzyme or the other requirements of the B_{12}-dependent transmethylase such as a reducing system and *S*-adenosylmethionine.)

D. Synthesis of *S*-Adenosylmethionine

1. Methionine Adenosyltransferase

(ATP: L-methionine *S*-adenosyltransferase, E.C. 2.5.1.6.)

$$\text{ATP} + \text{L-methionine} \rightleftarrows S\text{-adenosylmethionine} + P_i + PP_i$$

This ubiquitous enzyme catalyzes the only known route of biosynthesis of the intracellular alkylating agent, *S*-adenosylmethionine, from methionine and ATP; the other products of the reaction are inorganic phosphate and pyrophosphate.[49] Mutations in the *metK* locus, at 64 min on the *E. coli* chromosomal map, reduced transferase activity.[50-52] A temperature-sensitive *metK* mutant with a thermolabile adenosylmethioninetransferase activity has been described, confirming that *metK* is the structural gene for this enzyme.[53] The *metK* gene has been cloned and sequenced.[54,55] It codes for a polypeptide of 384 residues (M_r = 41,941). The deduced primary structure was confirmed by sequencing the 35 amino terminal residues of the purified protein, which is actually a homotetramer.[56] In addition to the transferase reaction, the purified enzyme catalyzes a tripolyphosphatase reaction stimulated by *S*-adenosylmethionine. The mechanism of the two reactions has been studied extensively and preliminary X-ray diffraction studies have been presented.[56-60]

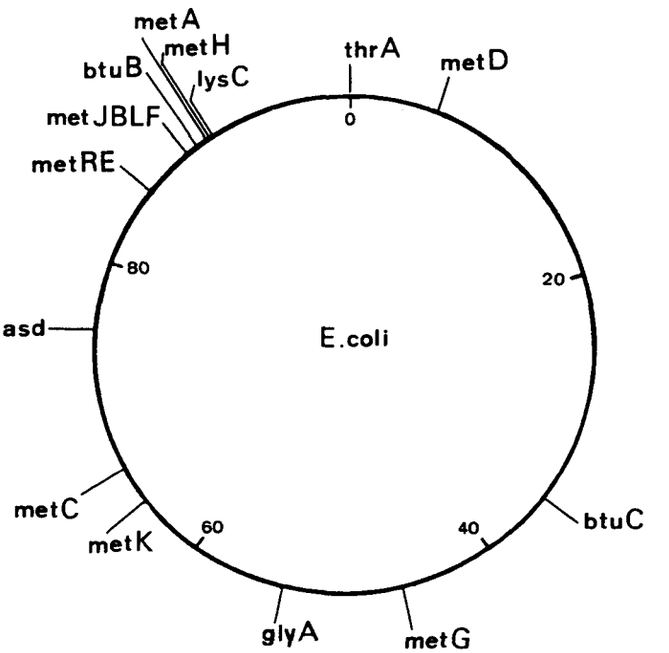


FIGURE 3. *E. coli* chromosomal map showing the *met* genes.[50] The *metR* gene was identified in *S. typhimurium*.[146]

Since *S*-adenosylmethionine is not taken up by *E. coli*, it has been impossible to obtain mutants that require this compound for growth. The finding that *S*-adenosylmethionine is required for regulating methionine biosynthesis was used to isolate *metK* mutations causing resistance to methionine analogs. These mutants show reduced *S*-adenosylmethionine synthesis in vitro and a lower level of intracellular *S*-adenosylmethionine. However, as all these mutants can grow without added *S*-adenosylmethionine, the block in its synthesis must be incomplete. To try to block *S*-adenosylmethionine synthesis more completely, double mutants were constructed by combining a temperature-sensitive *metK* mutation with a block (*metA*) in the synthesis of cystathionine, a precursor that supports only slow growth because of slow entry. These double mutants could grow on methionine but not on cystathionine at the nonpermissive temperature for *metK*, in line with the greatly reduced intracellular level of *S*-adenosylmethionine. This result indicates that it is possible to reduce intracellular *S*-adenosylmethionine concentrations to levels which are low enough to prevent growth of *E. coli*. Moreover, since the mutation responsible for the reduction in *S*-adenosylmethionine level is in the *metK* gene, this confirms that methionine *S*-adenosyltransferase is the enzyme that catalyzes *S*-adenosylmethionine biosynthesis in *E. coli*.[174]

III. GENES INVOLVED IN THE METHIONINE PATHWAY

In *E. coli* and *S. typhimurium*, the genes involved in the methionine pathway are scattered throughout the chromosome (Figure 3) and organized in independent units of transcription[61] (Table 1). Only the *metJBLF* genes are clustered and two of them, *metB* and *metL*, form an operon.[31,50,62] All of the *met* genes have been cloned and most of them have been sequenced. The regulation of expression of the genes by the Met repressor is discussed in Section IV. After giving the characteristics of each transcriptional unit and their nucleotide sequences (where known), we discuss their ribosome-binding sites, their 3′ regions, and lastly their promoters.

Table 1
GENE-PROTEIN CORRESPONDENCE

Gene				Protein	
Name	**Min**	**Regulation**		**Name**	**Specific inhibitor**
metA	91	Met	*metJ*	Homoserine succinyltransferase	Met + S-adenosylmethionine
metB	89	Met	*metJ*	Cystathionine-γ-synthase	—
metC	65	Met	*metJ*	Cystathionine-β-lyase	—
metE	86		*metJ*		
		Met + vitamin B_{12}	*metH*	Homocysteine methylase	
			metF	Vitamin B_{12} independent	—
			metR		
metF	89	Met + vitamin B_{12}	*metJ*	5,10-Methylenetetrahydrofolate reductase	—
			metH		
metH	91	Met + vitamin B_{12}	*metJ*		
			metR	Homocysteine methylase vitamin B_{12} dependent	—
metJ	89	Met	*metJ*	Methionine aporepressor	—
metK	64	Met	*metJ*	Methionine adenosyltransferase	—
metL	89	Met	*metJ*	Aspartokinase II-homoserine dehydrogenase II	—
metR	86	—	—	Methionine activator	—
asd	76	Lys, Thr, Met	—	Aspartate semialdehyde dehydrogenase	—
glyA	55	Ser, Met, Gly Ade, Gua, Thy		Glycine hydroxymethyltransferase	—
lysC	91	Lys		Aspartokinase III	Lys
thrA	0	Thr, Ile	*ileR*	Aspartokinase I-homoserine dehydrogenase I	Thr

Note: The position of the genes on the chromosomal map is indicated.[50] Regulation of expression of the genes is indicated as follows: first, the amino acid or vitamin or purine/pyrimidine bases which, when present in the growth medium, plays a role in gene expression is indicated. Second, the protein which plays a regulatory role is represented by the name of its gene. The expression of the *thrA* gene, not discussed in the text, is regulated by attenuation and the product of the *ileR* gene.[170,171]

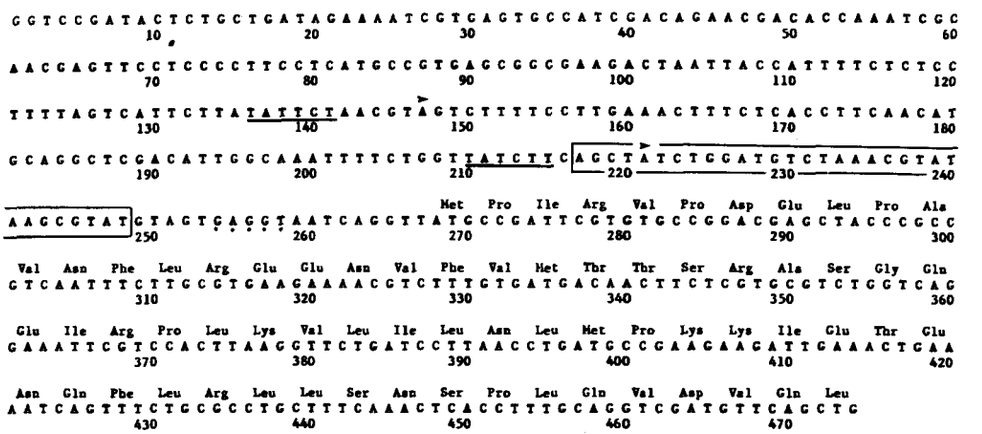


FIGURE 4. The nucleotide sequence of the noncoding strand of a DNA fragment containing the *metA* promoter regulatory region.[64] The inferred amino acid sequence is shown for the open reading frame. The following also applies to Figures 4 through 7: the "Pribnow box" sequences of the promoters are underlined; the mRNA start points are indicated by arrowheads; the Met repressor-binding site is boxed; putative ribosome-binding sites are indicated by dots; and regions of dyad symmetry are indicated by arrows.

A. Independent Transcriptional Units

1. metA Gene

The key enzyme in the methionine biosynthetic pathway is homoserine succinyltransferase, which is coded by the *metA* gene located at 91 min.[50] The *E. coli metA* gene has been cloned into a multicopy number plasmid from *metA* transducing phages.[26,63] The expression of *metA* was studied in wild-type and deregulated strains of *E. coli* K12 carrying the gene on high-copy number plasmids, and the results indicate that the synthesis of homoserine succinyltransferase is under the negative control of the regulatory *metJ* gene.[27]

The nucleotide sequence of a DNA fragment spanning the regulatory region and about one fourth of the *E. coli metA* gene has been reported (see Figure 4).[64] The study of the regulatory region of the *metA* gene indicates that transcription starts from a promoter which is under methionine control and is located next to a region which shares an extensive sequence homology with the operator regions of the *metBL*, *metF*, *metC*, and *metE* transcriptional units (see Section IV). However, there is a second transcription start signal for *metA* gene expression which is located 74 bp upstream from the first and which functions independently of the intracellular methionine concentration (Figure 4). Both promoters are expressed in vivo.[64]

2. metBL Operon and metC Gene

The biosynthesis of homocysteine in *E. coli* normally occurs by β-elimination from cystathionine catalyzed by cystathionine-β-lyase. Cystathionine is formed by the transsulfuration of *O*-succinylhomoserine by cysteine catalyzed by cystathionine-γ-synthase (Figure 2). Mutants deficient in cystathionine-β-lyase (*metC*) or cystathionine-γ-synthase (*metB*) are methionine auxotrophs. However, mutations (in a locus named *metQ*) were found which enable *E. coli* K12 to produce homocysteine in the absence of cystathionine-β-lyase.[65] In these *metC*, *metQ* mutant strains, cystathionine-γ-synthase directly catalyzes the formation of homocysteine from *O*-succinylhomoserine, bypassing the normal cystathionine intermediate. The function of the *metQ* gene product is still unknown. The fact that cystathionine-γ-synthase can physiologically replace cystathionine-β-lyase should be considered in the more general context of the homology of the two gene products discussed in Section V.

The *metB* and *metL* genes from *E. coli* and *S. typhimurium* were cloned with the *metJ*

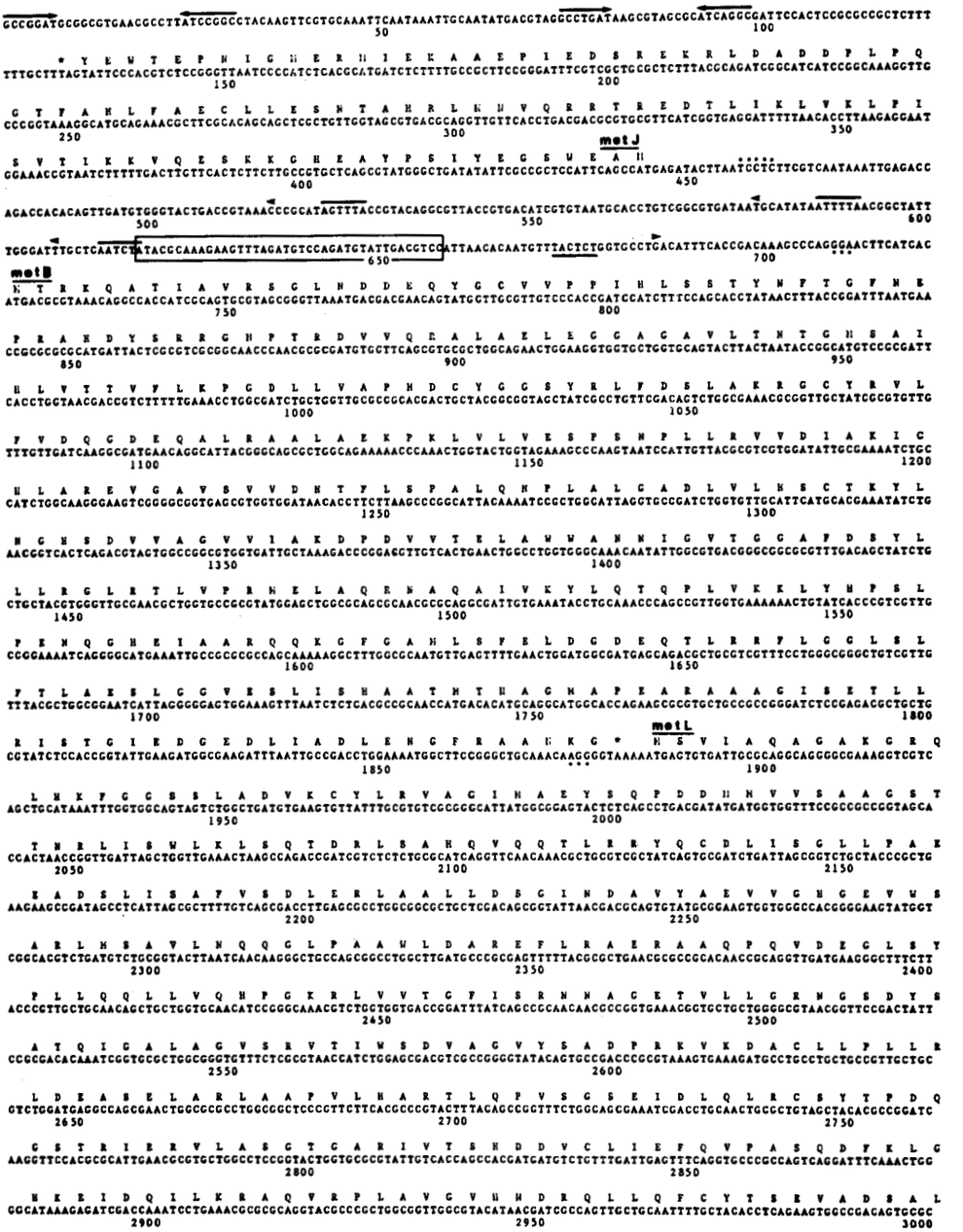


FIGURE 5. The nucleotide sequence of the *E. coli metJBLF* gene cluster.[13,31,39,91] The noncoding strand is presented except for *metJ*. The "Pribnow box" sequences of promoters are underlined except for *metJ* where they are overlined. The primary structure of the corresponding proteins as deduced from the nucleotide sequence is also indicated, the amino acids being represented by a one-letter code. Further details are given in legend to Figure 4.

regulatory gene.[66,67] The 1158-nucleotide-long *metB* gene coding for cystathionine-γ-synthase of *E. coli* is the first gene of the *metBL* operon.[31,62] The *metB* gene is transcribed divergently from the *metJ* gene and, consequently, a complex 276-bp regulatory region (vs. 264-bp region in *Salmonella*) is found between *metJ* and *metB*.[31,68] There is a single promoter for *metB*, whereas *metJ* is transcribed from three separate start points (see Section IV).[68,69] The entire sequence of the *E. coli metJBLF* cluster is shown in Figure 5.

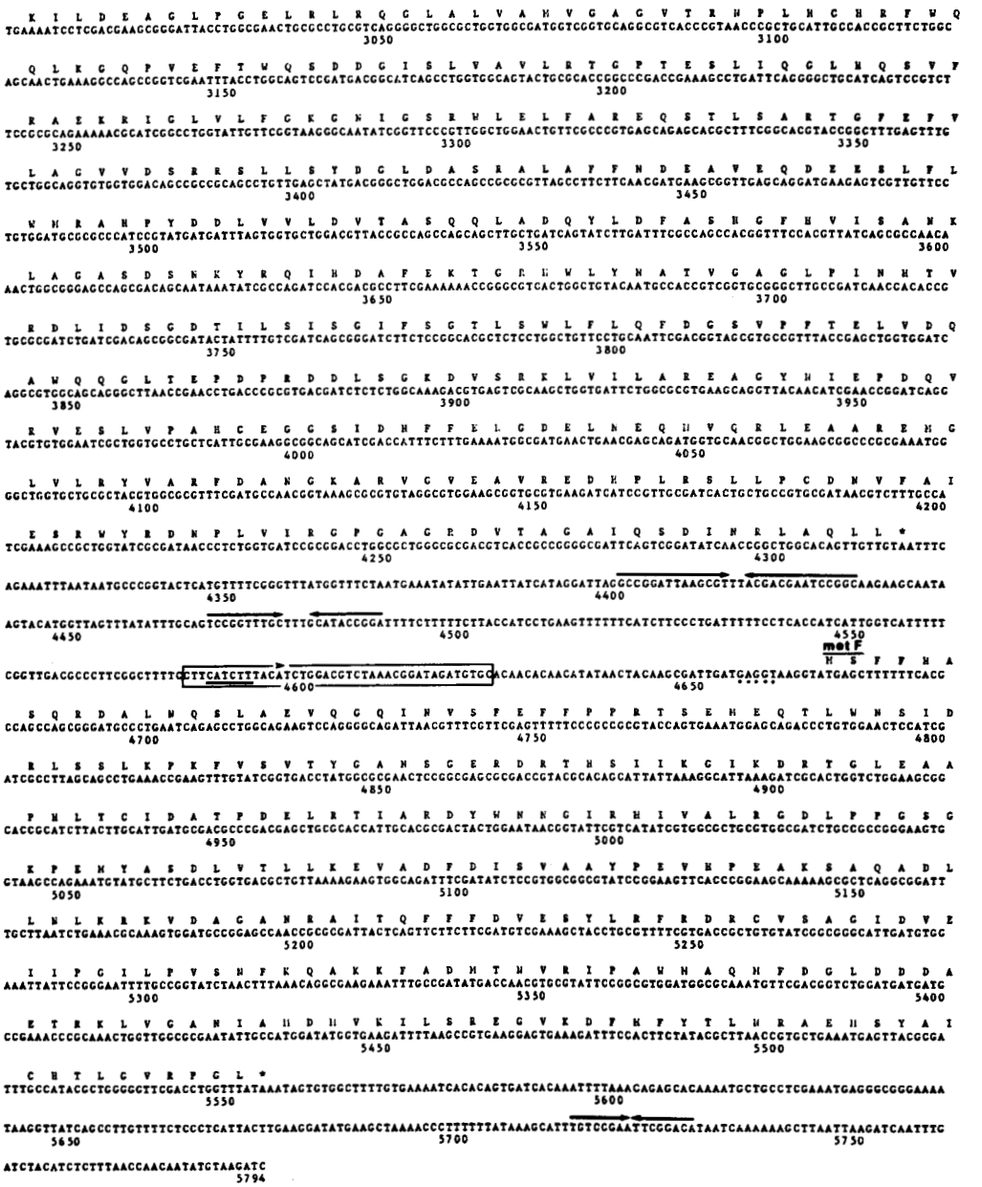


FIGURE 5 (continued)

The *E. coli metL* gene coding for aspartokinase II-homoserine dehydrogenase II is 2427 nucleotides long. Only two nucleotides separate the stop codon which terminates the *metB* gene from the translational origin of the *metL* gene.[13,31] It seems that the *S. typhimurium metL* gene is not part of an operon with *metB* since a Tn*5* insertion that inactivates *metB* does not affect *metL* complementation.[67] Nucleotide sequencing of the *metB-metL* intergenic region should indicate whether the *S. typhimurium* genes are organized differently from those of *E. coli*.

The *metC* gene, coding for cystathionine-β-lyase, has been cloned in a high-copy number plasmid and its 1185-nucleotide-long sequence was determined (Figure 6).[32,34,70] The promoter of the *metC* gene has not been identified, but operator-like sequences have been detected.

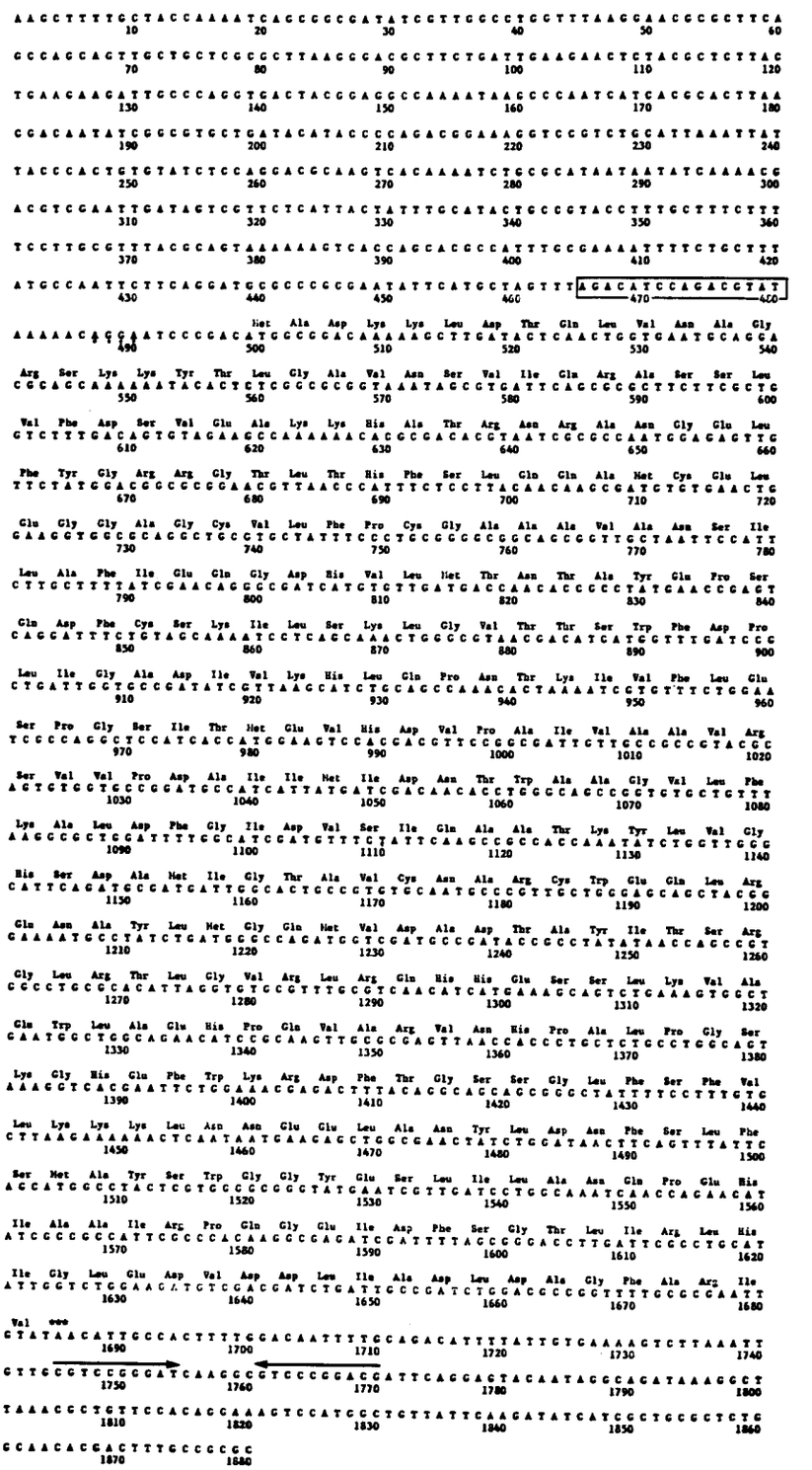


FIGURE 6. The nucleotide and deduced amino acid sequences of the *E. coli metC* gene.[34] For details, see legend to Figure 4.

3. metF, metE, and metH Genes

Expression of *metF*, *metE*, and *metH* genes is regulated not only by methionine, but also by vitamin B_{12} (Section IV). The *E. coli metF* gene coding for 5,10-methylenetetrahydrofolate reductase was cloned together with the *metJ* and *metBL* transcriptional units.[66] Its length is 888 nucleotides (Figure 5).[39] Both *metH* and *metE* code for a homocysteine transmethylase, the first being vitamin B_{12} dependent and the second being vitamin B_{12} independent. The *metE* genes of *E. coli* and *S. typhimurium* have been cloned and shown to code for a protein of approximately 95,000.[71,72] The sequence of the regulatory region of the *metE* gene has been determined[175] The *metH* genes of *S. typhimurium* and *E. coli* have also been cloned[73,176] and shown to encode a protein of approximately 125,000.

4. metK Gene

S-adenosylmethionine transferase catalyzes the biosynthesis of the intracellular alkylating agent *S*-adenosylmethionine.[49] No mutant completely lacking *S*-adenosylmethionine transferase has been reported, and even a strain with a transposon insertion in *metK* has residual *S*-adenosylmethionine transferase activity.[74] However, *metK* is indeed the structural gene for *S*-adenosylmethionine transferase, as discussed in Section II. The *E. coli metK* gene was shown to be located at 64 min, next to the *spe* genes encoding polyamine biosynthetic enzymes in the order *serA*, *speB*, *speA*, *metK*, *speC*.[54] The same 7.5-kb-long insert of a pBR322 derivative carries the *speA*, *metK*, and *speC* genes.[54] The nucleotide sequence of the *metK* gene indicates a length of 1152 nucleotides (Figure 7).[55]

5. glyA Gene

The conversion of serine to glycine produces most of the one-carbon units needed for methionine, purine, and thymine biosynthesis. Methionine plays a role in the regulation of the expression of glycine hydroxymethyltransferase, the enzyme responsible for both the synthesis of glycine from serine and the production of 5,10-methylenetetrahydrofolate. In *S. typhimurium* , a cumulative repression by serine, glycine, methionine, adenine, guanine, and thymine was proposed to regulate the synthesis of this enzyme.[75] This regulation does not occur in *metK* mutants, suggesting that a *metK* mutation affects not only the expression of the methionine biosynthetic enzymes but also that of glycine hydroxymethyltransferase.[76] The results in *E. coli* show that methionine limitation can lead to derepression of glycine hydroxymethyltransferase synthesis, but that the regulatory mechanism is different from the one which regulates the expression of the methionine genes.[77] Glycine hydroxymethyltransferase synthesis is thus partially regulated as if it were a methionine enzyme.[78] *S*-adenosylmethionine could be a corepressor of the synthesis of the *glyA* gene product, and a 22-bp dyad symmetry centered about the "Pribnow box" could be the target of an unknown repressor molecule (Figure 8).[79,80] No evidence for a transcription attenuation mechanism of regulation was obtained from the nucleotide sequence of the *E. coli glyA* control region.[80] The amino acid sequence, predicted from the DNA sequence, consists of 417 residues.[37]

6. asd Gene

The *asd* gene encodes aspartate semialdehyde dehydrogenase, an enzyme involved in lysine, threonine, and methionine biosynthesis. Its synthesis is multivalently repressed by these amino acids, the efficiency of derepression being greatest in the case of lysine limitation.[81,82] A regulatory role of glucose-6-phosphate also has been reported.[83] The gene was cloned and its nucleotide sequence (Figure 9) predicts a polypeptide chain of 367 amino acids, in good agreement with the molecular weight determined for the purified protein.[19,20,84,85] No characteristic features of a transcription attenuation signal were found in the region preceding the translational start signal. A large region of dyad symmetry that could play a role in regulation is located beween positions 160 to 236 (Figure 9).[20] Since

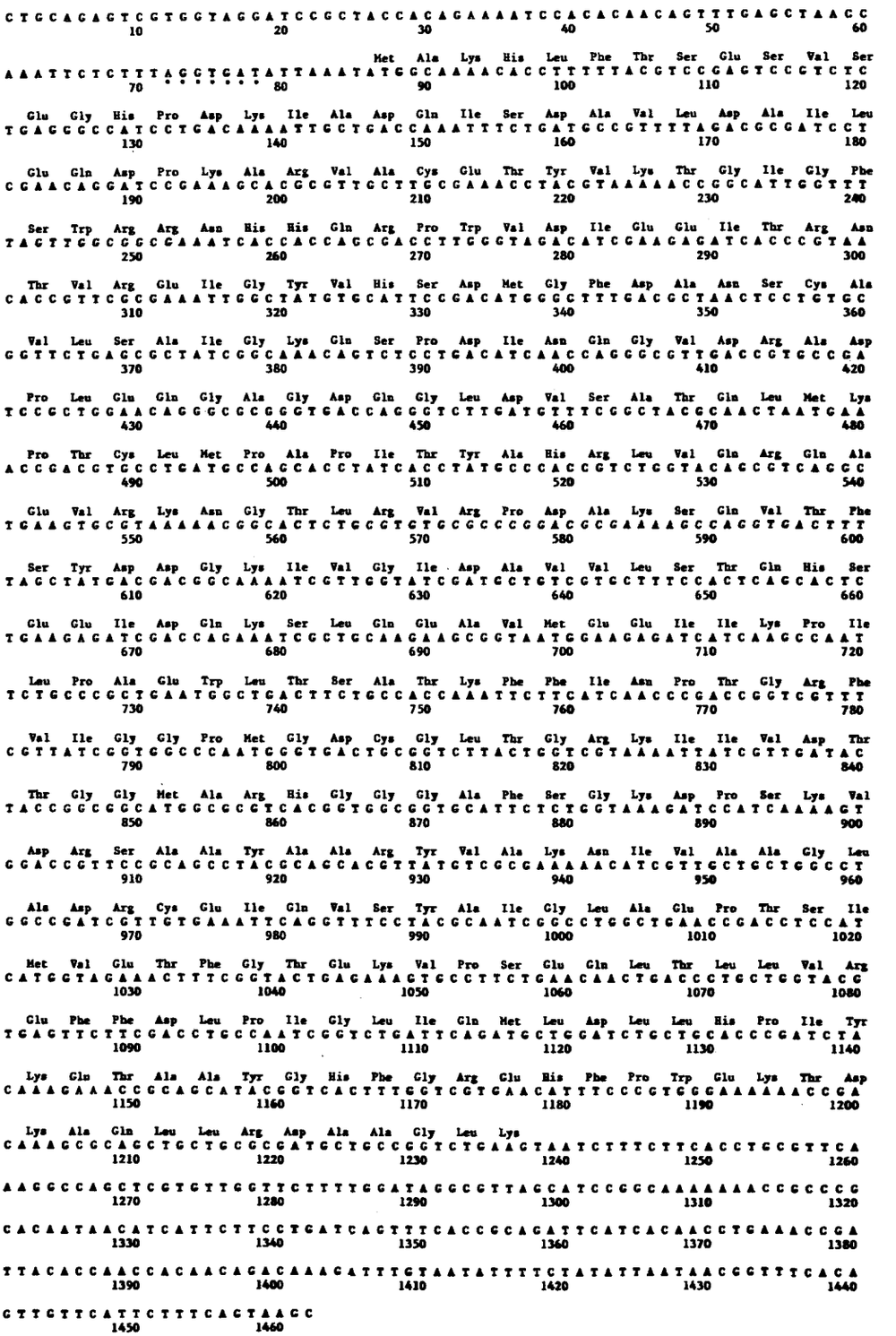


FIGURE 7. The nucleotide and deduced amino acid sequences of the *E. coli metK* gene.[55] For details, see legend to Figure 4.

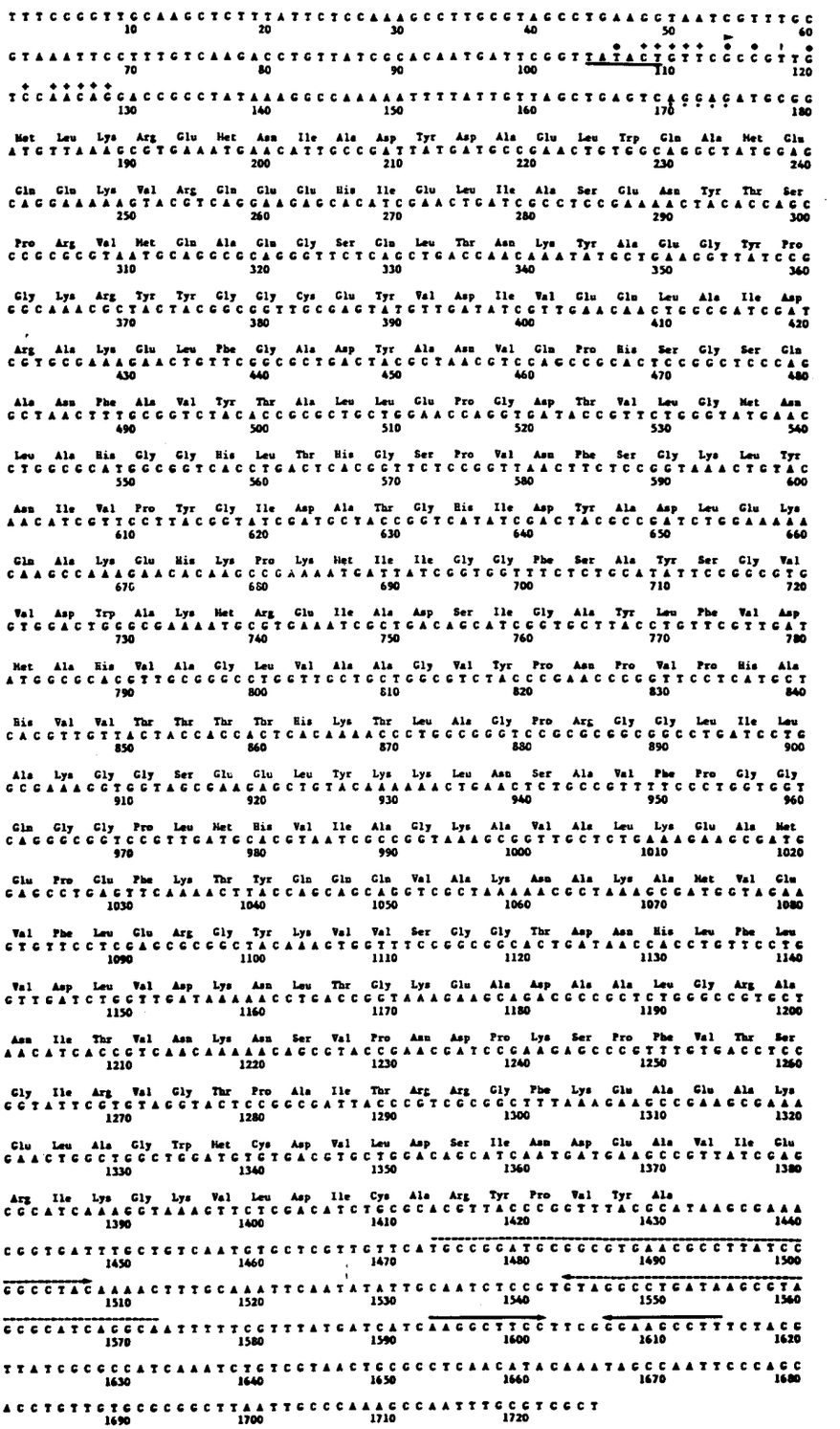


FIGURE 8. The nucleotide and deduced amino acid sequences of the *E. coli glyA* gene.[37] A 22-bp dyad symmetry around the "Pribnow box" is indicated by plus signs. The region of transcription termination is indicated by open arrows. For further details, see legend to Figure 4.

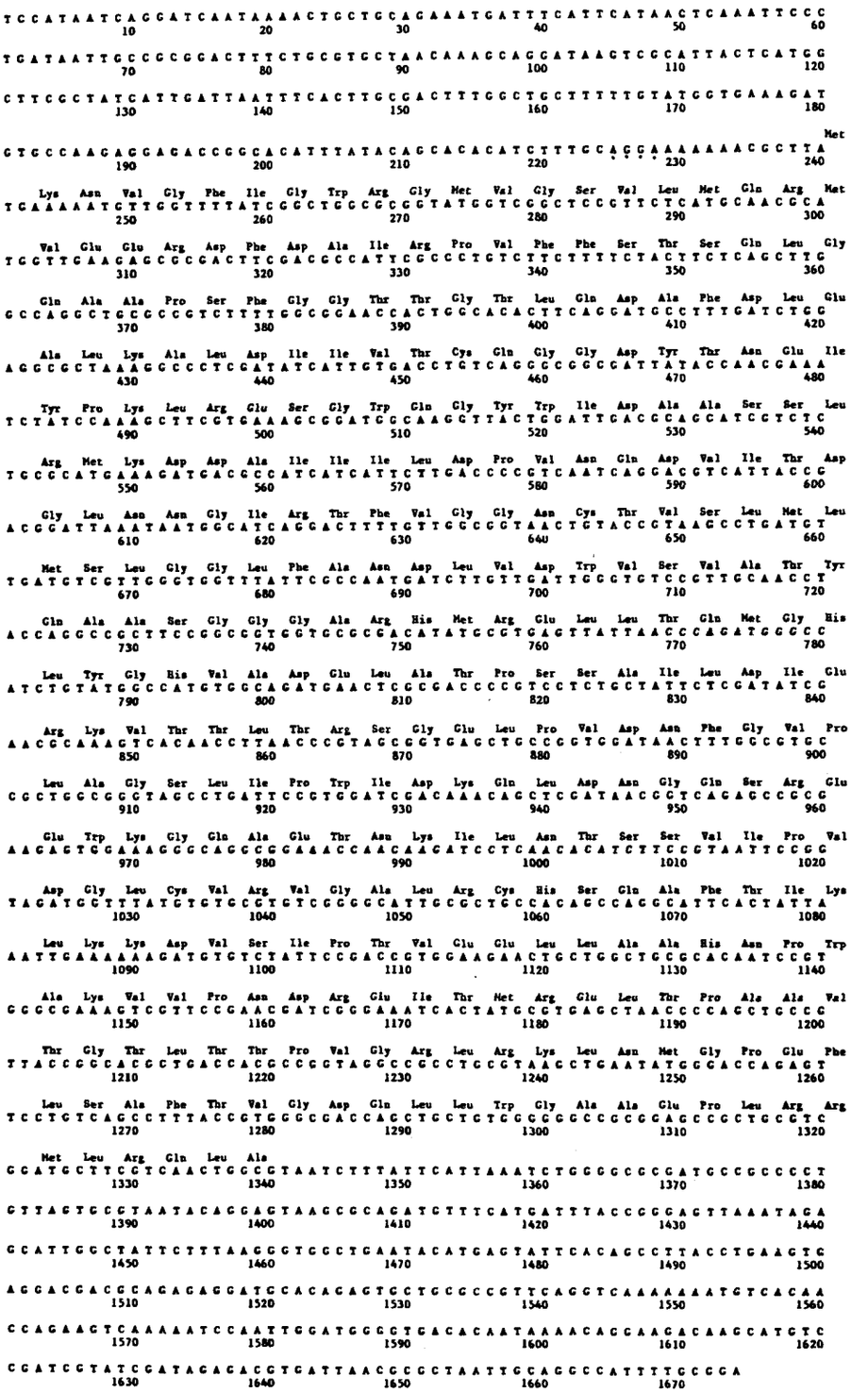


FIGURE 9. The nucleotide and deduced amino acid sequences of the *E. coli asd* gene.[20] For details, see legend to Figure 4.

FIGURE 10. Putative ribosome-binding sites of the *E. coli met* genes.[13,20,31,34,37,39,55,64,91] The sequence complementary to the 3'-OH extremity of 16S RNA is underlined and the ATG start codon is in boldface. The corresponding ribosome-binding sites of the *S. typhimurium metJ* and *metB* genes are identical.[68]

asd gene expression is regulated mainly by the availability of lysine, the identification of proteins involved in the regulation of the *asd* gene is part of studies underway concerning the lysine regulon.[16]

B. Ribosome-Binding Sites

There is now considerable evidence supporting the hypothesis that 16S RNA plays a direct role in the initiation of protein synthesis in *E. coli* and *S. typhimurium*.[86] In addition, the 5' ends of genes have been found to contain information besides the initiation codon and Shine and Dalgarno sequence.[87] Putative ribosome-binding sites for nine *met* genes are illustrated in Figure 10. In all cases, a purine-rich sequence complementary to the 3' end of 16S RNA is the only evidence for defining a ribosome-binding site. The extent of strict base pairing between mRNA and rRNA varies over a wide range from three nucleotides for *metB* to seven nucleotides for *metK*. In a study of the Shine and Dalgarno sequences of 124 genes, Stormo et al.[86] stipulated that at least three contiguous base pairs should be complementary to rRNA. The Shine and Dalgarno sequence of *trpR* is the only one which did not yield three contiguous base pairs.[86] It should be noted that in the case of *metL*, translation of the preceding gene (*metB*) should almost certainly affect the efficiency of initiation. Another striking variable is the distance between the ribosome-binding site and the ATG start codon (5 to 11 nucleotides). Further studies are needed before any conclusions can be drawn from these ribosome-binding site comparisons. The regulatory region between *metB* and *metJ* is rather complex. It has been reported that the *S. typhimurium metJ* gene is more efficiently transcribed than the *metB* gene, but that *metB* mRNA is more efficiently translated than *metJ* mRNA.[67] This suggests that translational efficiency plays an important role in maintaining the level of the *metJ* gene product.

C. Termination at the End of the Genes

There are only two methionine genes for which the 3′ end of the mRNA is known: the *metJ* regulatory gene and the *glyA* gene. In the other cases, only sequence comparisons have been made. The 3′ end of the *S. typhimurium metJ* mRNA was determined by the S1 mapping procedure and located about 40 bases distal to the 2 translation termination codons ending the *metJ* coding sequence.[67] The same procedure was used to locate the 3′ end of *glyA* mRNA at about 185 bp distal to the stop codon of the *E. coli glyA* gene.[37]

The proposed transcription termination region for the *glyA* gene is preceded by a G-C-rich sequence which, once transcribed, could form a stable stem-loop structure followed by an A-T-rich sequence within which transcription terminates. There is a long region of dyad symmetry and numerous smaller symmetrical regions between the stop codon and the site of proposed transription termination. These stem and loop structures show remarkable homology with intercistronic elements of other prokaryotic elements.[88] Indeed, a mutant was isolated with only 30% of the normal glycine hydroxymethyltransferase activity and the corresponding *cis*-acting mutation was located 35 bp after the *glyA* translation stop codon.[89] Those studies show that sequences distal to the *glyA* gene play an important role in the expression of the gene. Studies of retroregulation in λ phage support the role of 3′ secondary structures in upstream gene expression.[90]

Similar intercistronic elements had also been detected downstream from *metL* and *metJ*.[31,91] A ρ-independent terminator structure is indicated in Figure 5 following the translation stop codon of *metL*.

A region of dyad symmetry is located 20 bp after the stop codon of *metC*.[34] When transcribed, this region could form a stem and loop structure. However, it is not followed by a stretch of thymines and thus does not correspond to the characteristic structure of a ρ-independent termination signal. No typical ρ-independent terminator could be identified downstream of the structural *metF* gene.[39] Thus, there is no general rule for transcription termination of the *met* genes.

D. Promoter of *met* Genes

The promoters of the *met* genes are shown in Figure 11. The transcription start signals have all been determined by S1 mapping experiments. The consensus sequence of the −35 and −10 boxes for the *met* genes is TTGACN and TANNNT and the distance in between is almost invariably 17 nucleotides. These features correspond to the structure of promoter as defined by sequence comparison (TTGACA separated by 17 nucleotides from the "Pribnow box" TATAAT).[92] The transcription start signal is either a G or an A and is separated from the ATG start codon by 35 to 162 nucleotides. This region could be important for gene regulation. The promoters of the *E. coli met* genes are compared in Figure 11. Until now, only the regulatory regions of *metB* and *metJ* of *S. typhimurium* have been reported. The only differences between the *metB* and *metJ* −35 and −10 promoter sequences of the two organisms are one nucleotide change in the "Pribnow box" of *S. typhimurium metB* gene (TAATCT instead of TACTCT) and one nucleotide change in the −35 box of the third promoter of *S. typhimurium metJ* gene (GTGTCA instead of ATGTCA).

We should emphasize that the promoters and the operators of the *met* genes seem to overlap (see Section IV). It should be noted that for each particular gene the repressor-binding site is at a different location relative to the promoter, being around the −35 box, around the −10 box, or around the transcription start signals.

IV. REGULATION OF THE METHIONINE BIOSYNTHETIC PATHWAY

Growth of *E. coli* in the presence of methionine was shown to suppress methionine synthesis.[93-95] This was one of the first observations showing that synthesis of the enzymes

FIGURE 11. *E. coli* single or multiple promoter sequences of some methionine genes.[31,37,39,64,69,91] The sequences are aligned with respect to the first and the last T of the −35 and −10 boxes, respectively. The nucleotides identical to those of the canonical hexamers around −35 (TTGACA) and −10 (TATAAT) as well as the first transcribed nucleotide (+1) are in boldface. The number at the left of the "Pribnow box" indicates the position in the sequences given in the corresponding Figures (4, 5, and 8). The numbers in parentheses indicate the nucleotide distance between the transcription start signal and the A of the ATG start codon. In *S. typhimurium*, for which the unique *metB* and multiple *metJ* promoters are reported, the hexamers are identical except for two differences discussed in the text.[68] A few nucleotide differences are detected in the region between the −35 and −10 sequences; they do not modify the distance shown.

of a biosynthetic pathway can be inhibited by the end product of the pathway. Methionine represses the synthesis of the enzymes encoded by the *metA*, *metB*, *metC*, *metF*, and *metE* genes in both *E. coli* and *S. typhimurium*. Expression of *metL* gene involved in homoserine synthesis is also repressed by methionine.[4,96] We have also mentioned the role played by methionine in the regulation of expression of glycine hydroxymethyltransferase (Section III).

A. Regulation by Methionine and Vitamin B_{12}: Genetic Studies

Mutants resistant to inhibition by methionine analogs were first isolated in *E. coli*.[97] Subsequently, mutants resistant to α-methylmethionine, ethionine, and norleucine were selected in *S. typhimurium*.[98] These mutants fall into three classes. Those resistant to only α-methylmethionine carry *metA* mutations which alter feedback control of homoserine succinyltransferase and were discussed in Section II. The other two classes are mutated in *metJ* and *metK*.[98]

The *metJ* mutants, selected on the basis of their resistance to ethionine, overproduce methionine and are derepressed for the methionine biosynthetic enzymes and for methionine adenosyltransferase. The different derepression ratios for the various enzymes in *metJ* mutants confirm that the control of the expression of the methionine biosynthetic enzymes is not coordinate.[99] On the other hand, the levels of $tRNA^{met}$ and methionyl tRNA synthetase are unaltered in *metJ* mutants.[100] The *metJ* product was shown to be a protein by the isolation of suppressible nonsense mutations in the *metJ* gene.[101] The wild-type allele of the *metJ* gene is dominant, indicating that *metJ* encodes a *trans*-acting product in both *E. coli* and *S. typhimurium*.[100,102]

All of the *metK* mutants have reduced levels of methionine adenosyltransferase, although none of them are totally devoid of this essential enzymatic activity. There are two types of *metK* mutants.[52,98,103-105] One type excretes methionine and contains high levels of methionine biosynthetic enzymes that are not repressible (or are partially repressible) by methionine. The second type of *metK* mutants does not exhibit such properties, i.e., they have a normal

regulation of the methionine biosynthetic enzymes and possess methionine adenosyltransferases with elevated K_ms for methionine or temperature sensitivity or altered stability.[51,53,98,106] From the properties of the first group of *metK* mutants in which methionine regulation was altered, it was hypothesized that *S*-adenosylmethionine interacts with the aporepressor, encoded by *metJ*. The holorepressor then acts upon each of the *met* genes, whose operators have different affinities for the holorepressor.

It seems unlikely that methionyl-tRNA is involved in control of methionine synthesis since *metG* mutants of *S. typhimurium* and *E. coli* specifying altered methionyl-tRNA synthetase show normal regulation of the methionine biosynthetic enzymes.[100,107,108]

Two of the enzymes involved in the terminal steps of methionine synthesis — methylenetetrahydrofolate reductase (*metF*) and the B_{12}-independent methylase (*metE*) — are repressed in a noncoordinate manner by both vitamin B_{12} and methionine. The repression by vitamin B_{12} was first observed in *E. coli B*.[109] Based upon the observations obtained with three types of mutants — *metJ*, *metK*, and *metH* — it became clear that repression due to vitamin B_{12} and methionine is mediated by two different mechanisms.[110] The data also indicated that the holoenzyme form of the B_{12}-dependent methylase encoded by the *metH* gene might be involved in the repression by vitamin B_{12}.[110-112] This seems to be an unusual type of repression in which the coenzyme of a catalyst participating in a biosynthetic pathway is involved in the regulatory process. Mutations affecting regulation of the *metE* gene were generated in the presence of vitamin B_{12} by the insertion of Tn*5*.[74] In addition to Tn*5* insertions at the known regulatory loci, *metK* and *metJ*, Tn*5* insertions were also obtained at the *metH*, *metF*, and *btuB* loci.[113] The isolation of the *metH* mutant was consistent with the previous finding that the *metH* product is required for the repression of the *metE* gene by vitamin B_{12}.[110] The results with the *metF*::Tn*5* insertion suggest that a functional *metF* gene product was also needed for repression of *metE* by vitamin B_{12}.[74] The isolation of mutants with Tn*5* insertions in *btuB* (vitamin B_{12} uptake) can be explained by their inability to accumulate high intracellular levels of vitamin B_{12}.

At least 20 enzymes are required for the production of cyanocobalamin (vitamin B_{12}), and it was generally believed that enteric bacteria are unable to synthesize it *de novo*.[114,115] Indeed, the activities of one of the homocysteine methylases (the *metH* gene product) and of ethanolamine ammonia lyase (E.C.4.3.1.7) in vivo depend upon an exogenous source of the vitamin in *E. coli* and *S. typhimurium*. However, experiments on the in vitro biosynthesis of methionine by crude extracts suggested that *S. typhimurium* could synthesize cyanocobalamin to a "limited extent".[116] In addition, certain *E. coli metE* mutants do not require methionine for anaerobic growth.[117] Further data led to the discovery that *S. typhimurium* could synthesize cyanocobalamin *de novo* under anaerobic culture conditions.[118] Since no essential role is played by the two known cyanocobalamin-dependent enzymes which function aerobically when the vitamin is supplied exogenously, one could ask why *S. typhimurium* synthesizes vitamin B_{12} at all. It is possible that the efficiency of methionine formation is more critical in anaerobically grown cultures. The cyanocobalamin-independent methyltransferase is much less efficient than the cyanocobalamin-dependent enzyme, the turnover numbers of the purified *E. coli* enzymes being 14 and 780 (moles of methionine formed per minute and per mole of enzyme), respectively.[44,119]

In order to compensate for its inefficiency, the *metE*-encoded enzyme is synthesized in large amounts. Thus, under aerobic conditions, the *metE* enzyme represents 3 to 5% of total protein in *E. coli*.[44,116] In anaerobically grown cultures, the cost imposed by the *metE* enzyme on protein synthesis may be energetically prohibitive, so that it might be more advantageous for the cells to synthesize cyanocobalamin in order to produce methionine more efficiently.

B. Methionine Repressor

1. Isolation and Characterization of the metJ Gene Product

The product of the *metJ* gene is a 12-kdalton protein.[68,91,120] The observation that the

methionine regulon is turned off in some strains carrying plasmids with a functional *metJ* gene implies that these cells overproduce the MetJ protein.[120] However, the amount of MetJ protein produced by these strains is still very low. Bacterial strains carrying the *metJ* gene on a plasmid from which the *rop* gene has been deleted produce elevated levels of the MetJ protein (0.2% of the total protein), presumably because of the high plasmid copy number in the cells.[121,122] Smith et al.[121] prepared radiochemically pure MetJ protein from plasmid-bearing maxicells to use as a tracer in large-scale purification. The *metJ* gene product was then obtained in nearly homogeneous form (with a purification factor of about 600-fold). Sedimentation equilibrium experiments showed that the native MetJ protein is a dimer and the extinction coefficient of the monomer at 280 nm was found to be $15 \times 10^3\ M^{-1}\ cm^{-1}$.

Since the amount of Met aporepressor was still insufficient for physicochemical studies, the *metJ* gene was cloned under the control of a strong, inducible promoter.[123] The *metJ* gene product represents 2% of the total protein in strains carrying such a construction compared with 0.02% (or 600 dimeric molecules per cell) in a wild-type strain.[123] The Met aporepressor was readily detectable on a sodium dodecyl sulfate polyacrylamide gel in crude extracts of the hyper-producing strain. The 50-fold purification of Met aporepressor yielded pure material with an overall recovery of 23%.

2. Definitive Identification of the Methionine Repressor

Genetic and biochemical studies have indicated that the *metJ* gene product and *S*-adenosylmethionine are involved in the repression of the methionine biosynthetic pathway. The regulation of the expression of the *E. coli metF* gene, coding for 5,10-methylenetetrahydrofolate reductase, has been investigated in vitro with various concentrations of the Met aporepressor and *S*-adenosylmethionine.[124] A simplified DNA-directed in vitro system measured the formation of the first dipeptide (fMet-Ser) of the *metF* gene product. Up to 100 μM *S*-adenosylmethionine alone had no effect on dipeptide synthesis, whereas high levels of Met aporepressor alone significantly inhibited fMet-Ser formation in a system programmed with a plasmid carrying the *metF* gene. Low concentrations of Met aporepressor inhibited dipeptide synthesis only when *S*-adenosylmethionine was present. The same effect was observed when the synthesis of the entire *metF* gene product was studied. MetJ aporepressor and *S*-adenosylmethionine inhibit *metF* transcription rather than translation since they were without effect in a system programmed with *metF* mRNA.

The binding of Met aporepressor to *met* DNA was determined by a gel electrophoresis assay similar to that used to detect lac repressor-operator binding.[123,125] Increasing amounts of aporepressor were incubated with a 319-bp fragment of *metF* DNA containing the potential repressor-binding site and electrophoresed on an acrylamide gel. Free DNA and the aporepressor-DNA complex migrate differently. L-Methionine did not enhance the specific aporepressor-DNA interaction even at 10 nM, whereas the same concentration of *S*-adenosylmethionine increased the affinity of the MetJ protein for the repressor site. An approximate value of 1 nM has been determined for the dissociation constant (Kd) of the repressor-operator complex in the presence of *S*-adenosylmethionine. In its absence, the Kd was about one order of magnitude higher.

Equilibrium dialysis experiments showed that 2 mol of *S*-adenosylmethionine binds to 1 mol of dimeric aporepressor, in an apparently noncooperative manner, suggesting that the two corepressor-binding sites are identical and independent.[123] The affinity of *S*-adenosylmethionine for the aporepressor is low (Kd = 200 μM).

These in vitro studies, in agreement with genetic studies, led to the conclusion that the Met holorepressor is composed of the *metJ* gene product and *S*-adenosylmethionine as a corepressor.

3. Physicochemical Studies on the Methionine Repressor

Several properties of the Met aporepressor such as its relatively small size, its dimeric

structure, and the *S*-adenosylmethionine-directed interaction with specific DNA sequences make it attractive for physicochemical studies. The protein has been crystallized in a form adequate for X-ray diffraction studies from an ammonium sulfate solution at pH 5.75 to 6. The crystals diffract to a resolution of at least 3 Å and are suitable for full three-dimensional structure analysis.[177] Attempts are now under way to grow crystals of the aporepressor-*S*-adenosylmethionine and aporepressor-*S*-adenosylmethionine-operator complexes suitable for diffraction studies.[178] Preliminary two-dimensional NOESY and COSY NMR spectra measured with a 400-MHz spectrometer are well resolved, and assignment of many of the observed resonances should be possible.[179] The combination of NMR and crystallographic data should facilitate a detailed study of the structures of the methionine repressor and its complexes in solution and crystalline state.

Infrared (IR) spectroscopy was one of the earliest experimental methods used for estimating the secondary structure of polypeptides and proteins.[126] The use of the Fourier deconvolution technique, whereby the broad overlapping amide bands are narrowed by computational procedure, has allowed a more detailed analysis of protein secondary structure.[127] The IR spectra of Met aporepressor is clearly dominated by the strong amide I and amide II bands at 1660 and 1550 cm^{-1}, while the amide III band around 1300 cm^{-1} is much less prominent.[128] Analysis of the secondary structure of the Met aporepressor is restricted to the amide I mode, for which resolution enhancement by Fourier deconvolution (as well as by Fourier derivation) revealed five individual component bands. The position of these bands with their width, half height, and total areas (as integrated intensities) is in turn related to the population of the corresponding substructures, of which there are at least four types in the native protein. The band at 1653 cm^{-1} can be assigned unambiguously to α-helices (44%), while the component bands at 1625 and 1676 cm^{-1} are due to β-structures. The 1665-cm^{-1} band is most likely due to turns, while the band at 1639 cm^{-1} deserves special attention since it can be assigned to β-structures or un- (or less-) ordered peptide segments in which the amide NH group has been replaced by ND groups. Although these assignments do not depend on any transferred secondary structure values from model homopolypeptides or on statistical correlations, they are tentative and should be confirmed by X-ray analysis.

4. Autoregulation of the E. coli and S. typhimurium metJ Genes

Regulation of the *metJ* gene expression in both organisms was examined by measuring β-galactosidase activity in *E. coli* strains lysogenic for a λ phage carrying a *metJ-lacZ* gene fusion. The results indicated that the *metJ* gene is regulated by its own gene product.[91,129] The experiments with the *S. typhimurium metJ* gene were designed in such a way that regulation by methionine supplementation to the growth medium could also be demonstrated.[129] The *S. typhimurium metJ* gene uses two tandem promoters for transcription, pJ1 and pJ2, separated by 72 bp.[67] Deletion analysis permitted the individual assessment of the activity of promoters pJ1 and pJ2.[129] Promoter pJ1 activity was negatively regulated by the *metJ* gene product and methionine. Although the mechanism of pJ2 regulation remained unclear, evidence was presented suggesting that it was not negatively regulated in the same way as pJ1.[129] Analyzing *metJ* transcription in *E. coli*, Kirby et al.[69] found three transcripts — J1, J2, and J3 — the latter two corresponding to *S. typhimurium* J1 and J2 (Figure 5). Since the promoter sequences corresponding to J1, J2, and J3 are conserved in both organisms except for a single nucleotide change, it is likely that they function in both *E. coli* and *S. typhimurium*, and indeed a third promoter, pJO, corresponding to pJ1 of *E. coli*, was found recently in *S. typhimurium*.[69,130] The start sites of the *E. coli* J1, J2, and J3, as determined by S1 mapping, are nucleotides −607, −587, and −515, respectively, as shown in Figure 5.[69] The J1 transcript is most prominent in cells with *metJ* or *metK* mutations (i.e., derepressed cells), although a trace of it is present in wild-type *E. coli* K12 grown in minimal medium. It appears that transcription from the J2 promoter is also reduced in cells with a fully functional

met regulatory system, although not to the same extent as that from J1. The J3 transcript is not repressed. It has been suggested that transcription from J3 (and from the corresponding *S. typhimurium* J2 promoter) may be stimulated by MetJ protein binding. The results with *metJ-galK* fusions in *E. coli* confirm that *metJ* is autoregulated.[69]

In a simplified in vitro system that measures the formation of the first dipeptide of the gene product (fMet-Ala for the *metJ* gene), MetJ protein and *S*-adenosylmethionine partially repressed (40 to 60%) *metJ* gene expression, thereby confirming autoregulation.[131] A number of other regulatory genes have also been shown to be autoregulatory. The genes for which the transcription sites are known, such as *tyrR*, *trpR*, *araC*, *crp*, and *cI*, differ from *metJ* in that they do not use multiple promoters.[132-136] Tandem promoters have been reported for genes coding for enzymatic proteins *metA*, *glnA*, *carA*, and *gal*.[64,137-139] Whether the multiple promoters for the *metJ* system are unique among genes for regulatory proteins remains to be determined.

5. Isolation of Mutations in the metJ Gene

Plasmids carrying wild-type or mutant alleles of the *E. coli metJ* gene were constructed. High-copy number plasmids with a functional *metJ* gene can cause a methionine growth requirement, apparently because of overproduction of the gene product and repression of the methionine regulon. This property was used to isolate insertion mutations of the plasmid-borne *metJ* gene leading to recovery of prototrophy.[120]

Plasmids carrying previously isolated *metJ* mutations also were constructed.[120,140] The nucleotide changes responsible for some of the mutant phenotypes have been determined. The *metJ185* defect is due to an amber mutation transforming the third codon (Trp) into an amber codon, whereas the Ala codon at position 60 is changed into a Thr codon in the *metJ184* allele.[91,140]

C. Methionine Operators

1. Upstream Regions of the Structural Genes Involved in Methionine Biosynthesis

Since all the *met* genes are subject to MetJ protein-mediated repression by methionine, it is reasonable to assume that the repressor-binding sites are similar. Two DNA regions have been proposed as MetJ repressor-binding sites in *E. coli*. First, DNA sequences with a twofold symmetry (5′-ATCT---C--------G---AGAT-3′) in the regions upstream from the *metF* and *metBL* transcriptional units were suggested as possible binding sites for the *metJ* gene product.[31,39] Michaeli et al.[64] compared the 5′ region of the *metA* gene to those of *metF* and *metB* and found extensive homology, although no common axis of symmetry was found. In addition, Markham et al.[55] used this sequence to assign a repressor-binding site to the 5′-flanking region of the *metK* gene which encodes methionine adenosyltransferase, an enzyme that utilizes methionine as a substrate. However, the authors introduced a 13-nucleotide gap in the regulatory regions of the *metF* and *metB* genes in order to maximize the homology and to keep the same axis of symmetry.

A second possibility that applies to all the *met* operators (except that of *metK*) is that the repressor-binding site is composed of repetitions of an 8-nucleotide-long unit.[34] The consensus sequence of this unit is a perfect palindrome, 5′-AGACGTCT-3′, and the unit is present in two to five copies, depending on the *met* gene (Figure 12). The differences between the frequency of repetition and degree of homology could be related to the different extents of repression elicited by the *metJ* gene product: the ratios of derepressed vs. fully repressed levels were found to be 12 for *metC*, 40 for *metB*, 100 for *metF*, and 300 for *metA*.[42]

It should be noted that the *metB* and *metJ* genes are transcribed divergently and could share the same repressor-binding site. Very recently, the tandemly repeated palindrome 5′-AGACGTCT-3′ was shown to be present in the 5′-upstream region of the *metE* gene.[175] A

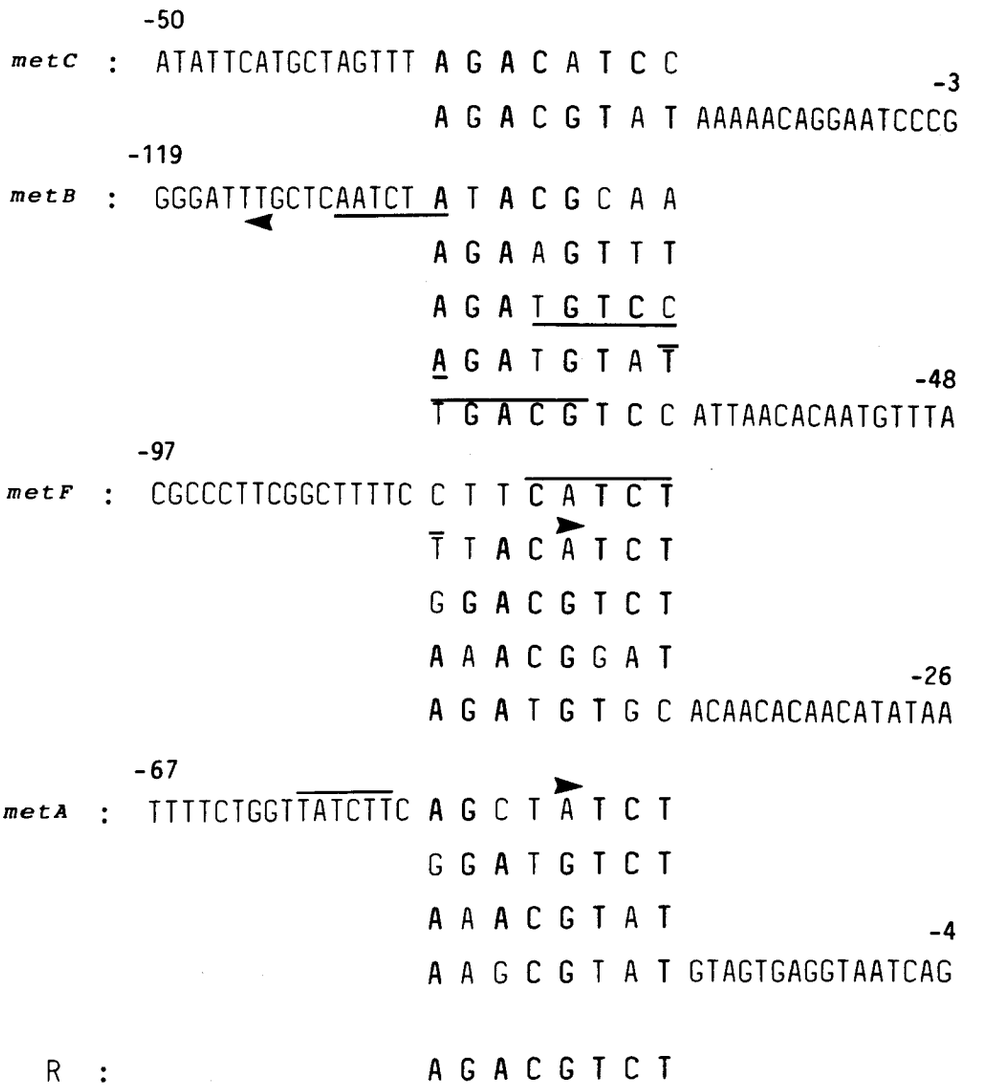


FIGURE 12. Comparison of the upstream regions of the *metC*, *metB*, *metF*, and *metA* genes.[31,34,39,64] The sequences 5′ to the structural *metC*, *metB*, *metF*, and *metA* genes are presented discontinuously and have been aligned in order to focus on the presence of the underlying repetitive palindromic unit. Nucleotides matching the consensus sequence presented in line R are in boldface. Numbers indicate positions relative to the adenine of the respective start codon taken as +1. The −10 promoter sequences are overlined and arrowheads indicate the transcription start signals. In the case of *metB*, the overlined hexamer is the −35 box; the two underlined promoter sequences represent the −35 and −10 boxes of the first promoter of *metJ*.[69]

repressor-binding site can be assigned tentatively to the region around the −35 box of the *glyA* promoter.[80,181]

2. Binding of the E. coli Met Repressor to DNA

Purified MetJ protein binds to a region of *E. coli* DNA between the *metB* and *metJ* genes, protecting 40 to 50 bp from cleavage by DNAase I, Fe-methidiumpropyl-EDTA/dithiothreitol, or Fe-EDTA/ascorbate/hydrogen peroxide.[121,141] The −35 sequence of the *metB* promoter, the entire *metJ1* promoter, and possibly part of the −35 part of the *metJ2* promoter

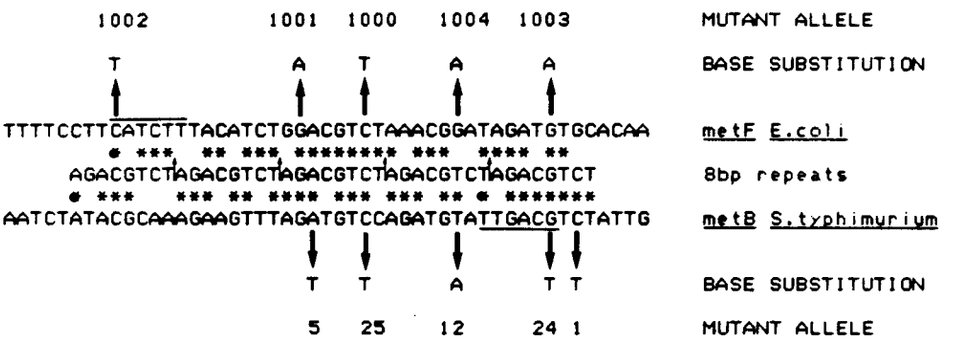


FIGURE 13. Mutations in the *met* regulatory regions. Part of the sequence of the regulatory region of the *E. coli metF* and the *S. typhimurium metB* genes is shown.[130,142] The nucleotide substitutions affecting the two genes are indicated. The −10 of the *metF* and the −35 of the *metB* genes are overlined and underlined, respectively. The sequence between the two regulatory regions represents the repetition of the AGACGTCT octamer, each repetition being delimited by a small arrow. The presence of an identical nucleotide between the *met* sequences and the consensus octamer is indicated by an asterisk.

lie within the protected region. In Figure 12, it can be seen that the protected region includes the repressor-binding site predicted by sequence comparison.[34]

The complex that binds to DNA seems to contain two or more molecules each of MetJ protein and *S*-adenosylmethionine since protection of DNA against chemical cleavage showed high cooperativity with respect to both components. *S*-adenosylmethionine might play a modulator role by promoting MetJ oligomerization.[141]

3. Isolation of Operator Constitutive Mutations

a. Mutations in the E. coli metF Regulatory Region

Sequence comparisons focused attention on DNA regions that might be the potential binding sites for the methionine repressor.[34] The results of the footprinting experiments supported this hypothesis.[121,141] A search for operator constitutive mutants was thus undertaken to find the target for the repressor protein using, as an example, the *E. coli metF* regulatory region.[142] Mutagenesis was performed in vivo by increasing the spontaneous rate of mutation through the use of a mutator allele or by the use of ethyl methane sulfonate or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in a strain carrying a plasmid bearing a *metF-lacZ* hybrid gene. The mutations carried by the recombinant plasmids were identified by increased β-galactosidase production, the *lacZ* gene being under the control of the *metF* promoter. Five mutations exhibiting all characteristics typical of operator constitutive mutations were localized to five distinct positions in the regulatory region of the *metF* gene (Figure 13). β-Galactosidase activity in a cell-free system programmed with DNA from the *metF-lacZ* gene with a wild-type operator was repressed 400-fold, whereas little or no repression was obtained with the altered operators. Moreover, the nucleotide changes affected the repetitive unit in such a way as to reduce its homology with the consensus sequence, AGACGTCT. Site-directed oligonucleotide mutagenesis has allowed the isolation of numerous additional mutations in the operator of the *metF* gene.[182]

b. Mutations in the S. typhimurium metB Regulatory Region

In order to genetically define the *S. typhimurium metJ* repressor-binding site, *cis*-acting up-mutations were selected in the *metJB* control region.[130] Mutations affecting the expression of *metB-lacZ* fusion were isolated in vivo. β-Galactosidase assays of extracts of the mutants showed elevated, partially regulated enzyme synthesis typical of mutations in a repressor-binding site. The mutations were located within or near the −35 region of the *metB* promoter,

and four out of the five mutations disrupted a region of dyad symmetry (Figure 13). It should be noted that most of the sequence forming the dyad symmetry can be generated by three repetitions of the above consensus octanucleotide 5′-AGACGTCT-3′. The fifth mutation alters the −35 sequence of the *metB* promoter in such a way that it perfectly matches the consensus sequence TTGACA. Similarly, a mutation was found altering the −10 sequence of the *E. coli metF* promoter so that it was closer to the consensus sequence TATAAT.[142]

D. Other Regulatory Mechanisms

No evidence for control by transcription attenuation typified by a region specifying a leader peptide and a terminator structure has been found in the 5′-upstream region of the *met* genes sequenced so far. On the other hand, there is evidence that positive control is involved in regulation of expression of some biosynthetic pathway genes.[143-145] Such seems to be the case in the regulation of the expression of two *met* genes. In *S. typhimurium*, the *metR* gene is adjacent to *metE* and its product appears to be necessary for expression of the *metE* and *metH* genes.[146] The *metR* locus was discovered during the search for methionine auxotrophs.[146] A class of auxotrophs with an unusual phenotype was further studied. Their behavior was analogous to that of *metF* mutants, although they carried normal *metE*, *metH*, and *metF* genes. The methionine auxotrophy of *metR* mutants was the result of insufficient production of both transmethylases. Since *metE* and *metH* at high-copy numbers restore *metR* mutants to prototrophy, the *metR* product is not a subunit necessary for transmethylase activity but is necessary for *trans*-activation of *metE* and *metH* genes expression.

The *metR* mutation was shown to be linked to *metE*, but outside the *metE* structural gene.[146] The location of the *metR* locus could be correlated with the reported existence of two *metE* complementation groups.[147] In fact, group I corresponds to mutations in the *metE* structural gene and group II to those in *metR*.[146] In addition, the necessary *trans*-activation of expression of the *metE* gene by the *metR* gene product could explain the very low amount of *metE* protein obtained *in vitro* compared with the high expression of *metE* obtained *in vivo*.[71] In conclusion, the *metE* gene is negatively regulated by the *metJ* and *metH* gene products and positively regulated by the *metR* gene product.

Regulation of the *metH* gene is much less well documented. It is not clear whether the *metH* gene expression is induced by vitamin B_{12} or if vitamin B_{12} stabilizes the gene product. (There is some doubt whether the *metH* gene is repressed to a significant extent by methionine. A limited positive effect is exerted on the expression of the *metH* gene by the *metR* gene product.) In addition, the *metH* gene product is *probably* itself a regulatory protein since it *seems to be* involved in repression of two genes of the folate pathway (*metE* and *metF*). Further mutational analysis and *in vitro* binding studies will be necessary to understand the interactions that occur in the different *met* control regions.

V. EVOLUTIONARY CONSIDERATIONS

A. Aspartokinases-Homoserine Dehydrogenases: Iso- and Bifunctional Enzymes

As discussed in Section II, the first step of the common pathway in *E. coli* K12 is catalyzed by three distinct aspartokinases, and the third step is likewise catalyzed by two distinct homoserine dehydrogenases. The discovery of two different bifunctional enzymes with the same activities immediately focused attention on the similarities between these two proteins and their evolutionary relationship. Enzymatic activities, molecular weights, amino acid compositions, proteolytic domains, and immunochemical reactivities were compared and led to the hypothesis that the two enzymes had a common ancestor.[3]

1. Amino Acid Sequence Comparisons

Determination of the nucleotide sequences of *thrA* and *metL*, which encode aspartokinases-

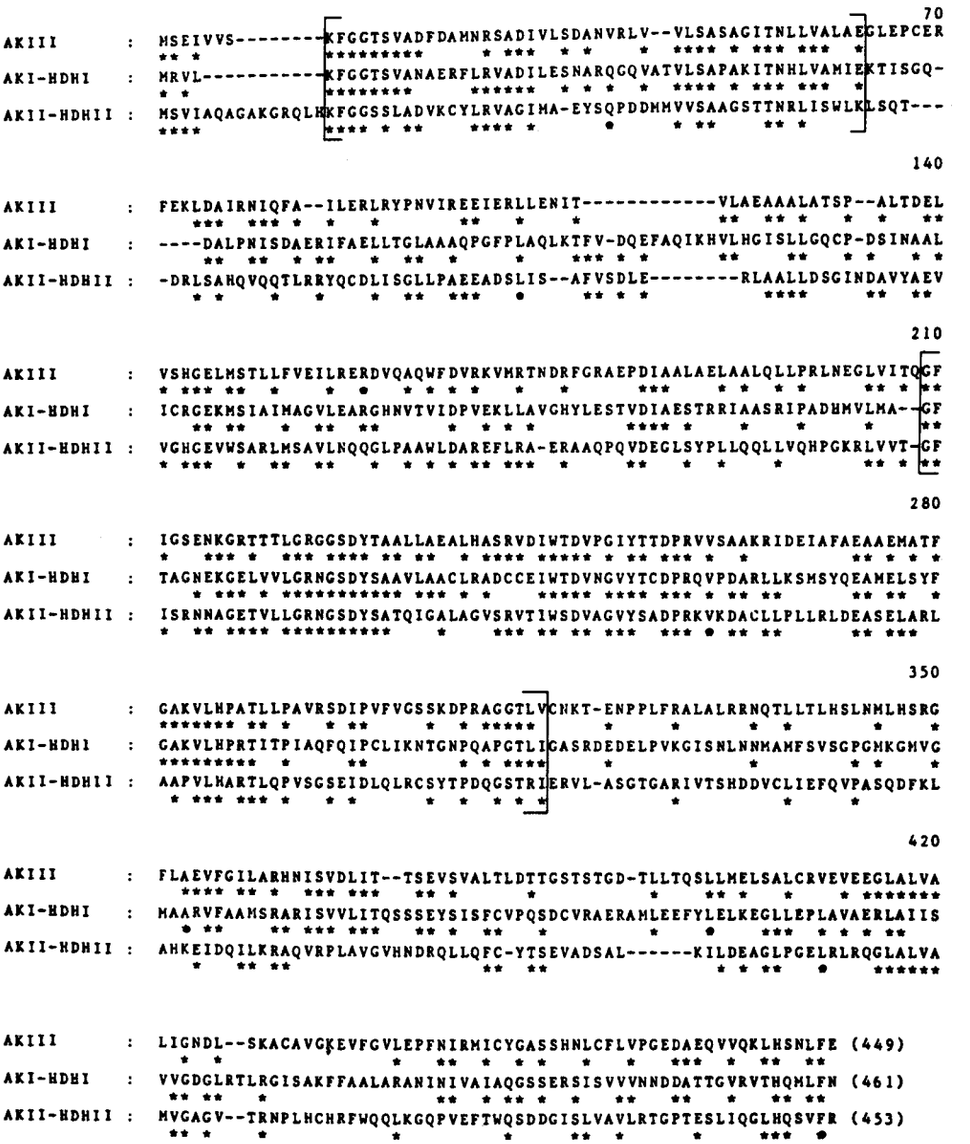


FIGURE 14. Comparison of the three aspartokinases of *E. coli*. The deduced amino acid sequence of aspartokinase III (AK III) and part of aspartokinase I-homoserine dehydrogenase I (AK I-HDH I) and aspartokinase II-homoserine dehydrogenase II (AK II-HDH II) are presented in the one-letter code.[13-15] Sequences have been aligned in order to maximize similarities by introducing gaps indicated by dashes (−). Identical residues are indicated by stars under the sequences. Numbers refer to the amino acid positions in the alignment, and numbers in parentheses refer to the position of the last presented amino acid in the original sequences. Brackets delimitate the two most conserved regions.

homoserine dehydrogenases I and II, respectively, allowed the amino acid sequences of the two proteins to be compared.[13,14] This comparison revealed extensive homology in the primary sequences (Figure 14) and thereby provided unequivocal evidence that these two bifunctional enzymes evolved from a common ancestor.[13]

Determination of the nucleotide sequence of the *lysC* gene, encoding *E. coli* lysine-sensitive aspartokinase III, revealed that the entire amino acid sequence of aspartokinase III

is homologous to the N-terminal part of the two bifunctional aspartokinases-homoserine dehydrogenases (Figure 14).[15] A model was then proposed describing the evolution of this family of enzymes, taking into account the internal homologies in each aspartokinase and homoserine dehydrogenase moiety, and the data were obtained by limited proteolysis experiments on aspartokinases-homoserine dehydrogenases I and II.[8,148] This model proposes that *lysC* could have been derived from *thrA* after the fusion of the aspartokinase and homoserine dehydrogenase coding sequences. A high degree of conservation of the amino acid sequences was observed for two segments of the three protein sequences (residues 16 to 63 and 209 to 315 in Figure 14). Limited proteolysis experiments on aspartokinases-homoserine dehydrogenases I and II have led to the conclusion that the native polypeptides have a triglobular structure: an N-terminal domain (corresponding to residues 1 to 245) which carries the aspartokinase activity, a central domain (corresponding to residues 250 to 500) involved in subunit interactions, and a C-terminal domain (corresponding to residues 500 to 820) endowed with the homoserine dehydrogenase activity.[8] Thus, part of the second, very conserved region in the primary sequence (residues 209 to 315) spans the central domain delineated by limited proteolysis (residues 250 to 500) and is probably involved in protein-protein contacts leading to the polymeric state, dimeric in the case of aspartokinase III and aspartokinase II-homoserine dehydrogenase II and tetrameric in the case of aspartokinase I-homoserine dehydrogenase I.

2. Comparison with Enzymes from Other Organisms

Whereas two isofunctional aspartokinases have also been reported in Bacillaceae, multiple aspartokinases generally are not found in organisms other than Enterobacteriaceae.[149] A different control pattern has been found in other bacterial genera, such as *Pseudomonas*, purple bacteria, and *Azotobacter*.[150-152] In most of these cases, regulation of aspartokinase activity is achieved through concerted feedback inhibition by threonine and lysine, and the total inhibition that was obtained argues for the existence of a single aspartokinase. Aspartokinase from *Rhodopseudomonas spheroides* is not inhibited by any of the end products of the pathway, either singly or in combination, while the aspartokinase of *Rhodospirillum tenue*, in addition to being inhibited by threonine and lysine, is subject to a concerted feedback inhibition by threonine *plus* methionine.[153,154] Inhibition by threonine seems to be a general feature of the regulation of the homoserine dehydrogenase activity in many organisms.

The other puzzling feature of the *E. coli* methionine biosynthetic pathway is the presence of bifunctional enzymes, aspartokinases-homoserine dehydrogenases I and II. In those cases where these enzymes have been at least partially purified from other microorganisms, aspartokinase was easily separated from homoserine dehydrogenase by chromatography or molecular sizing. A similar situation was encountered in the case of anthranilate synthase and anthranilate phosphoribosyltransferase, which are associated in a single polypeptide in *E. coli* and *S. typhimurium*, while they are separate polypeptides in *Pseudomonas putida*, *Bacillus subtilis*, and *Serratia marcescens*. Other examples are phosphoribosylanthranilate isomerase and indole glycerol phosphate synthase, which exist as two separate entities in *Saccharomyces cerevisiae* and as a single polypeptide chain in *E. coli*, or tryptophan synthase α and β of *E. coli*, which are fused in *S. cerevisiae*.[155] In all of these cases, the two activities catalyze consecutive reactions (thereby allowing a possible channeling of the metabolites from one enzyme to the other) irrespective of whether or not they are present in a single polypeptide. This is obviously not the case for aspartokinase and homoserine dehydrogenase. Thus, one may wonder whether the association of aspartokinase and homoserine dehydrogenase in *E. coli* resulted from a "fortuitous" mutational event that eliminated the stop codon between the two genes once they were clustered in an operon.

B. Metabolism of Cystathionine

Trans-sulfuration was the name originally given to the pair of reactions by which sulfur

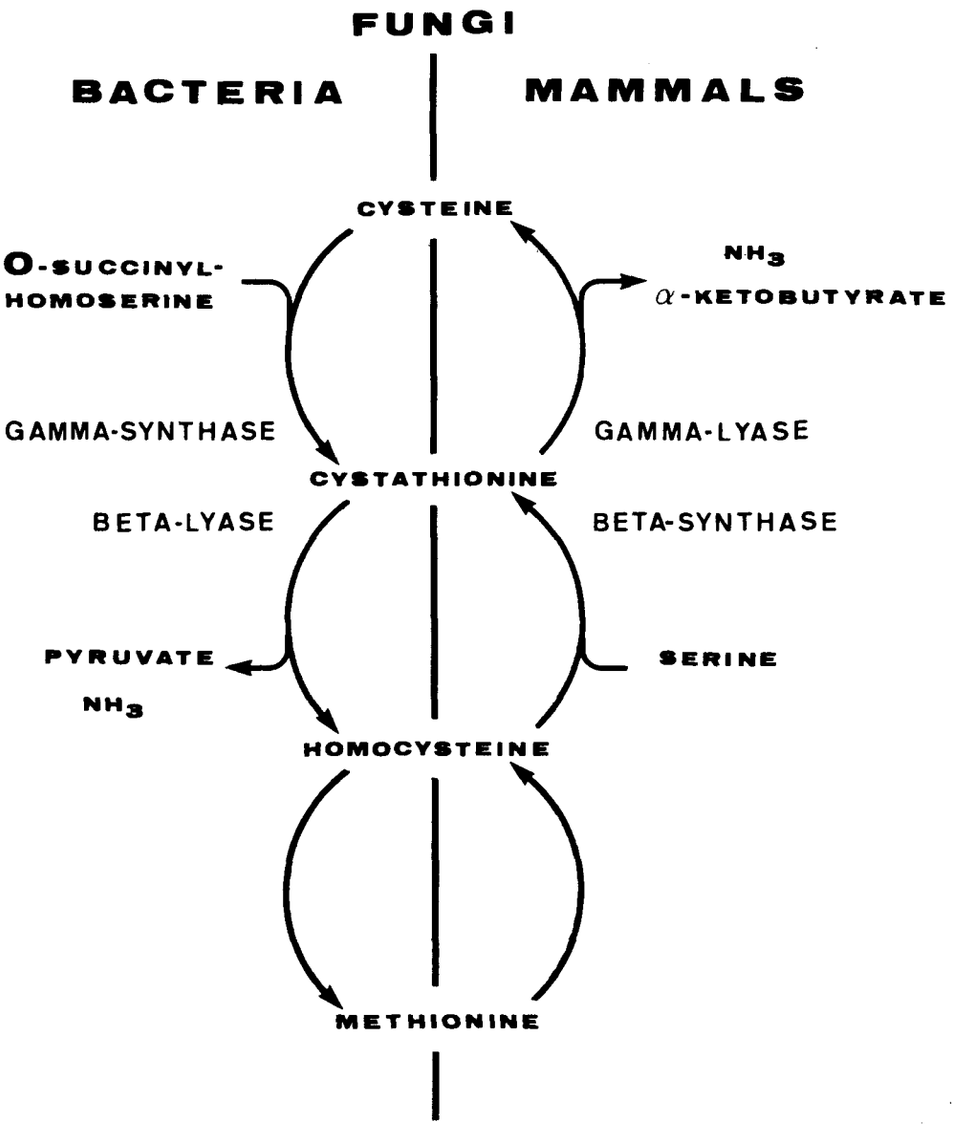


FIGURE 15. The trans-sulfuration process. The various steps involved in the transfer of the sulfur atom from cysteine to homocysteine or vice versa are indicated along with the enzyme names.

is transferred from homocysteine to cysteine via cystathionine. Later it was extended to include the reaction mediating the *comparable* transfer from cysteine to homocysteine in bacteria and to the reactions in fungi by which sulfur is transferred in both directions. The reactions catalyzed by cystathionine-γ-synthase, cystathionine-β-lyase (β-cystathionase), cystathionine-γ-lyase (γ-cystathionase), and cystathionine-β-synthase are schematically indicated in Figure 15.

1. Cystathionine-γ-Synthase and Cystathionine-β-Lyase

Cystathionine-γ-synthase and cystathionine-β-lyase have certain similarities in their biochemical properties. The two *E. coli* enzymes copurify in many chromatographic systems,[172] and cystathionine-β-lyase from *S. typhimurium* was obtained as a byproduct of cystathionine-

γ-synthase purification.[33] Both of these enzymes are pyridoxal phosphate dependent, and cystathionine-γ-synthase has a broad substrate specificity, catalyzing β-elimination in addition to γ-replacement reactions.[156] Moreover, cystathionine-γ-synthase catalyzes the reaction of *O*-succinylhomoserine with H_2S to give homocysteine, thus bypassing the cystathionine intermediate.[157] However, this reaction cannot provide a major alternative pathway in vivo since *E. coli metC* mutants (lacking cystathionine-β-lyase), although slightly leaky, have been selected as methionine auxotrophs.

An important insight into understanding similarities between cystathionine-γ-synthase and cystathionine-β-lyase came from the determination of the nucleotide sequences of the *E. coli metB* and *metC* genes and the comparison of the encoded polypeptides.[31,34] A high degree of homology (36%) was detected between the amino acid sequences of cystathionine-γ-synthase (*metB*) and cystathionine-β-lyase (*metC*) (Figure 16). From this comparison and the fact that the homology is uniformly distributed throughout the two amino acid sequences, it seems very likely that cystathionine-β-lyase and cystathionine-γ-synthase have a common evolutionary origin.

Studies using suicide substrates have allowed the identification of the lysine residue bound to pyridoxal phosphate cofactor in cystathionine-β-lyase and cystathionine-γ-synthase.[173] These lysine residues, indicated by an arrow in Figure 16, are present at the same place in conserved regions. This conservation of the amino acid sequences around the pyridoxal phosphate-binding site reinforces the hypothesis of a common evolutionary origin for cystathionine-β-lyase and cystathionine-γ-synthase.

2. Cystathionine-γ-Lyase and Cystathionine-β-Synthase

Cystathionine-γ-lyase (E.C.4.4.1.1), also designated γ-cystathionase or homoserine dehydratase, is involved in the transfer of sulfur from methionine to cysteine in mammals (see Figure 15). This enzyme catalyzes γ-elimination on a number of amino acid substrates, cystathionine, homoserine, and homocysteine, as well as α,β-elimination from cysteine and cystine.[156] Rat liver cystathionine-γ-lyase is composed of four identical subunits of M_r = 40,000 and contains four pyridoxal 5′-phosphates.[158] Fearon et al.[159] have reported the amino acid sequence of the peptide containing the active-site lysine residue which forms a Schiff base with pyridoxal phosphate. There is strong homology between the sequence of this peptide and the pyridoxal phosphate-binding sites of *E. coli* cystathionine-γ-synthase and cystathionine-β-lyase (Figure 16). From the similarities in the catalyzed reactions, the homology in the pyridoxal phosphate-binding sites, and the identical subunit molecular weight (M_r = 40,000) and oligomeric state (tetrameric) of these three enzymes, it seems most likely that cystathionine-γ-lyase, cystathionine-β-lyase, and cystathionine-γ-synthase have a common evolutionary origin.

Cystathionine-β-synthase (E.C.4.2.1.22), also identified as serine sulfhydrase (or sulfolyase) and cysteine (or alkylcysteine) synthetase, mainly catalyzes the formation of cystathionine from serine and homocysteine by a β-replacement reaction. It is also involved in the reversible L-cysteine synthesis from L-serine and H_2S.[156] Cystathionine-β-synthase is a pyridoxal phosphate enzyme with a molecular weight ranging from 2 × 47,000 to 2 × 60,000, depending upon the biological sources from which it has been purified. Rat liver cDNA encoding cystathionine-β-synthase has been recently cloned.[160] It will be interesting to see whether sequence analysis indicates that this enzyme belongs to the same family as cystathionine-β-lyase, -γ-lyase, and -γ-synthase.

3. Common Ancestor

The discovery that enzymes catalyzing different reactions in the same or even in different metabolic pathways probably have a common evolutionary origin prompts the following questions: (1) what were the biochemical properties of the ancestral enzyme and (2) what reactions, if any, was it able to perform?

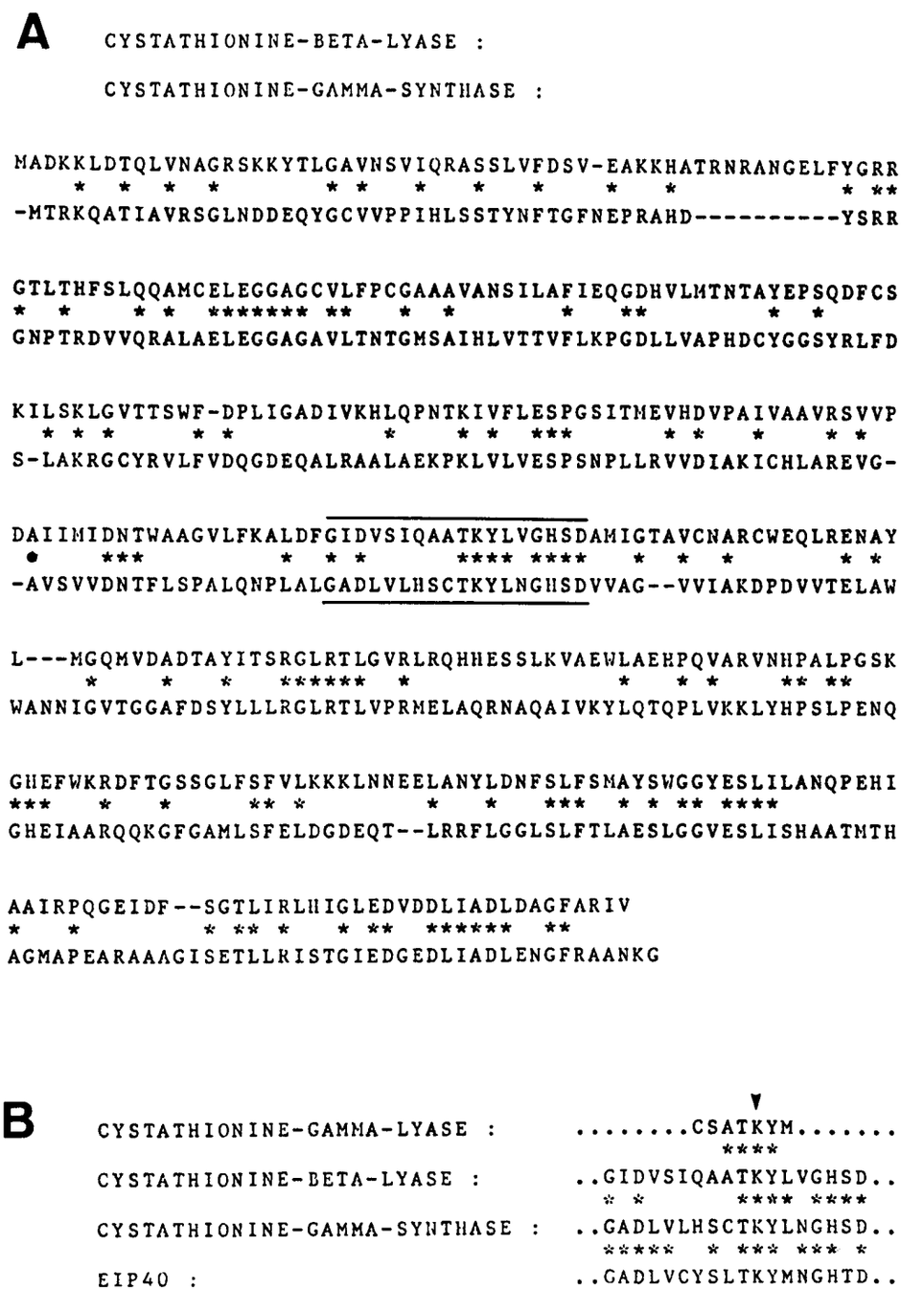


FIGURE 16. Comparison of cystathionine-β-lyase and cystathionine-γ-synthase. (A) The entire amino acid sequences of the two polypeptides are presented in the one-letter code and have been aligned by introducing gaps (−) in order to maximize identities indicated by stars.[31,34] Bars indicate the position of the sequence segments presented in (B) along with the sequence of the pyridoxal-binding site of cystathionine-γ-lyase and a segment of EIP40 sequence.[159,162] The arrows indicate the lysine residue to which pyridoxal phosphate binds in cystathionine-γ-lyase, -β-lyase, and -γ-synthase.

Considering the biochemical properties and the reactions catalyzed by present-day enzymes, is it possible to make an educated guess about the properties of the ancestral enzyme? Because cystathionine-β-lyase, cystathionine-γ-lyase, and cystathionine-γ-synthase are pyridoxal phosphate enzymes and since the lysine residues involved in the fixation of the cofactor are present in conserved regions of the protein sequences, it seems reasonable to speculate that the primitive enzyme was also pyridoxal phosphate dependent. Similarly, it is also likely that the ancestor was about 400 amino acid residues long and perhaps tetrameric.

Can knowledge of the substrates and products of the present-day enzymes provide insight into the substrates and products of the ancestor? From the two pathways shown schematically in Figure 15 and the fact that both of them are present in fungi, it seems probable that the ancestor could have been involved in the global trans-sulfuration process between cysteine and methionine and vice versa. The most important fact would have been the pyridoxal phosphate-dependent stabilization of the reactive intermediates of C_3 or C_4 amino acids, such as α-aminocrotonate or α-aminoacrylate, intermediates that could undergo α,β- or β,γ-elimination or β- or γ-replacement reactions. Whether or not the ancestor was directly involved in the synthesis of cysteine via the serine sulfhydrase activity of cystathionine-β-synthase is open to speculation. A process of gene duplication followed by mutations improving the specificity and the catalytic properties of the encoded proteins could have led to the present-day enzymes.

4. Further Speculation

While studying *Drosophila melanogaster* genes, whose transcription is specifically activated in response to the steroid hormone ecdysome, Cherbas and co-workers[161] cloned and sequenced the *eip40* gene. Comparison of the deduced amino acid sequence of the *eip40* product with protein sequences contained in libraries revealed a striking homology (54%) between EIP40 and cystathionine-γ-synthase, the *E. coli metB* gene product.[162] Interestingly, the lysine residue of cystathionine-γ-synthase to which the pyridoxal phosphate cofactor binds is conserved in the EIP40 sequence (Figure 16).

The function of EIP40 in the *D. melanogaster* developmental process has not been elucidated, but it is clear that its expression in Kc cells is very rapidly induced by ecdysone.[163] From the homology detected between EIP40 and cystathionine-γ-synthase, we can speculate that EIP40 could be a pyridoxal phosphate enzyme somehow involved in trans-sulfuration between cysteine and methionine. Since methionine is the direct precursor of *S*-adenosylmethionine, the universal methyl donor, induction of an enzyme involved in the metabolism of methionine could allow a rapid rise or fall in the size of the pool of methyl group donor molecules in the cell, thereby modifying the methylation level of some cellular compounds (DNA or proteins). As methylation has been shown to play a role in gene expression, variation in the size of the pool of methionine could allow the amplification of the ecdysone signal.

C. Concluding Remarks

One of the difficulties in understanding the mechanisms whereby multistep metabolic pathways have evolved is the apparent lack of any selective advantage conferred by individual steps of the pathway prior to the establishment of the whole pathway. Horowitz[164] proposed that evolution of such pathways proceeded in a stepwise manner, with individual steps being recruited in the reverse direction relative to the final pathway, i.e., the last step in the pathway was acquired first, the penultimate step next, etc. This hypothesis supposed that pristine life took place in a paradise where all compounds were readily available, an assumption that seems unlikely in view of the instability of some metabolic intermediates. Horowitz's hypothesis, while unlikely, remained attractive in view of (1) the overlap between products and substrates of the consecutive enzymes in a pathway, (2) the existence of

allosteric inhibition in which the affinity of the first enzyme of a given pathway for the end product of that pathway could be regarded as a kind of memory of the enzyme for its origin, and (3) the clustering of some biosynthetic genes in bacterial operons.

The determination of the nucleotide sequence of most of the *met* genes allowed this hypothesis to be tested directly by comparing the amino acid sequences of the encoded polypeptides. No convincing homology was detected between the primary sequences of the enzymes involved in the methionine biosynthetic pathway, with the exception of the *metB* and *metC* gene products. Furthermore, no homology was detected between the enzymes involved in the lysine or the threonine biosynthetic pathways, even though genes for threonine biosynthetic enzymes are clustered in an operon. These data therefore do not support Horowitz's hypothesis of retrograde evolution, although it can be argued that similarities between the primary sequences have been lost during evolution and specialization of the enzymes.

From consideration of the substrate ambiguity exhibited by contemporary enzymes, Ycas[165] and Jensen[166] suggested that primitive enzymes possessed a broad specificity, allowing them to utilize a wide range of structurally related substrates, thereby yielding small amounts of related products. This process would have provided a biochemically diverse environment in which *individual* enzyme recruitment would have improved the function of a slow, but already existing, multistep pathway. The homology detected between cystathionine-γ-synthase and cystathionine-β-lyase seems to be relevant to this hypothesis. The primitive enzyme probably catalyzed both kinds of reactions, or possibly the direct synthesis of homocysteine from *O*-succinylhomoserine and H_2S, a reaction which can now be performed readily by the cystathionine-γ-synthase. Keeping in mind that cystathionine-γ-synthase can use either *O*-succinylhomoserine or *O*-acetylhomoserine, a striking similarity appears between the biosynthetic pathways of homocysteine and cysteine from homoserine and serine, respectively, as previously noted by Ycas.[165] In particular, the reaction catalyzed by *O*-acetylserine sulfhydrylase (E.C.4.2.99.8) with *O*-acetylserine and H_2S seems almost equivalent to the reaction catalyzed by cystathionine-γ-synthase with *O*-acetylhomoserine and H_2S. In this respect, it is noteworthy that *O*-acetylserine sulfhydrylase is also a pyridoxal phosphate-dependent enzyme, composed of two identical subunits of M_r = 34,000.[168] Amino acid sequence data will help to elucidate whether this latter enzyme and cystathionine-γ-synthase share a common ancestor, providing an additional case for the involvement of an ancestral enzyme in two *different* biosynthetic pathways.[169]

Studies of the methionine biosynthetic pathway of *E. coli* have revealed the existence of isofunctional enzymes (aspartokinases I, II, and III), multifunctional enzymes (aspartokinases-homoserine dehydrogenases I and II), and homologous enzymes (cystathionine-γ-synthase and -β-lyase). This organism thus seems not to have been miserly with respect to gene duplications and rearrangements, providing work (and proportional fun) for investigators.

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