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DOMAIN 3 METABOLISM

Folate Biosynthesis, Reduction, and Polyglutamylation and the Interconversion of Folate Derivatives

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ABSTRACT Many microorganisms and plants possess the ability to synthesize folic acid derivatives de novo, initially forming dihydrofolate. All the folic acid derivatives that serve as recipients and donors of one-carbon units are derivatives of tetrahydrofolate, which is formed from dihydrofolate by an NADPH-dependent reduction catalyzed by dihydrofolate reductase (FolA). This review discusses the biosynthesis of dihydrofolate monoglutamate, its reduction to tetrahydrofolate monoglutamate, and the addition of glutamyl residues to form folylpolyglutamates. *Escherichia coli* and *Salmonella*, like many microorganisms that can synthesize folate de novo, appear to lack the ability to transport folate into the cell and are thus highly susceptible to inhibitors of folate biosynthesis. The review includes a brief discussion of the inhibition of folate biosynthesis by sulfa drugs. The folate biosynthetic pathway can be divided into two sections. First, the aromatic precursor chorismate is converted to paminobenzoic acid (PABA) by the action of three proteins. Second, the pteridine portion of folate is made from GTP and coupled to PABA to generate dihydropteroate, and the bifunctional protein specified by *folC*, dihydrofolate synthetase, or folylpolyglutamate synthetase, adds the initial glutamate molecule to form dihydrofolate ($H_2PteGlu_1$, or dihydropteroylmonoglutamate). Bacteriophage T4 infection of *E. coli* has been shown to cause alterations in the metabolism of folate derivatives. Infection is associated with an increase in the chain lengths in folylpolyglutamates and particularly the accumulation of hexaglutamate derivatives.

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In contrast to humans, for whom folate is a dietary requirement, many microorganisms and plants possess the ability to synthesize folic acid derivatives de novo, initially forming dihydrofolate. This folic acid derivative is not active in one-carbon transfer reactions and must first be reduced to the level of tetrahydrofolate, in which the N-5–C-6 and C-7–N-8 double bonds present in folic acid have been reduced (Fig. 1). All the folic acid derivatives that serve as recipients and donors of one-carbon units are derivatives of tetrahydrofolate, which is formed from dihydrofolate by an NADPH-dependent reduction catalyzed by dihydrofolate reductase (FolA). This chapter takes as its starting point the chapter on this topic that was published in the 1996 edition of *Escherichia coli and Salmonella: Cellular and Molecular Biology* (chapter 41), with updates on developments since that time.

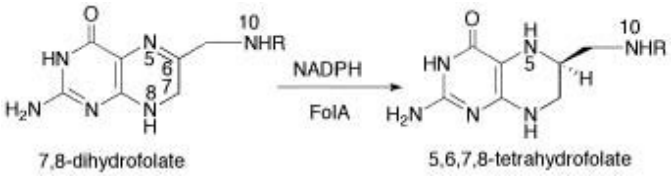

Figure 1 Reduction of dihydrofolate to tetrahydrofolate. NHR represents the *p*-Aminobenzoyl-glutamate substituent.

The product of de novo dihydrofolate biosynthesis is also a monoglutamate. However, the intracellular forms of folic acid are almost entirely polyglutamates, formed by the addition of two to seven glutamyl residues to the monoglutamate. In the derivative shown in Fig. 2, the bonds of the second and third glutamyl residues are to the γ-carboxyl group of the preceding glutamate, in contrast to peptide bonds where the linkage would be to the α-carboxyl group. During the exponential growth of *Escherichia coli*, the major folylpolyglutamates are triglutamates, with γ-linkages as shown in Fig. 2 (1). However, during stationary phase (1) or infection with T4 bacteriophage (2), polyglutamates with longer chains are formed. The polyglutamate tail is required for the intracellular retention of folic acid derivatives and plays an important role in the binding of folate cosubstrates to enzymes involved in one-carbon transfer or the interconversion of folic acid derivatives (3). Most folate-dependent enzymes will use both monoglutamate and polyglutamate forms of folic acid derivatives as substrates, but polyglutamate derivatives are usually better substrates, with lower Michaelis constants (K_m values) and/or with higher maximum initial velocities (V_{max}). However, the cobalamin-independent methionine synthase specified by the *metE* gene shows an absolute requirement for the polyglutamate form of its substrate methyltetrahydrofolate (4). Strains of *E. coli* with greatly reduced folylpolyglutamate synthetase activity are auxotrophic for methionine or vitamin B_{12} (vitamin B_{12} supplementation permits methionine synthesis using the cobalamin-dependent MetH protein that can use methyltetrahydrofolate monoglutamate as a substrate), and their growth is stimulated by the addition of glycine and thymine to the medium (5).

Tetrahydrofolate serves as a recipient for one-carbon units in various oxidation states that are attached to N-5 or N-10 or both. The major derivatives are illustrated in Fig. 3. 5,10-Methylenetetrahydrofolate contains a methylene group bridging N-5 and N-10 and serves as the source of one-carbon units for the de novo biosynthesis of thymidylate from uridylate in the reaction catalyzed by thymidylate synthase (ThyA) as shown in Fig. 4. Methylenetetrahydrofolate also provides a one-carbon unit in the biosynthesis of pantothenate (6). This cofactor can be reversibly converted to 10-formyltetrahydrofolate, which is used to supply one-carbon units in the de novo biosynthesis of purines. Two enzymes, glycinamide ribonucleotide (GAR) transformylase and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, use 10-formyltetrahydrofolate to introduce a carbon unit during the assembly of the purine ring. (There is also a glycinamide ribonucleotide transformylase that uses

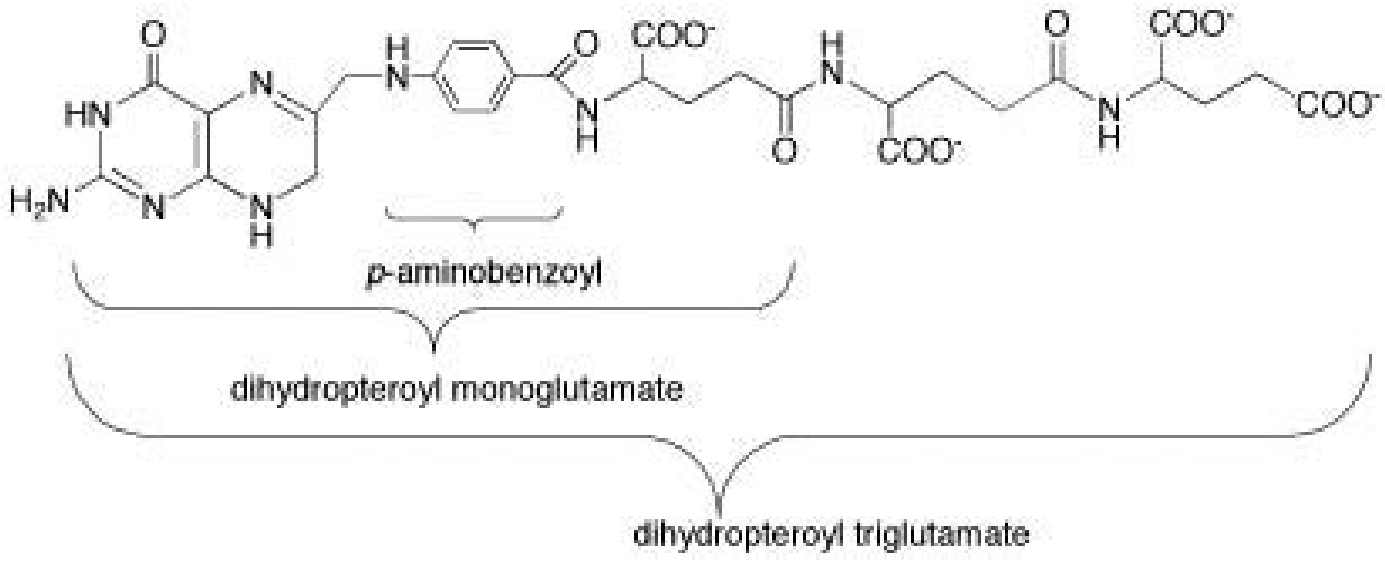

Figure 2 Composition of 7,8-dihydropteroyl triglutamate, a dihydrofolate polyglutamate.

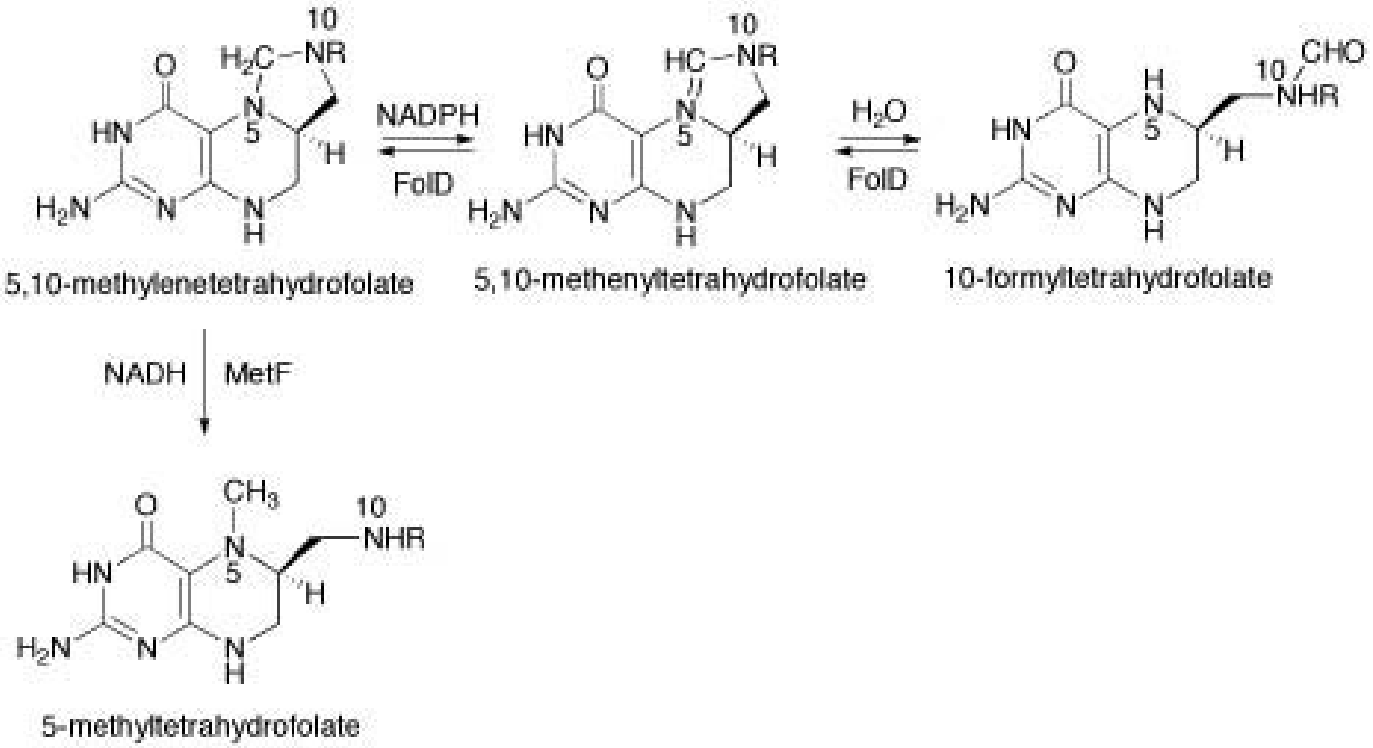

Figure 3 Major derivatives of tetrahydrofolate. NR and NHR represent the *p*-aminobenzoylglutamate substituent.

formate rather than formyltetrahydrofolate as the source of one-carbon units [7].) Formyltetrahydrofolate is also the one-carbon-unit donor for the formylation of fMet-tRNA^{fMet}. Methylenetetrahydrofolate can be reduced to form 5-methyltetrahydrofolate, which is used exclusively for the conversion of homocysteine to methionine in the terminal step of methionine biosynthesis.

Tetrahydrofolate serves as a recipient for one-carbon units generated during glycine cleavage to form a methylene group, CO_2 and NH_3 catalyzed by the glycine cleavage system (GcvT, H, and P and Lpd), and during the conversion of serine to glycine and a methylene group catalyzed by GlyA (serine hydroxymethyltransferase). In both cases, 5,10-methylenetetrahydrofolate is formed. *E. coli* does not contain a formyltetrahydrofolate synthetase, preventing the utilization of formate as a source of tetrahydrofolate-bound one-carbon units.

Folate derivatives also participate in other cellular processes that do not involve one-carbon transfers. Methenyltetrahydrofolate, an intermediate in the conversion of methylenetetrahydrofolate to 10-formyltetrahydrofolate, plays a role in the repair of pyrimidine dimers by DNA photolyase (8). 7,8-Dihydrofolate hexaglutamate, derived from the reaction catalyzed by thymidylate synthase, is involved in the assembly of the baseplate of T4 phage.

In this chapter, we will discuss the biosynthesis of dihydrofolate monoglutamate, its reduction to tetrahydrofolate monoglutamate, and the addition of glutamyl residues to form folylpolyglutamates. *E. coli* and *Salmonella*, like many microorganisms that can synthesize folate de novo, appear to lack the ability to transport folate into the cell and are thus highly susceptible to inhibitors of folate biosynthesis. This chapter will include a brief discussion of the inhibition of folate biosynthesis by sulfa drugs.

BIOSYNTHESIS OF DIHYDROFOLATE

The folate biosynthetic pathway can be divided into two sections that will guide the organization of this section of the chapter. First, the aromatic precursor chorismate is converted to *p*-aminobenzoic acid (PABA) by the action of three proteins. Second, the pteridine portion of folate is made from GTP and coupled to PABA to generate dihydropteroate, and the bifunctional protein specified

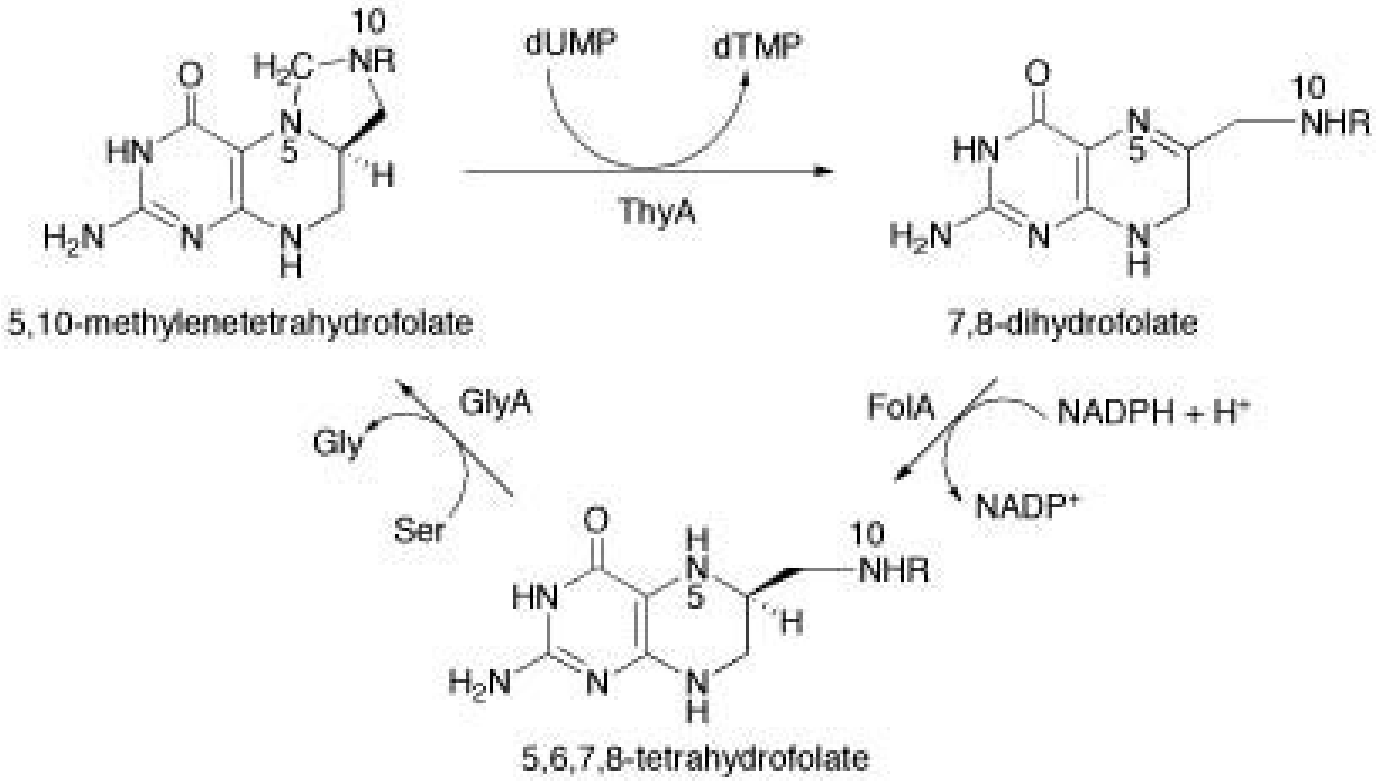

Figure 4 Folate-dependent reactions required for the synthesis of dTMP. NHR represents the *p*-aminobenzoylglutamate substituent.

by *folC*, dihydrofolate synthetase, or folylpolyglutamate synthetase, adds the initial glutamate molecule to form dihydrofolate ($H_2PteGlu_1$, or dihydropteroylmonoglutamate). Dihydrofolate is then reduced to tetrahydrofolate by dihydrofolate reductase (FolA or FolM), and the folylpolyglutamate synthetase function of FolC adds two additional glutamyl residues to form $H_4PteGlu_3$ (tetrahydropteroyltriglutamate). The reactions involved in the synthesis of the pteridine ring were extensively reviewed in the first edition of this work (9), which can be referred to for a more historical perspective. The overall synthesis of tetrahydrofolate is depicted in Fig. 5, along with the structures of the intermediates. The names of genes known to be involved in folate biosynthesis are also shown in Fig. 5. Since folate and its reduced derivatives cannot cross the inner membrane of *E. coli*, most of the folate biosynthetic pathway was first worked out biochemically by isolating intermediates and characterizing purified or partially purified proteins; much of this early work was accomplished in the laboratory of Gene Brown. In contrast, since PABA does freely enter *E. coli* cells, this part of the pathway was first identified genetically, with the biochemistry being completed later.

Biosynthesis of PABA from Chorismate

Although it was shown in 1950 that PABA was derived from the same pathway as the aromatic amino acids tyrosine, phenylalanine, and tryptophan, it was only in 1967 that Huang and Pittard (10) used mutagenesis techniques to obtain strains of *E. coli* that required PABA for growth. They identified two loci, which they named *pabA* and *pabB*. In the early 1980s, investigators in the laboratory of Brian Nichols cloned *pabA* and *pabB* (11, 12). Nucleotide sequence analysis of *pabA* and *pabB* revealed the corresponding deduced amino acid sequences to be 44 and 26% identical, respectively, to those of the homologous subunits of anthranilate synthase. Despite this similarity, the subunits of anthranilate synthase could not substitute for the analogous subunits in PABA synthase or vice versa.

In 1989, Nichols et al. (13) constructed two plasmid-containing strains that overexpressed the products of *pabA* and *pabB*. Studies with these partially purified enzymes showed that the conversion of chorismate to PABA occurs in two steps. PabA and PabB act upon chorismate and glutamine to form a diffusible intermediate, which is then converted to PABA by the action

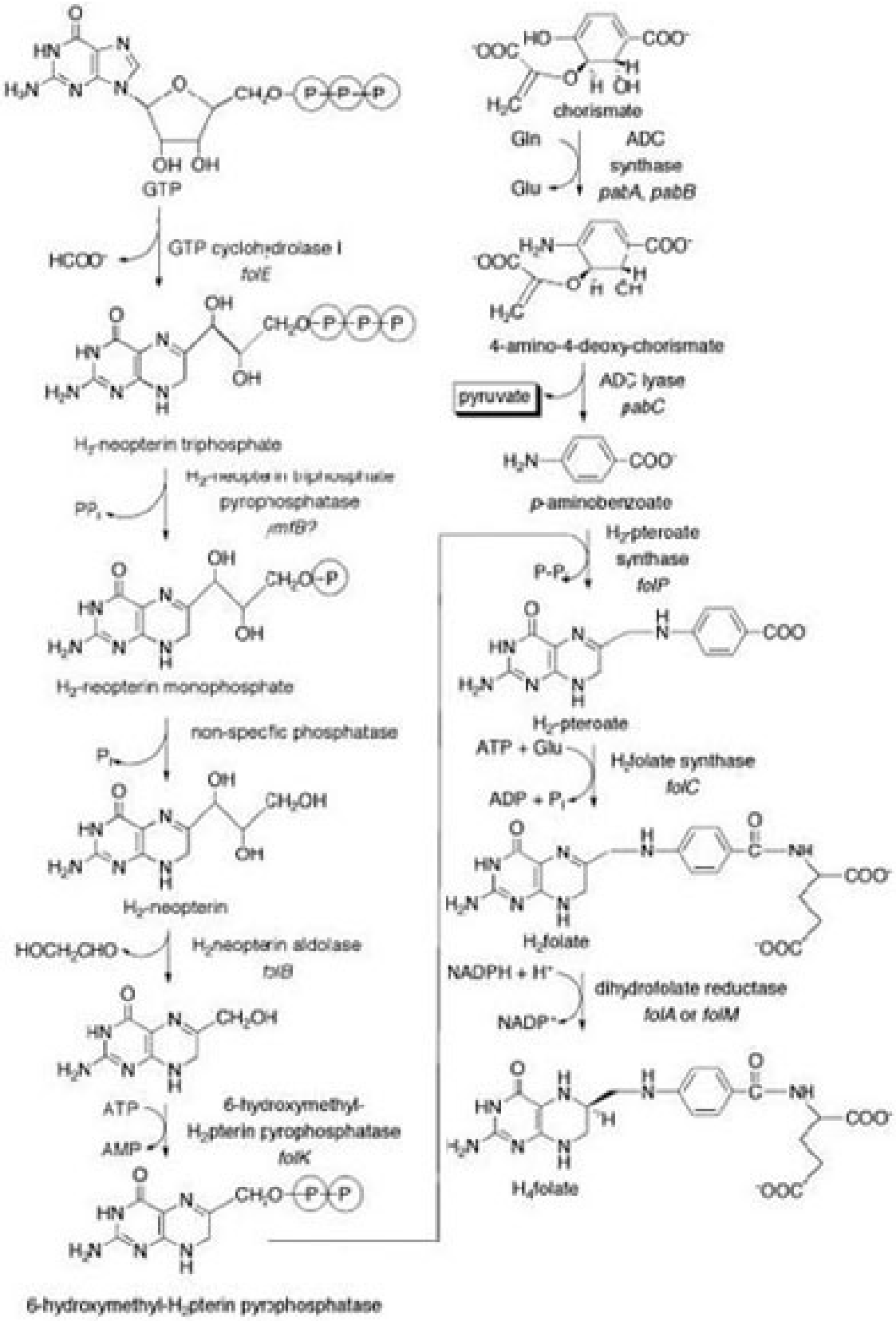

Figure 5 Sequence of reactions involved in the biosynthesis of dihydrofolic acid from GTP and chorismate.

of a third enzyme, at that time termed enzyme X. In 1985, Teng et al. (14) synthesized the proposed intermediate in the biosynthesis of PABA, 4-amino-4-deoxychorismate (Fig. 5). They found that cell extracts from a strain overexpressing PabB converted 4-amino-4-deoxychorismate to PABA at a rate commensurate with a biological function of this compound as an intermediate in PABA biosynthesis. This intermediate was subsequently identified as 4-amino-4-deoxychorismate (15, 16). Enzyme X was then renamed aminodeoxychorismate lyase, and the complex containing PABA synthase components I and II, the *pabA* and *pabB* gene products, was named aminodeoxychorismate synthase.

In summary, PABA is one of seven aromatic products derived from chorismate in *E. coli*. Chorismate is converted to PABA in two steps through the action of three gene products, PabA, PabB, and PabC. 4-Amino-4-deoxychorismate synthase, a heterodimer of PabA and PabB, converts chorismate and glutamine to aminodeoxychorismate and glutamate. Aminodeoxychorismate lyase aromatizes aminodeoxychorismate, releasing pyruvate and generating PABA.

Although the early literature is replete with references to PABA synthase, it is likely that these studies involved aminodeoxychorismate synthase components I and II, with enough contaminating lyase to support activity, since gel filtration experiments fail to separate the native synthase from the lyase, both of which elute with apparent molecular weights of about 50,000 (17).

Aminodeoxychorismate synthase (PabA, PabB)

Aminodeoxychorismate synthase or its components can catalyze the following reactions.

chorismate + glutamine → aminodeoxychorismate + glutamate (reaction 1)

chorismate + NH_3 ⟷ aminodeoxychorismate (reaction 2)

glutamine → glutamate + NH_3 (reaction 3)

Although reactions 1 and 3 require both aminodeoxychorismate synthase components I and II, the products of *pabB* and *pabA*, respectively, reaction 2 can be carried out by PabB alone.

Current research suggests that the physiological reaction occurs as follows. PabA acts as a glutaminase that generates ammonia from glutamine, although activation requires equimolar amounts of PabB (18). PabB then uses the ammonia to aminate chorismate, generating aminodeoxychorismate (18). PabC acts separately to generate PABA from aminodeoxychorismate (19). Research from the Nichols laboratory strongly suggests that free ammonia is not a physiological substrate for PabB (20). For reaction 2, the measured K_m for ammonia was 360 mM in the presence of PabA and 140 mM in the absence of PabA, whereas the K_m for glutamine in reaction 1 was about 1 mM. *pabA* mutants require more than 50 mM ammonia to grow (21). The hydrolysis of cellular glutamine will not generate enough ammonia to support the PabB-dependent amination of chorismate. In contrast, *trpG* mutants, which lack anthranilate synthase component II, require only 1 mM ammonia for growth (22).

Aminodeoxychorismate synthase is a heterodimer composed of a fairly loose association between PabA and PabB (21). The X-ray crystal structure of *E. coli* PabB was determined to 2.0-Å resolution (23). The monomer has a complex α/β fold similar to that of known anthranilate synthase structures. It is fascinating that in the region of PabB analogous to the regulatory region of anthranilate synthase there is a tightly bound tryptophan molecule. Since this tryptophan can be removed only by denaturing conditions and is within an area highly conserved among chorismate-dependent enzymes, it may possess a structural role. In 1989 Irving Crawford compared sequences of genes involved in homologous reactions and theorized that *pabB* and *trpE* arose from the duplication of a common gene ancestor, followed by divergence (24). The observation of a tryptophan binding pocket within a highly conserved region lends support to his theory.

The *pabA* genes from *E. coli* and *Salmonella enterica* serovar Typhimurium have been shown to specify polypeptides with a predicted molecular mass of 21 kDa (12, 25). Expression studies reveal that *pabA* from *E. coli* is expressed constitutively from a monocistronic transcript (26, 27). However, it has also been reported that *pabA* is expressed from a transcript including *fic*, the gene upstream of *pabA* (28).

The *pabB* genes from *E. coli* (11) and *Salmonella* serovar Typhimurium (29) have been isolated, and their nucleotide sequences have been analyzed. The *E. coli pabB* gene consists of 1,359 nucleotides specifying a protein of 453 residues with a predicted molecular mass of ~51 kDa.

Properties of aminodeoxychorismate lyase (PabC)

4-Amino-4-deoxychorismate lyase catalyzes the elimination of pyruvate from 4-amino-4-deoxychorismate and the aromatization of the resulting product to generate PABA. The enzyme was identified in 1989 (13), partially purified in 1990 (16), and further purified in 1991 (19). The enzyme was shown to be a homodimer with ~25-kDa subunits (13, 19, 30). Sequence analysis of the cloned *pabC* gene identified an open reading frame specifying a protein of 29,700 Da (31). The purified, overexpressed protein was shown to contain a pyridoxal phosphate cofactor (31). In 2000, Nakai et al. reported the X-ray crystal structure of aminodeoxychorismate lyase at 2.2-Å resolution (32). They confirmed that the enzyme was a homodimer with a two-fold crystallographic axis, and they observed that the polypeptide chain had two domains, one large and one small, connected by a loop. The pyridoxal 5-phosphate cofactor is bound at the interface of the two domains.

Transport of PABA

Little is known about the transport of PABA, although it is clear that PABA is excreted from wild-type cells and can support the growth of strains that are PABA auxotrophs (10).

Biosynthesis of the Dihydropteroic Acid from GTP and PABA

As shown in Fig. 5, the synthesis of dihydropteroic acid uses GTP as the initial substrate and requires the sequential action of GTP cyclohydrolase I, dihydroneopterin pyrophosphatase, a phosphomonesterase, dihydroneopterin aldolase, 6-hydroxymethyl-dihydropterin pyrophosphokinase, and dihydropteroate synthase. This figure also provides the structural gene designations where known.

GTP cyclohydrolase I (FolE)

GTP cyclohydrolase I, so named to differentiate this enzyme from GTP cyclohydrolase II, which catalyzes the first step in the biosynthesis of riboflavin from GTP, was identified in *E. coli* and subsequently characterized by Brown and coworkers (33, 34, 35). The enzyme catalyzes the overall reaction shown in reaction 4.

GTP + $2H_2O$ → H_2neopterin triphosphate + formate (reaction 4)

The reaction occurs in four steps, which include an Amadori rearrangement (Fig. 6). The intermediates in

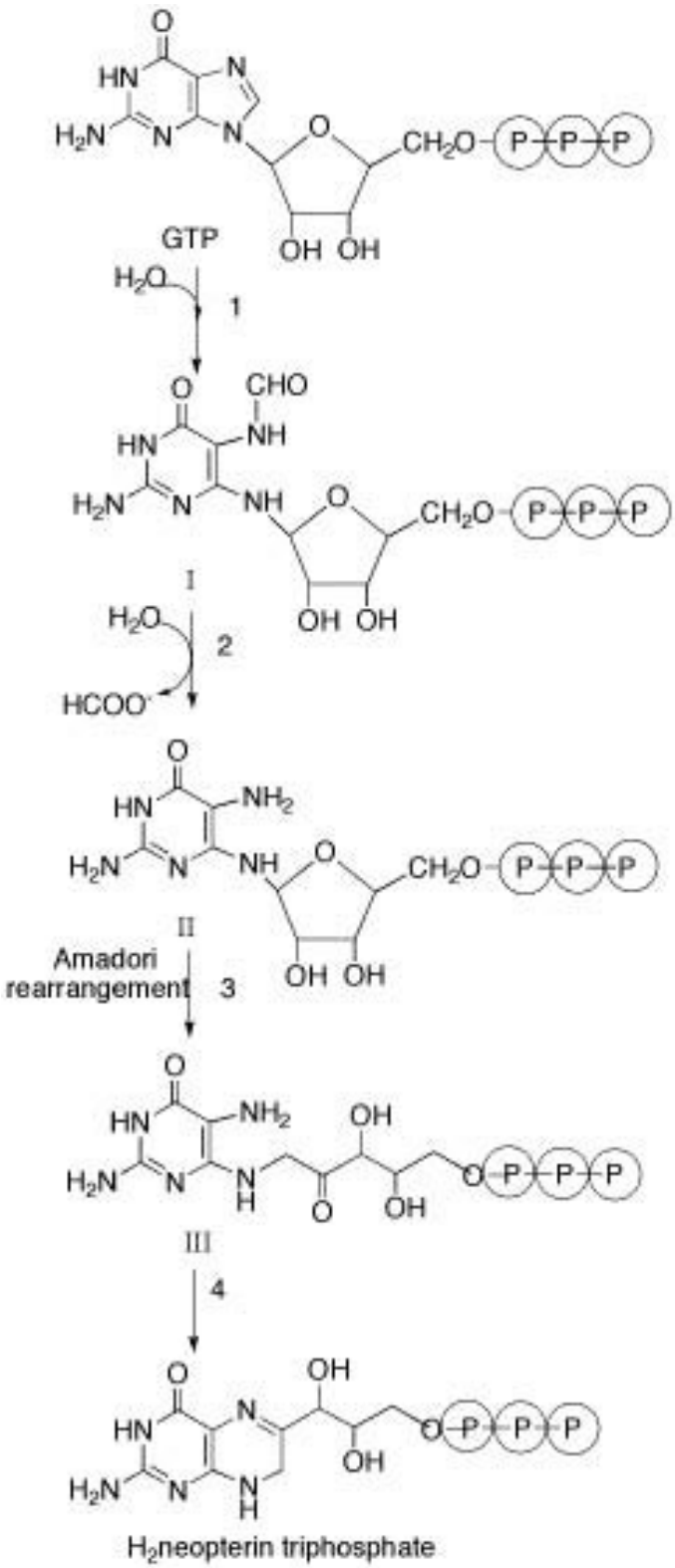

Figure 6 Reactions and noncovalently bound intermediates involved in the conversion of GTP to dihydroneopterin triphosphate in the presence of the enzyme GTP cyclohydrolase I. H_2neopterin, dihydroneopterin.

the GTP cyclohydrolase I-catalyzed reaction are noncovalently bound, and those in the GTP cyclohydrolase II-catalyzed reaction have been proposed to be covalently bound to the protein (36). The enzyme was purified 3,900-fold to homogeneity by Yim and Brown (30), who determined that subunits of the enzyme have a molecular mass of ~25 kDa and that the enzyme binds one molecule of GTP per subunit and has a K_m of 20 nM for GTP. Unlike many other enzymes that catalyze the committing step in a biochemical pathway, GTP cyclohydrolase I exhibits no feedback inhibition by end products.

The X-ray crystal structure of *E. coli* GTP cyclohydrolase I has been solved by Nar et al., who determined that the torus-shaped enzyme is composed of 10 identical subunits arranged as a pentamer of tightly associated dimers (37). Subsequently, the structure of the enzyme bound to the substrate analogue, dGTP, was determined. The enzyme possesses 10 identical active sites, each of which is at the interface of three subunits. Site-directed mutagenesis of active-site residues was used to relate enzyme activity to catalytically important residues (38). Directed mutagenesis of His-179 yielded a GTP cyclohydrolase I that catalyzed a conversion of GTP to the intermediate 2-amino-5-formylamino-6-ribofuranosylamino-4(3*H*)-pyrimidinone 5′-triphosphate, as determined by multinuclear magnetic resonance. The wild-type enzyme converts this intermediate into product dihydroneopterin triphosphate at a rate equivalent to that of the generation of the product from GTP (39). Crystallographic studies of the human GTP cyclohydrolase, and the discovery of an essential zinc molecule in that enzyme, led to the reinvestigation of the *E. coli* protein. The use of EDTA in earlier preparations precluded this observation of a metal cofactor. In the *E. coli* enzyme, the zinc molecule is bound to Cys-100, Cys-118, and His-113 on each subunit (40). Single-turnover quenched-flow experiments were performed, and the rate constant for the reversible opening of the GTP imidazole ring was determined to be 0.9 s^{-1}, in comparison to the rate constant for the release of formate (2.0 s^{-1}) and the rate constant for the formation of product dihydroneopterin triphosphate (0.03 s^{-1}). Surprisingly, the opening of the imidazole ring is not the rate-limiting step for this complex reaction (41).

The gene coding for *E. coli* GTP cyclohydrolase I, *folE*, has been cloned (42), the corresponding amino acid sequence has been determined (43), and the gene has been mapped at 45 min on the *E. coli* chromosome (~2,250 kbp) (44). The open reading frame contains 669 nucleotides, specifying a protein with a predicted molecular weight of 25,873. Recently, it has been found that *folE* is regulated by the *metJ* repressor (45). Using DNA arrays and a strain with a deletion of the methionine repressor, *metJ*, Marines et al. (45) created an in vitro assay for transcription. They observed a fourfold derepression of *folE* in the *metJ* knockout strain compared to that in cells transformed with the wild-type repressor. MetJ is a ribbon-helix-ribbon DNA binding protein that binds two *S*-adenosylmethionine molecules as corepressors. It makes sense that *folE* would be sensitive to the methionine regulon since methionine synthase requires methylenetetrahydrofolate as a methyl donor for the methylation of homocysteine.

Dihydroneopterin triphosphate pyrophosphatase (FolQ?)

Suzuki and Brown (46) proposed that two separate enzyme-catalyzed steps were necessary in the conversion of dihydroneopterin triphosphate to dihydroneopterin. The first step was proposed to be catalyzed by dihydroneopterin triphosphate pyrophosphatase, and the removal of the third phosphate group was proposed to be catalyzed by nonspecific phosphate monoesterases. However, more recently it has been proposed that the release of pyrophosphate occurs nonenzymatically under the influence of divalent cations (47).

E. coli dihydroneopterin triphosphate pyrophosphatase catalyzes the removal of pyrophosphate from dihydroneopterin triphosphate to yield dihydroneopterin monophosphate. This enzyme was purified ~50-fold and partially characterized by Suzuki and Brown (46). The molecular weight of the enzyme is 17,000. The enzyme requires a magnesium ion for activity and will not recognize nucleoside triphosphates as substrates. The K_m for dihydroneopterin triphosphate is 11 μM. The gene specifying dihydroneopterin triphosphate pyrophosphatase has not yet been identified. However, Klaus and his colleagues recently identified *ylgG* as the gene specifying a pyrophosphohydrolase in *Lactococcus lactis* that corresponds to part of a multicistronic folate synthesis operon and that exhibits high activity towards dihydroneopterin triphosphate (48). A BLAST search of the *E. coli* K-12 genome identifies *ymfB* as the closest homologue. This gene specifies a protein annotated as a putative bifunctional thiamine pyrimidine pyrophosphate hydrolase and shows 33% identity and 53%

similarity to *ylgG*. If YmfB is shown to catalyze the conversion of dihydroneopterin triphosphate to dihydroneopterin monophosphate, it should be renamed FolQ, as has the YlgG protein from *Lactococcus lactis*.

Phosphomonoesterase

Suzuki and Brown (46) showed that several phosphomonoesterases in *E. coli* could catalyze the removal of the phosphate group from dihydroneopterin monophosphate, but no single enzyme showed specificity for dihydroneopterin monophosphate.

Dihydroneopterin aldolase (FolB)

Dihydroneopterin aldolase reacts with dihydroneopterin to generate 6-hydroxymethyl-7,8-dihydropterin (6-CH_2OH-[H_2]pterin) and glycolaldehyde. The enzyme from *E. coli* is remarkably heat stable, which greatly aided in the purification of the enzyme. The molecular weight was estimated to be ~100,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration (49, 50). The K_m for dihydroneopterin is 9 μM. The enzyme is highly specific for dihydroneopterin and will not use neopterin or the mono- or triphosphate of dihydroneopterin. The product 6-CH_2OH-dihydroneopterin is a good inhibitor, with a K_i of 1.7 μM. The *ygiG* gene specifying this protein was expressed, and the purified protein was characterized by Haussman et al. and designated FolB (51). They determined that the subunit molecular weight was 12,500 and that the active protein is a homo-octamer, consistent with the results of the earlier studies. Interest in this enzyme as a potential antibiotic target has led to several studies of potential inhibitors (52, 53). Although a structure of the *E. coli* enzyme has not been reported, an X-ray structure of the enzyme from *Staphylococcus aureus* has been reported (54). This enzyme has 27% identity to the *E. coli* enzyme. The enzyme is a homo-octomer, and the active site is located between two adjacent subunits. Mechanistic studies of the *S. aureus* enzyme confirm the mechanism initially proposed for the *E. coli* enzyme, which involves the abstraction of a proton from the β-hydroxy group and rearrangement to form an enamine intermediate that collapses to the product (54).

6-Hydroxymethyl-7,8-dihydropterin pyrophosphokinase (FolK)

Hydroxymethyldihydropterin pyrophosphokinase (HPPK) converts 6-CH_2OH-dihydropterin to its pyrophosphate ester with ATP as the donor of the pyrophosphate group (55). The enzyme activity requires Mg^{2+}. Talarico et al. (56) have purified HPPK more than 10,000-fold to homogeneity. The native enzyme had a mass of 25 kDa as determined by gel electrophoresis in the presence and absence of sodium dodecyl sulfate and thus must be monomeric. Kinetic studies with the pure protein yielded K_m values of 1.6 μM for 6-CH_2OH-dihydropterin and 17 μM for ATP.

The X-ray crystal structure of HPPK was determined by several groups; first, it was crystallized as an apoenzyme (57) and then in the ternary complex with ATP and a pterin analogue (58). Both structural studies revealed that the first two-thirds of the protein contain four strands of antiparallel β ?sheet with helical connections. Further structural studies including nuclear magnetic resonance and X-ray analysis of HPPK revealed that the binding of Mg-ADP resulted in notable changes in the relative conformation of the three loops implicated in catalysis. While both loops 2 and 3 are relatively flexible and are thought to be involved in ligand recognition and catalysis, the largest change was associated with loop 3, in which some amino acids moved more than 17 Å away from the active site (59, 60, 61). Loop 3 is not necessary for protein folding or the binding of Mg-ATP but is necessary for catalysis (62). Based on the body of data from structural, kinetic, and thermodynamic experiments, HPPK employs a bi-bi mechanism in which ATPbinds first, followed by 6-hydroxymethyl-7,8-dihydropterin (63).

The *folK* gene was subsequently cloned, its sequence was determined, and the gene product was overexpressed (64). The open reading frame of the *folK* gene is 477 bp in length, coding for a protein with a predicted molecular weight of 17,945. The open reading frame for *folK* is just 8 nucleotides downstream of *pcnB*, a gene involved in controlling plasmid copy number (65), and *pcnB* and *folK* may be transcribed as a polycistronic mRNA molecule.

Dihydropteroate synthase (FolP)

Dihydropteroate synthase catalyzes the condensation of PABA and 6-CH_2OH-dihydropterin pyrophosphate to form dihydropteroate (H_2pteroate). The reactants and products for this reaction in *E. coli* were elucidated in the laboratory of Gene Brown (55). Although *p*-aminobenzoylglutamate (PABAGlu) may be used instead of PABA as the substrate to generate dihydrofolate, dihydropteroate synthase uses PABA ten times more

efficiently than PABAGlu. For the purified enzyme from *E. coli*, the K_m for PABA is 2.5 μM, whereas the K_i for PABAGlu as a competitive inhibitor is 1.3 mM (66). This observation, together with the fact that it proved impossible to isolate a PABAGlu-requiring strain and no enzyme was ever found in any organism that could condense PABA and glutamate, led researchers to conclude that the biological substrate for dihydropteroate synthase is PABA and that dihydropteroate is the true intermediate in the synthesis of dihydrofolate.

Talarico et al. purified dihydropteroate synthase more than 700-fold from a wild-type strain, obtaining an apparently homogenous enzyme (56). The native enzyme is a dimer with ~30-kDa subunits. Kinetic studies of the pure enzyme yielded K_m values for 6-CH_2OH-dihydropterin pyrophosphate and PABA of 1.9 and 0.5 μM, respectively.

Dallas et al. cloned, determined the sequence of, and overexpressed the *folP* gene encoding dihydropteroate synthase, which lies at 71.5 min (67, 68). The open reading frame of the gene contains 846 bp and is predicted to code for a polypeptide of 282 amino acids with a mass of 30,314 Da.

Biosynthesis of 7,8-Dihydrofolate–Dihydrofolate Synthetase (FolC)

Dihydrofolate synthetase catalyzes the synthesis of dihydrofolate from dihydropteroate, glutamate, and ATP and requires $Mg^{2+}$for activity. The enzyme was partially purified and the reaction in *E. coli* was first characterized by Griffin and Brown (69). In *E. coli*, dihydrofolate synthetase and folylpolyglutamate synthetase are activities contained on a single bifunctional protein (70, 71). In 1985, Bognar et al. cloned the *folC* gene and expressed the *folC* gene product ~400-fold over levels in a wild-type strain (5). They purified the overexpressed dihydrofolate synthetase activity to homogeneity. Dihydrofolate synthetase activity copurified with folylpolyglutamate synthetase activity. The enzyme was shown to be a monomer of ~45 kDa. Kinetic experiments were performed, and the K_m values for dihydropteroate, ATP, and glutamate were found to be 0.6 μM, 6.9 μM, and 3.9 mM, respectively.

The recent determination of the crystal structures of *E. coli* FolC bound to various substrates and analogues revealed that although the ATP binding site and the active-site structures are similar to those of the enzyme from *Lactobacillus casei*, the dihydropteroate binding site structure was very different (72). Because the *L. casei* enzyme is considered to be a model for human folylpolyglutamate synthetase, these differences may be exploitable in the design of specific inhibitors of *E. coli* FolC.

The *folC* gene in *E. coli* is essential, as evidenced by the observation that the chromosomal gene can be inactivated only if a second *folC* gene is present in *trans* (73). The availability of strains with *folC* deleted from the chromosome permitted the isolation and characterization of plasmid-carried mutant *folC* gene products in the absence of the wild-type enzyme. Such mutants are described in papers from the Bognar laboratory (73, 74).

Intervention by Sulfa Drugs

Sulfonamide derivatives (Fig. 7) are structural analogues of PABA and compete with PABA for condensation with 6-hydroxymethylpterin pyrophosphate in the reaction catalyzed by dihydropteroate synthase (75, 76). The pterin-sulfonamide adducts are thought not to be inhibitory to cellular function and passively diffuse out of the cell (75, 76). Since sulfonamide derivatives compete with PABA for dihydropteroate synthase, the cell becomes depleted of dihydropteroate and reduced folates derived from dihydropteroate (76, 77, 78). Mechanisms of development of sulfonamide resistance include alterations in cellular transport properties (75, 79, 80), modification of the inhibitor (75, 76), overproduction of PABA (81), and mutation of dihydropteroate synthase.

The mechanism of sulfonamide resistance that has been best characterized is mutation to form a sulfonamide-resistant dihydropteroate synthase. Several groups have isolated resistant dihydropteroate synthases from strains of *E. coli* that are resistant to sulfathiazole (68, 80, 82, 83). In all cases so far characterized, the resistant dihydropteroate synthase has a decreased ratio of the K_m for PABA to the K_i for sulfanilamide, allowing the enzyme

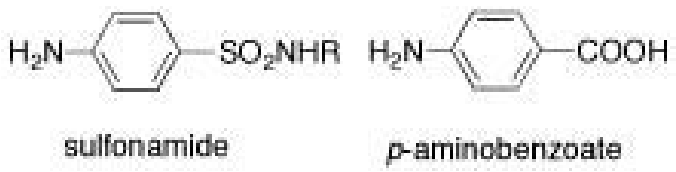

Figure 7 Comparison of sulfonamide and PABA. NHR represents the *p*-aminobenzoylglutamate substituent.

to discriminate more effectively between the natural substrate and the inhibitor (68, 82). In two cases, the mutant *folP* has been sequenced and found to contain a Phe-28-to-Leu change (68) or a Pro-64-to-Ser mutation (84). In addition, sulfonamide resistance conferred by R-plasmids isolated from resistant pathological strains of *E. coli* has been shown to involve resistant dihydropteroate synthases (85). Two different dihydropteroate synthase genes from R-plasmids, *sulI* and *sulII*, have been identified (86). Sequence analysis revealed that these genes share 50% identity but are not closely related to *folP* from the *E. coli* chromosome (87, 88, 89).

REDUCTION AND POLYGLUTAMYLATION OF FOLIC ACID DERIVATIVES

Reduction of Dihydrofolate to Tetrahydrofolate

The chromosomally carried gene for dihydrofolate reductase in *E. coli* (*folA*) specifies a monomeric protein with a molecular weight of ~18,000. This protein is very sensitive to inhibition by trimethoprim and its analogues, forming the basis for effective treatments directed against gram-negative organisms. Resistance to trimethoprim is often associated with the production of a plasmid-carried dihydrofolate reductase that is structurally unrelated and is a tetramer with ~8-kDa subunits.

Dihydrofolate reductase (FolA)

The major chromosomally encoded enzyme responsible for the reduction of dihydrofolate (H_2folate) to the physiologically active tetrahydrofolate (H_4folate) is the *folA* gene product. The enzyme dihydrofolate reductase catalyzes the reaction shown below.

$$NADPH + H^+ + H_2\text{folate} \leftrightarrow NADP^+ + H_4\text{folate} \text{ (reaction 5)}$$

Although dihydrofolate reductase from many organisms, including some strains of *E. coli*, will also carry out a very slow reduction of folic acid to dihydrofolate (90), strains of *E. coli* do not transport folates, and this reaction presumably does not occur in vivo. The *E. coli* enzyme is needed to reduce newly synthesized dihydrofolate to tetrahydrofolate. Dihydrofolate is also formed in the reaction catalyzed by thymidylate synthase.

$$dUMP + CH_2\text{-}H_4\text{folate} \rightarrow dTMP + H_2\text{folate} \text{ (reaction 6)}$$

In this reaction, CH_2-H_4folate serves a dual function, providing both a one-carbon unit (the methylene group) for the methylation of dUMP and the reducing equivalents necessary to convert a methylene to a methyl group. Dihydrofolate reductase must therefore reduce dihydrofolate as rapidly as it is formed during the de novo biosynthesis of dTMP in order to prevent the depletion of cellular stores of tetrahydrofolate derivatives.

Since *E. coli* dihydrofolate reductase is a rather small protein of considerable importance as a target for chemotherapy, it has been extensively characterized and its structure is known to high resolution. Excellent reviews and original papers can be consulted for detailed information about the structure and mechanism of the enzyme (90, 91, 92).

The enzyme is usually assayed spectrophotometrically by measuring the consumption of NADPH at 340 nm during the reduction of dihydrofolate to tetrahydrofolate (93). The absorbance changes at 340 nm are due both to the oxidation of NADPH and to the reduction of dihydrofolate and are associated with a molar extinction change at 340 nm of 12,300 cm^{-1} (90). This method is not suitable for the measurement of activity in crude extracts, for which a radioisotopic method of activity measurement should be employed (94).

The wild-type chromosomal gene for dihydrofolate reductase (*folA*) in an *E. coli* K-12 strain was cloned into pBR322 following enrichment of the gene by bacteriophage Mu-mediated transposition (95). The predicted amino acid sequence was 160 residues long and had an estimated molecular weight of 17,998. *folA* lies at 1 min on the *E. coli* chromosome (96).

Although it might have been expected that a deletion mutation leading to the complete loss of *folA* expression would be lethal, strains containing a deletion of the *folA* gene have been isolated from *thyA* strains independently in two different laboratories (97, 98). These strains are auxotrophic for thymine, adenine, pantothenate, glycine, and methionine and are viable only in the *thyA* background. The auxotrophies are those expected for a strain that is unable to produce sufficient reduced forms of tetrahydrofolate for one-carbon transfer reactions. The requirement of a *thyA* background is more perplexing. *thyA* is the structural gene for thymidylate synthase, and a *thyA* mutant would therefore fail to generate dihydrofolate from tetrahydrofolate. However, if there were no tetrahydrofolate derivatives in Δ*folA* strains, then thymidylate synthase activity should not affect the

phenotype. A solution to this dilemma appeared with the observations of Hamm-Alvarez et al. that tetrahydrofolate derivatives are present in the strains with deletions of the *folA* gene (99). Although the identity of the enzyme responsible for the formation of tetrahydrofolate derivatives in *folA* mutant strains is not known, it may be the recently identified dihydropteridine reductase of *E. coli* (100, 101), which has dihydrofolate reductase activity, or it may be FolM (see below).

Dihydrofolate reductase (FolM)

A second chromosomally encoded gene product capable of catalyzing the dihydrofolate reductase reaction was identified, cloned, and partially characterized by Giladi et al. (102). Knowing that a number of groups had independently gathered evidence supporting the notion of a second dihydrofolate reductase in *E. coli*, these researchers looked for candidate genes. Pteridine reductase (PTR1) is a short-chain dehydrogenase-reductase that is found in parasitic trypanosomatids and possesses the ability to reduce folate to tetrahydrofolate using NADPH (103). Giladi et al. performed a BLAST search of wild-type *E. coli* using the *Leishmania* PTR1 protein sequence as a query and identified *ydgB* as a homologous gene that also exhibits conservation in corresponding amino acids known to be essential for activity. Using PCR, they cloned the 723-bp gene and demonstrated that cloned *ydgB* could complement a *folA* mutant strain. They were unable to generate a *folA ydgB* double mutant. Strains overexpressing *ydgB* were highly resistant to trimethoprim. A plasmid containing *ydgB* with a histidine tag at the amino terminus of the gene product was used to generate protein that could be purified by nickel affinity chromatography. Kinetic analysis of the 26-kDa purified protein yielded K_m values for dihydrofolate and NADPH of 9.5 and 1.9 μM, respectively. The enzyme could not reduce folic acid over a pH range of 4.7 to 7, and its ability to reduce biopterin was only 10% of its ability to reduce dihydrofolate. Methotrexate is a competitive inhibitor with a K_i of 5.9 μM; trimethoprim does not inhibit FolM at concentrations of up to 138 mM. Because of its product's enhanced ability to reduce dihydrofolate over biopterin, *ydgB* was renamed *folM*. The FolM gene is located at 36 min on the *E. coli* chromosome. The physiological role of FolM may not be completely understood, however, as the V_{max} of FolM is only about 20% that of FolA and *folA* mutants are fully complemented only by the overexpression of FolM.

R-plasmid-carried dihydrofolate reductase

Shortly after trimethoprim was introduced as a clinical drug, bacterial strains that were resistant to trimethoprim and contained an R-plasmid that specified a trimethoprim-insensitive dihydrofolate reductase were isolated (104). Two types of resistant dihydrofolate reductase appeared to be synthesized: type I enzymes exhibiting a several-thousand-fold reduction in sensitivity to trimethoprim and type II enzymes with complete insensitivity to trimethoprim. The type II enzymes are especially interesting because they are genetically and structurally unrelated to chromosomally encoded dihydrofolate reductases. The R67 type II enzyme is a tetramer of identical 8.4-kDa subunits (105), and the active site is located in a pore passing through the center of the protein with residues from each monomer contributing to the active site (106). The evolutionary origin of the enzyme specified by the R-plasmid is not known.

Formation of Folylpolyglutamates

Folylpolyglutamate synthetase—the FolC gene product

The monoglutamate form of dihydrofolate that is the product of the de novo biosynthetic pathway discussed above is a substrate for dihydrofolate reductase, and the tetrahydrofolate monoglutamate product is itself a substrate for serine hydroxymethyltransferase and the enzymes that interconvert methylenetetrahydrofolate with other tetrahydrofolate cofactors. The addition of glutamyl residues by folylpolyglutamate synthetase probably occurs after the reduction of newly synthesized dihydrofolate to tetrahydrofolate and its conversion to other tetrahydrofolate derivatives. The *folC* gene product is a bifunctional enzyme that also catalyzes the addition of the glutamyl residue to dihydropteroate to form dihydrofolate [see "Biosynthesis of 7,8-Dihydrofolate–Dihydrofolate Synthetase (FolC)," above]. The two reactions catalyzed by FolC are shown below.

H_2pteroate (H_2Pte) + ATP + L-glutamate → H_2folate (H_2PteGlu$_1$) + ADP + HPO_4^{2-} (reaction 7)

H_4PteGlu$_n$ + ATP + L-glutamate → H_4PteGlu$_{n+1}$ + ADP + HPO_4^{2-} (reaction 8)

FolC adds successive glutamyl residues to H_4PteGlu$_1$ by linking each glutamyl residue to the γ-carboxyl group of the terminal glutamyl residue (Fig. 2). Thus, the isopeptide bonds formed are not the normal amide bonds

to the α-carboxyl group of glutamate and are not hydrolyzed by peptidases or proteases that are specific for α-carboxyl group-linked peptide bonds. The preferred monoglutamate substrate is 10-CHO-H_4PteGlu$_1$, and H_2PteGlu$_1$ is a very poor substrate (Barry Shane, personal communication). The preferred diglutamate substrate is 5,10-CH_2-H_4PteGlu$_2$. Although the enzyme forms triglutamates and tetraglutamates, the formation of longer-chain folates occurs very slowly, particularly when the glutamate concentration is low (107). The X-ray structure of *E. coli* FolC has recently been determined (72).

A folylpolyglutamate synthetase that forms α-linked peptide bonds

Studies on the in vivo distribution of polyglutamate chain lengths of folate derivatives in *E. coli* during exponential growth in Luria broth (1) indicated that mono- and triglutamate derivatives were most abundant, with tetra-, penta-, and hexaglutamate derivatives also found in order of decreasing abundance (Fig. 8). However, in stationary phase, cells were found to contain longer-chain folylpolyglutamates, with the predominant chain length now consisting of six or seven glutamyl residues. Since the FolC enzyme predominantly forms triglutamyl residues both in vivo (108) and in vitro (99), the in vivo distribution of folylpolyglutamates was puzzling. Ferone and his colleagues have shown that there is a second folylpolyglutamate synthase in *E. coli* that adds additional glutamyl residues to triglutamate derivatives, but this enzyme adds residues to the α-carboxyl group rather than the γ-carboxyl group of the terminal glutamyl residue (109, 110). Conditions governing the expression of this second folylpolyglutamate synthase have not been studied, nor is the gene encoding this protein identified. A BLAST search of the *E. coli* W3110 genome for homologues of the FolC gene yielded limited similarities to *ypdE*, annotated as a predicted peptidase gene, and *mpl*, encoding UDP-*N*-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase. It has not yet been determined whether the long-chain folylpolyglutamates produced in stationary phase contain any α-linkages.

H_2PteGlu$_6$ and the assembly of T4 phage

Bacteriophage T4 infection of *E. coli* has been shown to cause alterations in the metabolism of folate derivatives. Infection is associated with an increase in the chain lengths in folylpolyglutamates and particularly the accumulation of hexaglutamate derivatives (2, 111). Dihydropteroylhexaglutamate (H_2PteGlu$_6$) was shown to be a structural component of the phage baseplate (112), and six molecules are present in the baseplate. Four folate-related proteins specified by the T4D phage genome are gene product 29, a protein with folylpolyglutamate synthetase activity; gene product 28, a protein with folylpolyglutamate hydrolase (γ-glutamyl carboxypeptidase) activity (113); the *td* gene product, thymidylate synthase; and the *frd* gene product, dihydrofolate reductase. In cells infected with T4D gp28 mutants, there was a marked increase in the amount of very large folylpolyglutamates and up to 8% of the cell folates contained as many as 12 to 14 glutamate residues (114, 115). The phage-induced folylpolyglutamate synthetase used dihydrofolate derivatives almost as well as tetrahydrofolate derivatives (113), in contrast to FolC, for which H_2PteGlu$_1$ is a very poor substrate, while the phage-induced hydrolase appeared to degrade long-chain dihydropteroylpolyglutamates to the hexaglutamate. H_2PteGlu$_6$ was proposed to play a critical role in linking

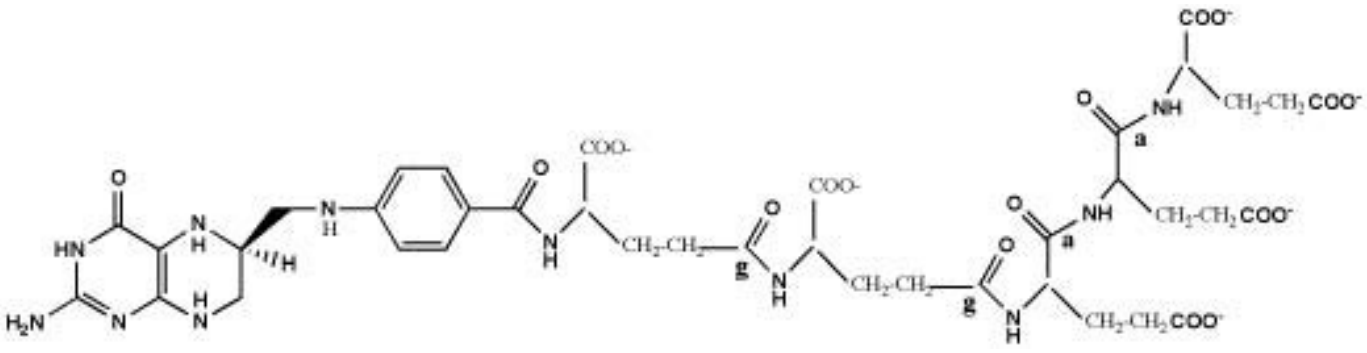

Figure 8 Composition of a long-chain folylpolyglutamate with mixed α- and γ-glutamate linkages. The first two glutamates are linked through their γ-carboxyls, while subsequent glutamyl residues are linked through their α-carboxyls. g, γ-linked peptide bonds; a, α-linked peptide bonds.

together the six wedge-shaped elements that form the baseplate (116). The pteridine portion of the folate was proposed to bind to a site on the phage dihydrofolate reductase, which was thought to be a component of the central plug. The long, flexible polyglutamate was proposed to form a flexible bond between the proximal end of the phage long tail fiber and the baseplate. A 12-Å cryoelectron microscopy image of the structure of the bacteriophage T4 baseplate has now been published (117) in which none of the folate-associated gene products have been located. Furthermore, an immunoblot assay with antibody raised to highly purified recombinant T4 phage dihydrofolate reductase and thymidylate synthase suggested that these proteins are not part of the virion structure (118). Thus, it is not clear whether dihydrofolate hexaglutamate is a structural component of the T4 phage baseplate or an adventitious contaminant.

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