Biosynthesis of Pteridines in *Escherichia coli*

**STRUCTURAL AND MECHANISTIC SIMILARITY OF DIHYDRONEOPTERIN-TRIPHOSPHATE EPIMERASE AND DIHYDRONEOPTERIN ALDOLASE**

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An open reading frame located at 69.0 kilobases on the *Escherichia coli* chromosome was shown to code for dihydroneopterin aldolase, catalyzing the conversion of 7,8-dihydromonapterin to 6-hydroxydihydromethyl-7,8-dihydropoterin in the biosynthetic pathway of tetrahydrofolate. The gene was subsequently designated *folB*. The FolB protein shows 30% identity to the paralogous dihydroneopterin-triphosphate epimerase, which is specified by the *folX* gene located at 2427 kilobases on the *E. coli* chromosome. The *folX* and *folB* gene products were both expressed to high yield in recombinant *E. coli* strains, and the recombinant proteins were purified to homogeneity. Both enzymes form homo-octamers. Aldolase can use L-threo-dihydroneopterin and D-erythro-dihydroneopterin as substrates for the formation of 6-hydroxymethylhexahydromonapterin, but it can also catalyze the epimerization of carbon 2' of dihydroneopterin and dihydromonapterin at appreciable velocity. Epimerase catalyzes the epimerization of carbon 2' in the triphosphates of dihydroneopterin and dihydromonapterin. However, the enzyme can also catalyze the cleavage of the position 6 side chain of several pteridine derivatives at a slow rate. Steady-state kinetic parameters are reported for the various enzyme-catalyzed reactions. We propose that the polarization of the 2'-hydroxy group of the substrate could serve as the initial reaction step for the aldolase as well as for the epimerase activity. A deletion mutant obtained by targeting the *folX* gene of *E. coli* has normal growth properties on complete medium as well as on minimal medium. Thus, the physiological role of the *E. coli* epimerase remains unknown. The open reading frame *ygiG* of *Hemophilus influenzae* specifies a protein with the catalytic properties of an aldolase. However, the genome of *H. influenzae* does not specify a dihydroneopterin-triphosphate epimerase.

The pathway of tetrahydrofolate biosynthesis is an important drug target in bacteria and protozoal parasites (1–3). Thus, the sulfonamides inhibiting dihydropterate synthase were the first synthetic agents that could be used with high efficiency against a wide variety of microbial and protozoal parasites. Even after more than 60 years, they remain important therapeutic agents (1, 4). More recently, trimethoprim was found to act as an inhibitor of bacterial dihydrofolate reductase (5). The combined application of trimethoprim and a sulfonamide is an important protocol for the treatment of bacterial infections (5–8).

Tetrahydrofolate is biosynthesized by plants and many microorganisms. The first committed step catalyzed by GTP cyclohydrolase I is a mechanistically complex ring expansion reaction conducive to the formation of dihydroneopterin triphosphate (Compound 1) (Fig. 1) (9). A pyrophosphatase and a phosphatase have been proposed to convert Compound 1 to dihydroneopterin (Compound 3) in two consecutive steps (10), but firm evidence for the involvement of specific enzymes in the conversion of Compound 1 to Compound 3 has not been obtained. Recently, it has been proposed that the release of pyrophosphate from Compound 1 can be catalyzed of two-valent ions such as Mg$^{2+}$ and Ca$^{2+}$ (11).

The conversion of Compound 3 to 6-hydroxyethyl-7,8-dihydropterin (Compound 4) is catalyzed by dihydroneopterin aldolase (12). Compound 4 is converted to dihydrofolate by the subsequent condensation with 4-aminobenzoate and glutamate by the catalytic action of dihydropterate synthase and dihydrofolate synthetase, respectively (13).

An enzyme catalyzing the epimerization of the folate precursor, dihydroneopterin triphosphate, under formation of 7,8-dihydromonapterin (Compound 2) had been observed in *Escherichia coli* by Heine and Brown (14). Recently, the primary structure of this enzyme has been determined and has been found to show similarity to the dihydroneopterin aldolase domain of a multifunctional folate biosynthetic enzyme from *Pneumocystis carinii* (15). On the other hand, the epimerase is similar to a reading frame at 69.0 kb$^1$ of the *E. coli* chromosome designated *ygiG* (EMBL accession number L12966) in the close vicinity of the bacitracin resistance gene. This reading frame specifies the dihydroneopterin aldolase of *E. coli*, which is a potential target for the development of enzyme inhibitors with antibacterial and/or antiprotozoal activity.

This paper reports on structural and mechanistic similarities of dihydroneopterin-triphosphate epimerase and dihydroneopterin aldolase. It also shows that the single *Hemophilus influenza* homolog of these proteins (specified by the unannotated open reading frame *ygiG_haein*) is an aldolase-type enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—6-Hydroxymethylpterin, D-dihydroneopterin, L-dihydromonapterin, D-dihydromonapterin, L-neopterin, L-monapterin, D-dihydromonapterin monophosphate, D-neopterin, and dihydrobiopterin

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$^1$ The abbreviations used are: kb, kilobase(s); PIPES, 1,4-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; bp, base pair(s).
Tetrahydrofolate

**FIG. 1. Pteridine biosynthetic pathways in *E. coli*.** A, GTP cyclohydrolase I; B, dihydroneopterin-triphosphate epimerase; C, dihydroneopterin aldolase. Compound 1, 7,8-dihydro-δ-neopterin 3′-triphosphate; Compound 2, 7,8-dihydro-λ-neopterin 3′-triphosphate; Compound 3, 7,8-dihydro-neopterin; Compound 4, 6-hydroxymethyl-7,8-dihydropterin.

were purchased from Dr. B. Schirecks (Jona, Switzerland). Synthetic oligonucleotides were purchased from MWG (Ebersberg, Germany). Restriction enzymes and T4 DNA ligase were obtained from Pharmacia (Freiburg, Germany), Life Technologies, Inc. (Eggenstein, Germany), and New England Biolabs Inc. (Beverly, MA). Goldstar DNA polymerase was obtained from Eurogentec (Seraing, Belgium). GTP cyclohydrolase I was purified from a recombinant *E. coli* strain as described earlier (9).

**Microorganisms and Plasmids—**The bacterial strains and plasmids used in this study are summarized in Table I.

**Assay of Epimerase Activity—**The epimerase substrate, dihydroneopterin triphosphate, was produced enzymatically from GTP. Reaction mixtures containing 100 mM Tris-HCl, pH 7.5, 0.1 M KCl, 50 mM GTP, 5 mM dithiothreitol, and 0.05 mg/ml recombinant *E. coli* GTP cyclohydrolase I were incubated for 4 h at 37 °C. Protein was then removed by ultrafiltration using a YM-30 membrane (Amicon Inc.).

An aliquot of this reaction mixture (20 μl) was added to 80 μl of a solution containing 80 mM Pipes, pH 6.2, and 8 mM MgCl₂. The epimerase reaction was started by the addition of protein solution. The reaction mixture was incubated at 55 °C for 10 min. The reaction was terminated by the addition of a solution (30 μl) containing 1% I₂ and 2% (w/v) KI in 1 M HCl. The samples were incubated at room temperature for 5 min. Excess iodine was reduced by the addition of 2% ascorbic acid (10 μl, w/v). A solution (210 μl) containing 1.5 mM Tris-HCl, pH 8.5, 4.8 mM ZnCl₂, 4.5 mM EDTA, and 0.5 units of alkaline phosphatase (Boehringer Mannheim, grade II) was added. The mixture was incubated at 37 °C for 90 min. Trichloroacetic acid (50 μl, 40%, w/v) was added. Aliquots of 10 μl were analyzed by HPLC using a reversed-phase column (4 × 250 mm) of Nucleosil RP18 and an eluent containing 0.5% (w/v) H₃BO₃, pH 4.7. The effluent was monitored fluorometrically (excitation, 365 nm; emission, 446 nm). The flow rate was 2 ml/min. The retention volume was 7 ml for γ-erythro-neopterin and 9 ml for l-erythro-neopterin (monoprotein).

**Assay of Dihydroneopterin Aldolase—**The assay is based on a method described earlier (12). Assay mixtures contained 100 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, 70 μg dihydroneopterin, and protein. The mixture was incubated at 70 °C for 10 min. The reaction was terminated by the addition of 30 μl of 1 M HCl containing 1% I₂ and 2% (w/v) KI. The samples were incubated at room temperature for 5 min. Excess iodine was reduced by the addition of 2% ascorbic acid (10 μl, w/v). The samples were analyzed by reversed-phase HPLC using a column (4 × 250 mm) of Nucleosil RP18 and an eluent containing 30 mM HCOOH and 7% MeOH. 6-Hydroxymethylpterin was detected fluorometrically (excitation, 365 nm; emission, 446 nm). The retention volume was 6.5 ml.

**Construction of Expression Plasmids for the folX and folB Genes—**The expression plasmids for the *folX* and *folB* genes were constructed as described by Richter et al. (16). The *folX* and *folB* genes of *E. coli* were amplified by PCR with chromosomal DNA from *E. coli* XL1-Blue as template. The *folB* gene of *H. influenzae* was amplified by PCR using the plasmid GHIFLOI as template (Table I). The *folX* gene was amplified with the oligonucleotides EpiExanti as forward primer and EpiExanti as reverse primer (Table II). The *folB* gene of *E. coli* was amplified with ECAL1 as forward primer and ECAL2 as reverse primer (Table II), and the *folB* gene of *H. influenzae* was amplified with HAELD1 as forward primer and HAELD2 as reverse primer (Table II). Each amplification product served as template for a second PCR with the universal forward primer kEcoRI (Tables II and III). The reverse primer EpiExanti was used for the *folX* gene. The reverse primer ECAL2 was used for the *folB* gene of *E. coli*, and the reverse primer HAELD2 for the *folB* gene of *H. influenzae*. The PCR products (*folB* of *H. influenzae* and *folX* of *E. coli*) were cleaved with EcoRI and BamHI and ligated in the expression vector pNCO113, which had been digested with EcoRI and BamHI, whereas the *folB* gene of *E. coli* was cleaved with PstI and EcoRI and then ligated into the expression vector pNCO113, which had been digested with PstI and EcoRI. The expression plasmids designated pEPI (containing the *folX* gene of *E. coli*), pEAL (containing the *folB* gene of *E. coli*), and pHAL (containing the *folB* gene of *H. influenzae*) (Table I) were transformed into *E. coli* MAK705 (Table I and Fig. 2). Kanamycin (20 μg/ml) and ampicillin (180 μg/ml) were added for the maintenance of the plasmids in the host strain.

**Construction of the Vector Used for the Deletion of the *folX* Gene—**The *folX* gene and the adjacent regions were amplified by PCR with EpiKpn as forward primer and EpiPst as reverse primer (Table II) using chromosomal *E. coli* XL1 DNA as template. The amplification product was cleaved with KpnI and PstI and then ligated into the vector pMAK705, which had previously been digested with KpnI and PstI. The vector contains the temperature-sensitive pSC101 origin of replication and a chloramphenicol resistance gene. The resulting plasmid, pMAK705E, was digested with EcoRV. The *folX* gene contains two internal EcoRV sites, and digestion results in the excision of a DNA fragment of 105 bp. A kanamycin resistance cassette was amplified by PCR using the forward primer kan-Rbs-1 and the reverse primer Kanad (Table II) with the plasmid pREP4 (Table I) as template. The EcoRV-digested plasmid pMAK705E was T-tailed with Taq polymerase, and the product was ligated with the kanamycin resistance cassette, yielding the plasmid pMAK705EK (Table I and Fig. 2).

**Replacement of the Chromosomal *folX* Gene with Its Inactivated Variant—**The replacement of the chromosomal *folX* gene is a modification of the methods described by Hamilton et al. (17), Russel and Model (18), and Fermé and Swedberg (19). The plasmid pMAK705EK was transformed into the recA⁺ *E. coli* strain SK6600. The plasmid replicates at 30 °C, but not at 44 °C. Growth at 44 °C on plates that contain chloramphenicol selects clones with the plasmid integrated into the chromosome by homologous recombination. This results in a tandem arrangement of the intact *folX* gene adjacent to the inactivated *folX* gene.
gene with the kanamycin insert. PCR screening was performed using two pairs of primers in order to check whether the integration of the inactivated \( \text{folX} \) gene had occurred by homologous recombination. The forward primer Epi01, complementary to the \( E.\ coli \) chromosome in the direct vicinity of the 5'-end of the \( \text{folX} \) insert, in combination with the reverse primer kan02, complementary to the \( \text{folX} \) insert, should result in a fragment of \( \sim 1.2 \) kb. The forward primer kan01, complementary to the kanamycin gene, and the reverse primer Epi02 (Table II), complementary to the \( E.\ coli \) chromosome in the direct vicinity of the 3'-end of the \( \text{folX} \) insert, should result in the amplification of a 1.4-kb fragment.

One of 12 clones screened with this method showed the correct pattern of amplificates. The resolution of this cointegrate was achieved by overnight incubation of the bacterial strain in LB liquid medium containing 20 \( \mu \)g/liter chloramphenicol at 30 °C. Cells were then diluted 10^5-fold and grown on plates containing chloramphenicol at 30 °C. Plasmid isolation of the resulting colonies showed that 50% of the colonies now contained an intact \( \text{folX} \) gene on the plasmid and therefore the defective \( \text{folX} \) gene on the chromosome. Curing of the clones was achieved by growing them at 44 °C on LB plates. The resulting clones were sensitive to chloramphenicol. Epimerase activity could not be
detected in cell extracts of the mutants. PCR amplification using the forward primer Episense and the reverse primer Epianti (Table II) resulted in amplificates of ~1400 bp, whereas the intact folX gene yields amplificates of ~300 bp. For the comparison of growth rates, SK6800 cells with intact or defective folX genes were grown on LB medium as well as on M9 minimal medium supplemented with vitamin-free casamino acids (Gibco BRL).

**Purification of Recombinant Dihydronicotin aldolase—**E. coli strain M15(pREP4) carrying the expression plasmid pEAL was grown in LB medium containing 20 μg/ml kanamycin and 180 μg/ml ampicillin at 37 °C. Isopropyl-β-D-thiogalactopyranoside was added to a concentration of 0.1 mM, and incubation was continued for 2 h. Cells were harvested by centrifugation; washed with 50 mM Tris-HCl, pH 8.0; and stored at −20 °C.

Frozen cell mass (8.3 g) was suspended in 50 ml of 50 mM Tris-HCl, pH 8.0, containing 0.2 mg/ml DNase and 0.02 mg/ml RNase. Bacteria were lysed by ultrasonic treatment, and cell debris was removed by centrifugation. The supernatant was heated to 95 °C in a water bath for 3.5 min and was then cooled in ice water. The precipitate was removed by centrifugation. The supernatant was loaded on a Sepharose Q column (2.5 × 60 cm; Pharmacia). The column was developed with a linear gradient of 0–1 M NaCl. Fractions were combined and concentrated by ultrafiltration (YM-30 membrane). Concentrated fractions (0.1 ml) were further purified on a gel filtration on a Superdex 200 column (2.6 × 60 cm; Pharmacia). The column was developed with 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl at a flow rate of 3 ml/min. Dihydronicotin aldolase from *H. influenzae* could be purified using the same procedure.

**Purification of Recombinant Epimerase—**E. coli strain M15(pREP4) harboring the expression plasmid pEpi was grown in LB medium containing 150 μg/ml ampicillin and 20 μg/ml RNase. Cells were disrupted by ultrasonic treatment. The suspension was centrifuged at 4 °C and 4000 × g, and the supernatant was heated to 95 °C in a water bath for 4 min. The precipitate was removed by centrifugation. The supernatant was loaded on a Sepharose Q column (2 × 11 cm; Pharmacia) that had been equilibrated with 20 mM Tris-HCl, pH 8.0. The column was developed with a linear gradient of 0–1 M NaCl. Fractions were combined and concentrated by ultrafiltration (YM-30 membrane). Concentrated fractions (8 ml) were further purified by gel filtration on a Superdex 200 column (2.6 × 60 cm; Pharmacia). The column was developed with 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl at a flow rate of 3 ml/min. Dihydronicotin aldolase from *E. coli* was purified by chromatography as described under “Experimental Procedures.” A typical experiment described under “Experimental Procedures.” A typical experiment.
dihydroneopterin. The kinetic parameters were similar to those of the \textit{E. coli} aldolase.

\textbf{Recombinant Expression of Epimerase—} The \textit{folX} gene of \textit{E. coli} specifying dihydroneopterin-triphosphate epimerase was expressed under the control of the \textit{lac} promoter and T5 operator. The recombinant protein was purified by a sequence of ion-exchange chromatography, heat treatment, and gel permeation chromatography (Table V). The purified protein migrated as a single band of 14 kDa on SDS-polyacrylamide gels.

\textbf{Quaternary Structure—} The molecular masses of the proteins under study were determined by sedimentation equilibrium analysis. The molecular masses are summarized in Table VI and suggest that the three proteins under study form homo-octamers.

\textbf{Kinetic Properties of Aldolases—} In light of the sequence similarity between epimerase and aldolase, it was in order to perform a detailed comparison of their catalytic and kinetic properties. Initial enzyme experiments were performed at the temperature optimum of the enzymes studied (70 °C for dihydroneopterin aldolase and 55 °C for dihydroneopterin-triphosphate epimerase) (Tables IV and V). The more detailed kinetic analyses reported in Table VII were performed at 37 °C.

Products were determined by fluorescence-monitored HPLC after dehydrogenation and dephosphorylation as required. The results shown in Table VII and Fig. 4 indicate that the aldolases specified by the \textit{folB} genes of \textit{E. coli} and \textit{H. influenzae} catalyze the cleavage of dihydroneopterin. The velocity was 127 \(\mu\text{mol/mg/h}\) in comparison with 234 \(\mu\text{mol/mg/h}\) as determined by Mathis and Brown (12). The remarkable difference can be explained by the pH at which Mathis and Brown determined their kinetic properties. Their activities were measured at pH 9.6, but we have found a dramatic decrease in activity at pH > 8.5.

However, the enzyme could also catalyze the epimerization of position 3' of dihydroneopterin, yielding dihydromonapterin. The rates of the forward and reverse reactions were similar. Moreover, we found that the epimer dihydromonapterin could also serve as a substrate for aldol cleavage of the side chain, yielding hydroxymethyl喋啶opterin. All cleavage and epimerization reactions catalyzed by the enzyme have similar velocities. The kinetic properties of the aldolase from \textit{H. influenzae} are shown in parentheses in Fig. 4.

The naturally occurring substrate of the aldolases in the biosynthetic pathway of tetrahydrofolate is dihydroneopterin. However, the epimer dihydromonapterin can be converted to 6-hydroxymethyl喋啶opterin without prior epimerization to dihydroneopterin as the velocity of the cleavage reaction for both epimers is at least six times higher than the epimerization reaction of both substrates (Table VII). It should be noted that the \textit{folB} gene of \textit{E. coli} catalyzes the epimerization reaction more efficiently than the \textit{H. influenzae} protein. Table VII indicates that in \textit{H. influenzae}, the velocity of epimerization, as well as the velocity of the formation of hydroxymethyl喋啶opterin from dihydromonapterin, is considerably lower as compared with the \textit{E. coli} enzyme.

\begin{table}[h]
\centering
\caption{Purification of recombinant dihydroneopterin aldolase from \textit{E. coli}}
\begin{tabular}{|l|c|c|c|c|}
\hline
Procedure & Total activity & Specific activity & Purification factor & Yield \\
\hline
Cell extract & 89.5 & 179 & 1 & 100 \\
Heat treatment & 73.4 & 256 & 1.43 & 82 \\
Sepharose Q & 66.5 & 487 & 2.72 & 74 \\
Superdex 200 & 63.6 & 525 & 2.92 & 71 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Purification of recombinant dihydroneopterin-triphosphate epimerase from \textit{E. coli}}
\begin{tabular}{|l|c|c|c|c|}
\hline
Procedure & Total activity & Specific activity & Purification factor & Yield \\
\hline
Cell extract & 51.3 & 171 & 1 & 100 \\
Sepharose Q & 47.0 & 196 & 1.15 & 92 \\
Heat treatment & 38.3 & 284 & 1.66 & 75 \\
Superdex 200 & 34.1 & 461 & 2.7 & 66 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Properties of the enzymes studied}
\begin{tabular}{|l|l|l|l|}
\hline
Parameter & Epimerase & Aldolase \\
\hline
\textit{E. coli} & \textit{H. influenzae} & \\
\hline
Predicted subunit mass (Da) & 13,988 & 12,456 & 13,577 \\
Predicted octamer mass (Da) & 111,608 & 99,648 & 108,618 \\
\(\tilde{v}\) (ml/g) & 0.7322 & 0.7297 & 0.7300 \\
Molecular mass (kDa) & 100 & 104 & 104 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Kinetics of epimerase and aldolase at 37 °C}
\begin{tabular}{|l|l|l|l|l|}
\hline
Substrate & Product & Type of reaction & \(K_m\) & \(V_{\text{max}}\) \\
\hline
\textit{E. coli} dihydroneopterin aldolase & 6-HMP & Cleavage & 64 & 127 \\
H\textsubscript{Neo} & 6-HMP & Epimerization & 45 & 20.3 \\
H\textsubscript{Neo} & H\textsubscript{Mona} & Cleavage & 36 & 158 \\
H\textsubscript{Mona} & H\textsubscript{Neo} & Epimerization & 57 & 15.6 \\
\textit{H. influenzae} dihydroneopterin aldolase & 6-HMP & Cleavage & 21 & 242 \\
H\textsubscript{Neo} & H\textsubscript{Mona} & Epimerization & 43 & 1.67 \\
H\textsubscript{Mona} & H\textsubscript{Neo} & Cleavage & 16 & 9.9 \\
E. \textit{coli} dihydroneopterin-triphosphate epimerase & H\textsubscript{Neo} & Epimerization & 19 & 1.43 \\
H\textsubscript{Neo} & H\textsubscript{Mona} & Epimerization & 149 & 0.95 \\
H\textsubscript{Mona} & H\textsubscript{Neo} & Epimerization & 68 & 0.67 \\
\hline
\end{tabular}
\end{table}

The monophosphate of dihydroneopterin can be cleaved by the aldolases of \textit{E. coli} and \textit{H. influenzae}, but the velocity is <1% as compared with the unphosphorylated substrates. The triphosphates of dihydroneopterin and dihydromonapterin were neither cleaved nor isomerized by the aldolases under study.

\textbf{Kinetic Properties of Epimerase—} As shown in Table VII and Fig. 4, dihydroneopterin-triphosphate epimerase uses the triphosphates of dihydroneopterin and dihydromonapterin efficiently as substrates for epimerization. A value of 0.77 was estimated for the equilibrium constant on the basis of the steady-state kinetic parameters. Dihydroneopterin and dihydromonapterin can also serve as substrates for epimerization, but the reaction velocities of the unphosphorylated substrates are slower by more than a factor 700. However, the unphosphorylated substrates are also slowly converted to hydroxymethyl喋啶opterin. The velocity of the aldolase-type reaction catalyzed by epimerase is <1% as compared with the cleavage of dihydroneopterin by the FolB protein. The cleavage reactions cannot be attributed to a contamination of recombinant dihydroneopterin aldolase with dihydroneopterin-triphosphate epimerase specified by the chromosomal \textit{folX} gene since aldolase and epimerase are efficiently separated by the anion-exchange
The role of the FolB protein in the pathway of tetrahydrofolate biosynthesis is well understood. However, the biological role of the FolX protein in the pathway of tetrahydrofolate biosynthesis is not clear. We therefore constructed a folX deletion mutant of E. coli using the method described by Kushner and co-workers (17). PCR analysis of chromosomal DNA confirmed that the chromosomal gene of the mutant carried an insertion of 807 bp after bp 149 of the folX gene. Cell extracts of the mutant were devoid of epimerase activity. Since the enzyme assay used is very sensitive, any residual activity below the detection limit would have been <0.5% as compared with the E. coli wild-type strain. The folX mutant showed normal growth properties on minimal medium as well as on complete medium. Thus, the biological role of epimerase remains unknown.

**DISCUSSION**

The enzymes specified by the folX and folB genes of E. coli are similar in terms of their primary structure and quaternary structure. The sequence similarity extends to dihydroneopterin aldolases of several other microorganisms (Fig. 3). Some of these proteins are large multidomain proteins catalyzing several steps in the biosynthesis of tetrahydrofolate.

It appears safe to assume that the folB gene is involved in the pathway of tetrahydrofolate biosynthesis, where it is responsible for the shortening of the position 6 side chain of the pteridine precursor. On the other hand, the role of the dihydroneopterin-triphosphate epimerase is still unknown. Growth tests of E. coli with a defective folX gene showed no apparent phenotype.

Pteridines with the threo configuration of the trihydroxypropyl side chain that is generated by the epimerization reaction have been found in a variety of organisms (29, 30), but their metabolic role is unknown. Even if these stereoisomers have a specific metabolic function, a specific epimerase may not be required for their formation since, at least in E. coli and H. influenzae, they can be formed by the aldolase specified by the folB gene.

H. influenzae has a folB gene (i.e. the unannotated reading frame ygiG_haein) specifying a protein whose catalytic properties are similar to those of the FolB protein of E. coli. However, the genomes of H. influenzae and Bacillus subtilis appear to be devoid of folX genes specifying a pteridine epimerase, in line with the hypothesis that the epimerase is not an essential protein in bacteria.

A hypothetical reaction mechanism is shown in Fig. 5. We propose tentatively that both reaction types can be initiated by protonation of N-5 followed by deprotonation at the acidic C-1’ of dihydroneopterin- or dihydromonapterin-type substrates. Epimerase- as well as aldolase-type reactions can be catalyzed by both the FolB and FolX proteins. This raises the question of a common transition state for both types of reaction. A retroal- dol cleavage of the C–C bond between C-1’ and C-2’ is proposed to be the crucial reaction step for both enzyme reactions. Epimerization at C-2 would result from reversal of the cleavage reaction without stereoenzymatic control. The different product ratios of FolB- and FolX-type proteins could result from differences in compartmentalization between the different exit pathways originating from the common transition state.

Epimerization could occur if the reaction products resulting from cleavage of the C-6a-C-7 carbon bond, instead of being released from the enzyme, react under regeneration of a pteridine, albeit without stereo control. Partitioning between the reaction pathways for cleavage and isomerization could then depend on the compartmentalization of the cleavage fragments at the active site. This would imply that in the case of epimerase, the release of the cleavage products from the enzyme could be hindered by the protein conformation, and release would occur only after religation of the cleavage fragments.

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