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NOTES

Dihydroneopterin Triphosphate Epimerase of *Escherichia coli*: Purification, Genetic Cloning, and Expression

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The enzyme catalyzing the epimerization at position 2′ of dihydroneopterin triphosphate was purified by a factor of about 10,000 from cell extract of *Escherichia coli*. The cognate gene was cloned, sequenced, expressed, and mapped to kb 2427 on the *E. coli* chromosome.

In the course of studies on the dihydrofolate pathway in *Escherichia coli*, Heine and Brown (4) detected an enzyme catalyzing the epimerization of carbon 2′ of the side chain of dihydroneopterin triphosphate (compound 2 [Fig. 1]). The enzyme has been purified about 200- to 300-fold (3, 4) but has not been studied in detail.

Purification of epimerase. The assay of 7,8-dihydroneopterin triphosphate epimerase used in this study is a modification of a published procedure (3). A suspension of *E. coli* wild-type strain DSM 613 cells (500 g) in 2 liters of 50 mM Tris hydrochloride buffer, pH 8.0, was passed through a French pressure cell (Aminco). The crude cell extract was heated to 80°C for 5 min. The supernatant was placed on a column of DEAE-cellulose DE 52 (8 by 11 cm; Whatman) which was developed with a gradient of 0 to 0.4 M NaCl containing 20 mM Tris hydrochloride, pH 8. Fractions were combined, and sodium chloride was added to a final concentration of 2 M. The solution was placed on a column of phenyl-Sepharose 6FF (high sub, 4.5 by 8 cm; Pharmacia) which was developed with a gradient of 2.0 to 0 M NaCl containing 20 mM Tris hydrochloride, pH 8. Fractions were combined and concentrated by ultrafiltration (YM 30 membrane; Amicon).

Further purification was achieved by preparative polyacrylamide gel electrophoresis with a gel prepared with 6% acrylamide, 0.45% bisacrylamide, and 750 mM Tris hydrochloride, pH 8.5 (40 mA, 24 h). Fractions containing epimerase were combined and dialyzed against 20 mM Tris hydrochloride, pH 7.2. The solution was placed on a Resource Q column (6 ml; Pharmacia) which was developed with a linear gradient of 0 to 0.4 M NaCl containing 20 mM Tris hydrochloride, pH 7.2. Fractions were combined and concentrated by ultrafiltration. The solution was placed on a column of Superdex 75 (1.6 by 60 cm; Pharmacia) which was developed with 20 mM Tris hydrochloride, pH 8.0, containing 100 mM NaCl. Fractions were combined and applied to a column of Mono Q (1 ml; Pharmacia) which was developed with a linear gradient of 0 to 0.4 M NaCl containing 20 mM Tris hydrochloride, pH 8.0.

This procedure resulted in approximately 10,000-fold puri-

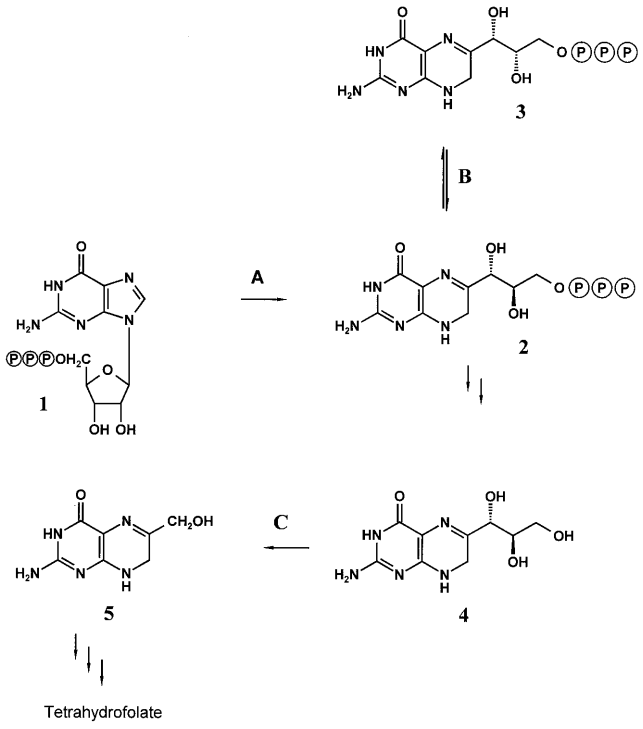

FIG. 1. Pterinic biosynthetic pathways in *E. coli*. A, GTP cyclohydrolase I; B, dihydroneopterin triphosphate epimerase; C, dihydroneopterinaldolase. 1, GTP; 2, dihydroneopterin triphosphate; 3, dihydromonapterin triphosphate; 4, dihydroneopterin; 5, 6-hydroxymethyldihydropterin.

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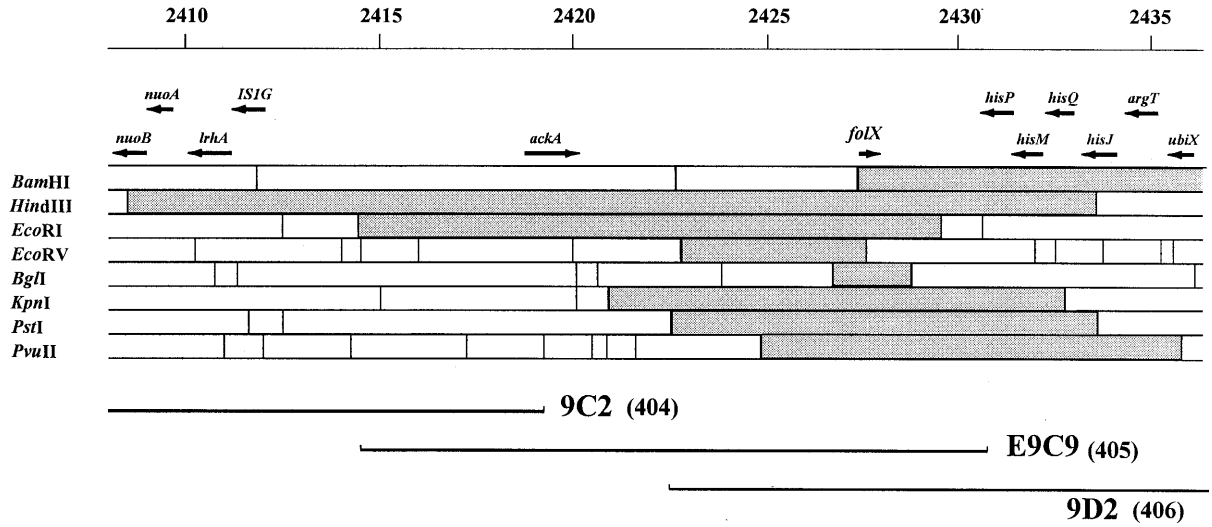

FIG. 2. Part of the restriction map of the *E. coli* chromosome. Grey bars, restriction fragments which have been mapped by Southern analysis. Clones used for construction of the restriction map (1) are represented by horizontal bars. Each clone name is indicated at the right of the bar. Numbers in parentheses are the serial numbers of the clones.

fication of the enzyme (Table 1). The protein migrated as a single band during sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating an approximate mass of 14 kDa.

Molecular cloning, sequence determination, and mapping. Automated Edman sequencing of the protein and of peptide fragments obtained after digestion with AspN protease yielded six partial amino acid sequences accounting for a total of 84 amino acid residues. Degenerate DNA primers were designed on the basis of the protein sequence data. PCR experiments using chromosomal *E. coli* DNA as a template resulted in the amplification of a DNA segment with an approximate length of 210 bp which was cloned and sequenced. This DNA fragment specified 71 amino acid residues at the N-terminal part of the putative epimerase protein.

The DNA fragment was used for Southern hybridization of chromosomal *E. coli* DNA digested with each of the restriction enzymes used for construction of the *E. coli* physical map (5). The data were consistent with the location of the *folX* gene, specifying epimerase, at kb 2427 on the *E. coli* chromosome between the genes *ackA* and *hisP* (Fig. 2).

Primers complementary to sections of the genes *folX*, *hisP*, and *ackA* were used for PCR amplification with chromosomal

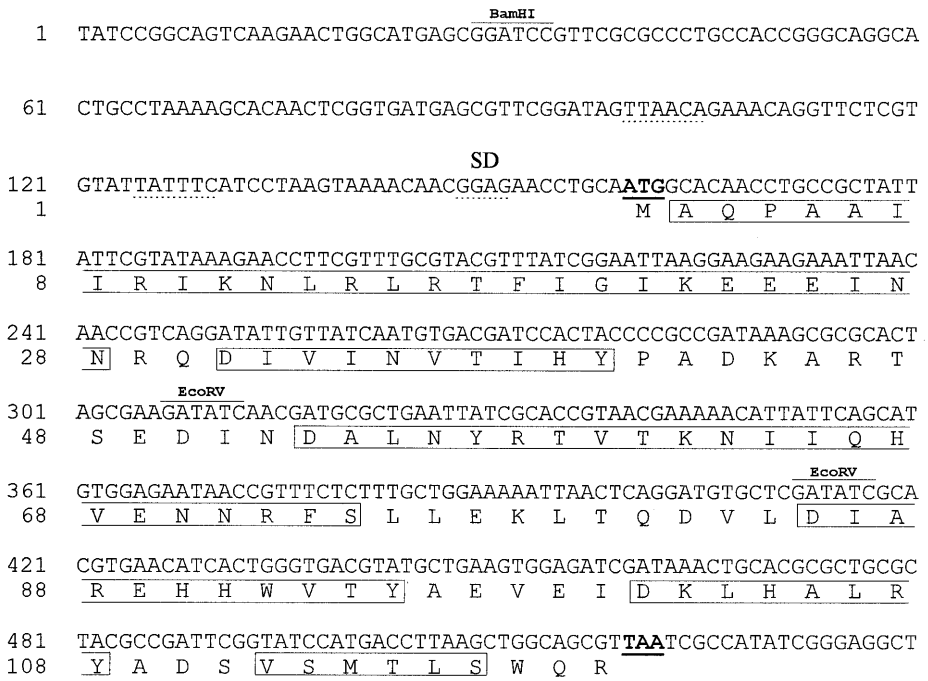

FIG. 3. Nucleotide sequence of the gene coding for dihydroneopterin triphosphate epimerase. The deduced amino acid sequence is also shown, and peptide fragments obtained by Edman degradation are boxed. The start and stop codons are in boldface type and underlined. The putative ribosome binding site (SD) and the hypothetical promoter region are indicated with dotted lines. Restriction sites identified by sequencing are overlined.

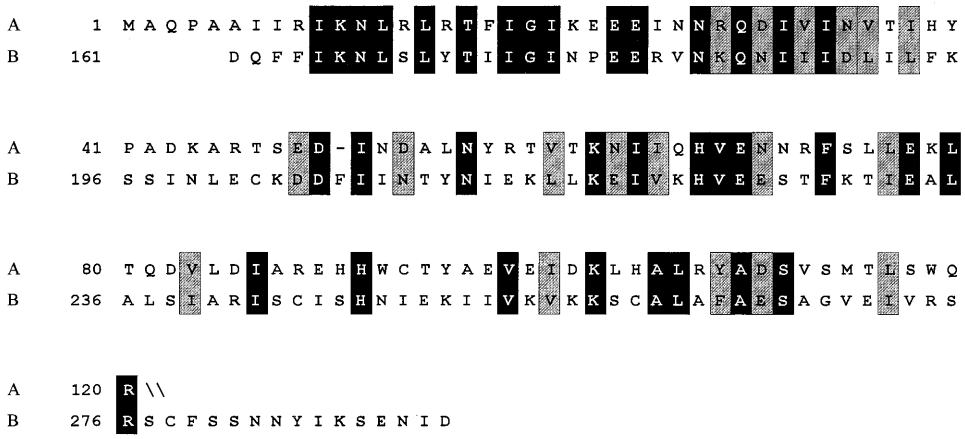

FIG. 4. Sequence alignment of dihydroneopterin triphosphate epimerase of *E. coli* (A) and the putative dihydroneopterin aldolase domain of the multifunctional Fas protein of *P. carinii* (B). Identical residues are in black boxes; similar residues are in grey boxes. The dash indicates a gap; diagonal lines indicate end of sequence.

E. coli DNA as a template and afforded amplificates of 2.6 and 4.7 kb which were partially sequenced, yielding the sequence of the entire *folX* gene and the adjacent regions.

The sequence of the *folX* gene, specifying epimerase, is shown in Fig. 3. The predicted amino acid sequence is also shown. The open reading frame consists of 360 bp specifying 120 amino acid residues. Edman sequencing of the intact epimerase had yielded alanine as the N-terminal amino acid. It follows that the N-terminal methionine is removed by post-translational processing in the *E. coli* wild-type strain.

A *Bam*HI site precedes the *folX* open reading frame, and two *Eco*RV sites are located inside the open reading frame (Fig. 3). These restriction sites are in close agreement with the *E. coli* physical map and confirm the location of the epimerase gene at kb 2427 on the revised physical map of the *E. coli* chromosome (2). This corresponds to a position of about 50 min (1).

Expression of the *folX* gene. The *folX* open reading frame was placed under the control of the *lac* operator and a T5 promoter in the expression plasmid pNCO113 (courtesy of A. van Loon, Hoffmann-La Roche AG, Basel, Switzerland). Recombinant *E. coli* cells harboring this plasmid formed a peptide of 14 kDa. Cell extracts of the recombinant strain showed epimerase activity about 2,000 times above the level of wild-type *E. coli*. The recombinant protein was purified by anion-exchange chromatography, gel filtration, and heat treatment. The purified protein had a specific activity of 650 μmol mg^{-1} h^{-1}.

Sequence comparison shows that dihydroneopterin triphosphate epimerase of *E. coli* is similar to the putative dihydroneopterin aldolase domain of the multidomain Fas protein of *Pneumocystis carinii*, catalyzing several steps of tetrahydrofolate biosynthesis (8) (Fig. 4). In light of the sequence similarity between the epimerase and the dihydroneopterin aldolase domain, we checked the recombinant epimerase for aldolase activity, which was less than 5 nmol mg^{-1} h^{-1} (i.e., less than 10^{-5} times the epimerase activity of 650 μmol mg^{-1} h^{-1}). The dihydroneopterin aldolase of *E. coli* has been highly purified by Mathis and Brown (6, 7) and was clearly different from the epimerase. Despite the sequence homology with the Fas protein, the enzyme studied in this investigation is clearly not the dihydroneopterin aldolase of *E. coli*. The metabolic roles of epimerase and its metabolic product in *E. coli* are unknown.

TABLE 1. Purification of dihydroneopterin triphosphate epimerase

Procedure	Total activity (μmol h^{-1})	Sp act (nmol mg^{-1} h^{-1})	Purification factor	Yield (%)
Cell extraction	4,320	16.5	1	100
Heat treatment	4,134	21.4	1.3	96
DEAE-cellulose	6,458	75.7	4.6	150
Phenyl-Sepharose	3,005	392	23.8	70
Gel electrophoresis	2,338	2,840	172	54
Resource Q	1,930	24,300	1,503	45
Superdex 75	709	79,000	4,850	16
Mono Q	302	161,000	9,760	7

Nucleotide sequence accession number. The sequence of the *folX* gene has been deposited in the EMBL database under accession number X96709.

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