Nucleotide sequence of dihydrofolate reductase type VI

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Summary. The complete sequence of the type VI dihydrofolate reductase (DHFR) gene from plasmid pUK672 was determined. The structural gene coded for a polypeptide of 157 amino acids which had a deduced mol. wt of 17 424. Comparison with amino-acid sequences of the type I, type V and *Escherichia coli* K12 chromosomal DHFRs showed that there was 63%, 61% and 31% homology respectively. Putative RNA polymerase and ribosomal binding sites were identified proximal to the initiation codon and a feature consistent with transcription termination was present distal to the coding region. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis showed that the enzyme had a subunit mol. wt of 17 500.

Introduction

High-level resistance to trimethoprim (Tp) (MIC > 1000 mg/L) is mediated by dihydrofolate reductases (DHFRs) which are resistant to the drug. The genes encoding these enzymes have been demonstrated in plasmids and transposons by which means they have also been found integrated into the bacterial chromosome.¹ Several such enzymes have been described in gram-negative bacteria and classified according to their biochemical properties. These are the types I(a and b), II(a, b and c), V, VI and VII.²

The nucleotide sequences of the types I, II and V DHFRs have been determined,^{3–5} and this has revealed considerable homology between enzymes of the same group, e.g., >78% between type IIa, b and c.^{6, 7} There is no sequence homology between the type I and type II, and the type II and type V genes.^{3, 5} The type V and type I DHFR genes have several similar features. Both genes code for a polypeptide of 157 amino acids and there is 75% amino-acid homology.⁵ However, these genes do not hybridise with one another.⁸ The type I and type V DHFR genes have regions which are homologous with the chromosomal DHFR gene of *Escherichia coli* K12.⁵

The type VI DHFR was originally found in *Proteus mirabilis* J120 isolated in South Africa.⁹ It has unique biochemical properties and, so far, has been found only in that country. The mol. wt, as determined by Sephadex column chromatography, was 10 000. This suggested that it might be a functional subunit of the type II DHFRs (mol. wt 9500), but hybridisation experiments with a type II probe failed to substantiate this.¹⁰

This paper describes the sequence of the type VI DHFR gene and its relationship to other DHFRs.

Materials and methods

Bacterial strains

The gene encoding the type VI DHFR was isolated from *P. mirabilis* strain J120 (pUK672). The cloning vectors used were pUC18 and bacteriophages M13 mp18 and mp19, which were propagated in *E. coli* K12 strains JM103 and JM101 respectively.¹¹

DNA isolation and purification

Plasmid DNA was purified by use of a Circleprep Kit (Bio 101 Inc., La Jolla, CA, USA) according to the manufacturer's instructions. DNA fragments were purified by separation in, and extraction from, low-melting-point agarose as previously described.¹⁰

Cloning procedure

All cloning procedures with the vectors pUC18 and bacteriophages M13 mp18 and mp19 were performed according to the methods recommended by the supplier (Boehringer Mannheim). Purified plasmid DNA (pUK672) was partially or fully digested with *Eco*RI and ligated into suitably restricted plasmid pUC18 with T4 DNA ligase. The hybrid plasmids were transformed into competent *E. coli* K12 JM103 and clones containing the DHFR gene were selected on a medium containing trimethoprim lactate (Wellcome) 100 mg/L, ampicillin 100 mg/L, isopropyl- β -D-galactoside and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). Plasmid DNA from each clone was screened by the method of Maniatis *et al.*¹² Similarly, DNA fragments from plasmid pUK672

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were ligated with suitably digested replicative form bacteriophage DNA and transformed into *E. coli* K12 JM101.

Sequence determination

Sequencing was performed by the chain termination method of Sanger *et al.*¹³ with an M13 sequencing kit (Boehringer) according to the manufacturer's instructions. The sequence was determined from both strands with the exception of *c.* 200 bp at the 5' end, the sequence of which was determined from one strand only. The sequence around the *Hind*III restriction site was confirmed with a 20-bp oligonucleotide primer which was complementary to the negative strand of the insert from position 266–285. The sequence was analysed with the Mount sequence analysis package (Genetics Software Centre, Department of Molecular and Cellular Biology, Biosciences West, University of Arizona, Tucson, AZ 85721, USA).

Results and discussion

Isolation of the type VI DHFR gene

After partial digestion with *Eco*RI, the plasmid pUK672 was ligated with suitably restricted pUC18 and the hybrid plasmid was transformed into *E. coli* K12 JM103. A clone expressing trimethoprim resistance was found to contain a vector plasmid with an insert of *c.* 9.0 kb. The insert DNA was excised from the vector, purified and digested a second time with *Eco*RI. The fragments were recloned into pUC18. Clones expressing trimethoprim resistance were screened and the vector with the smallest insert (*c.* 1.5 kb) was identified. Attempts to remove the *c.* 1.5-kb fragment from the plasmid revealed that the internal *Eco*RI site and the adjacent *Xba*I site of the vector had been damaged. However, the *Pst*I site was functional and was used together with the second *Eco*RI site to remove the insert.

Digestion of the *c.* 1.5-kb insert with a number of restriction enzymes showed that single sites for both *Xba*I and *Hind*III were present and that the fragments generated by each enzyme were large enough to contain the DHFR gene. These fragments were cloned into pUC18, but only the *Pst*I/*Xba*I insert expressed trimethoprim resistance. This insert, which was *c.* 1.0 kb in size, also contained the *Hind*III site. The biochemical properties of the enzyme expressed by this gene were identical to those expressed by the gene in *P. mirabilis* J120.

For sequencing, three fragments were cloned into bacteriophages M13 mp18 and mp19. These were the *c.* 1.0-kb *Pst*I/*Xba*I fragment, the *c.* 600-bp *Pst*I/*Hind*III fragment and the *c.* 400-bp *Hind*III/*Xba*I fragment (fig. 1).

Fig. 1. Restriction map of the 1026-bp insert containing the type VI DHFR gene (cross-hatched area). Arrows indicate the direction and extent of sequencing.

Nucleotide sequence of the type VI DHFR gene

Because the type VI enzyme had been previously assigned a mol. wt of 10 000, a gene of about 240–300 bp was expected. Analysis of the sequence of the 1026-bp insert yielded only one open reading frame (ORF) larger than 240 bases. This was 471-bp long and encoded a peptide of 157 amino-acid residues with a calculated mol. wt of 17 424. The nucleotide and predicted amino-acid sequences are shown in fig. 2. The proposed type VI structural gene was found to be bounded by an initiation codon (ATG) at position 336 and a termination codon (TAA) at position 807. At position 646 there was a *Hind*III restriction site. Other restriction sites are shown in fig. 1.

Analysis of the 5' and 3' nucleotide sequence

Analysis of the DNA sequence upstream of the ORF showed a set of possible –35 and –10 promoter sequences. The most highly conserved base pairs of the –35 consensus sequence in *E. coli* promoters are –TTGACA–.¹⁴ The hexamer –TAGACA– found at position 260 of this sequence was identical in five of the six base pairs and could, therefore, have fulfilled this function. Harley and Reynolds,¹⁵ in an analysis

PstI															
CCTGCAGTTGCCATGTTTTACGGCAGTGAAGAGATAGCGAGCGCTGATGTCCGGCGGTGC															60
TTTTGCCGTTACGCACCACCCCGTCAGTAGATGAACAGGAGGGACAGCTGATAGAAACAG															120
AAGCCACTGGAGCACCTCAAAAAACCATCATACACTAAATCAGTAAGTTGGCAGCATCA															180
CCCTATTTAACTTAATTCAAATATTTTAGAAAACCTAGTGATAGTAATACCTTAGGATAT															240
TTGTTAAAACTATATAGAGTAGACAAATGAGCGTTTAGTCGGCAGAAATATGCGCGATGA															300
-35 -10															
GCGCACTATTAAATTGTTAGCCCTCAGGAGGAAAA ATG AAA ATA TCT CTT ATG															353
GCA GCT GTT TCC GAG AAT GGA GTA ATT GGC TCT GGA TTG GAT ATA															398
Ala Ala Val Ser Glu Asn Gly Val Ile Gly Ser Gly Leu Asp Ile															
CCT TGG CAT GTA CAA GGC GAG CAG CTC CTA TTC AAA GCC ATG ACT															443
Pro Trp His Val Gln Gly Glu Gln Leu Leu Phe Lys Ala Met Thr															
TAC AAT CAA TGG CTT CTA GTT GGT CGT AAA ACC TTC GAC TCA ATG															488
Tyr Asn Gln Trp Leu Leu Val Gly Arg Lys Thr Phe Asp Ser Met															
GGT AAA CTT CCG AAT AGA AAA TAT GCA GTG GTT ACT CGT TCT AAA															533
Gly Lys Leu Pro Asn Arg Lys Tyr Ala Val Val Thr Arg Ser Lys															
ATT ATC TCG AAT GAC CCT GAT GTT GTG TAT TTC GCA AGT GTT GAA															578
Ile Ile Ser Asn Asp Pro Asp Val Val Tyr Phe Ala Ser Val Glu															
TCG GCA TTA GCT TAC CTA AAC AAT GCG ACA GCA CAT ATC TTT GTT															623
Ser Ala Leu Ala Tyr Leu Asn Asn Ala Thr Ala His Ile Phe Val															
TCT GGT GGT GGT GAA ATA TAT AAA GCT TTA ATC GAT CAA GCA GAT															668
Ser Gly Gly Gly Glu Ile Tyr Lys Ala Leu Ile Asp Gln Ala Asp															
GTT ATC CAT CTT TCA GTG ATT CAC AAG CAT ATC TCT GGC GAT GTG															713
Val Ile His Leu Ser Val Ile His Lys His Ile Ser Gly Asp Val															
TTT TTT CCT CCA GTT CCA CAG GGC TTC AAG CAA ACA TTT GAG CAA															758
Phe Phe Pro Pro Val Pro Gln Gly Phe Lys Gln Thr Phe Glu Gln															
AGT TTC AGT TCA AAT ATT GAT TAC ACG TAC CAA ATT TGG GCA AAG															803
Ser Phe Ser Ser Asn Ile Asp Tyr Thr Tyr Gln Ile Trp Ala Lys															
GGC TAA CAATCTGTTTAAGAGTGATTGCGAACGCGTGGAATTTTACTATGCGTTGCG															860
Gly TER															
TTTAGTGTTTAAGGTGGTATGCGGAGGCTTCGGTATTGCGTTGCTCACACCTTAACAGGG															920
CGTTATGTGTCTCTTGGATTAGGTGAAATAAACTATGTTGAATATCATTAAATTTATTC															980
XbaI															
TTATTTCTTAATGCTTTCAGTGATTTTATCTATATTGCTCTAGA															1026

Fig. 2. Nucleotide sequence of the 1026-bp insert. The DHFR coding region extends from base 336 to base 806. The deduced amino-acid sequence is written below the DNA sequence. The -35 and -10 regions and the ribosomal binding site are underlined. The inverted repeat sequence is overlined.

of 253 *E. coli* promoters, found that the optimum distance between the -35 and -10 regions was 17 ± 1 bp. Taking this into consideration, the hexamer -AGAAAT-, which was 18-bp downstream from the proposed -35 sequence, could have formed the -10 region. However, this sequence differed in three of the six base pairs found most commonly in *E. coli* -10 sequences, -TATAAT-. If these regions did function as the RNA polymerase binding site, they were further upstream than expected. However, longer leader sequences have been noted previously although their function is not known.^{4,16} There was a ribosomal binding site (-AGGA-) at an appropriate distance from the proposed initiation codon.¹⁷

A possible transcription termination site was present downstream of the coding region. An inverted repeat sequence centred at position 845/6 could be involved in the formation of a stem and loop structure in which the stem contains 8 bp and the loop 16 bases.

Other features consistent with *rho*-independent termination were not present.

Comparison of amino-acid sequences

The types I, V and VI DHFR genes encode polypeptides of 157 amino acids, whereas the gene for the *E. coli* chromosomal DHFR encodes a polypeptide of 159 residues.¹⁸ These sequences are aligned in fig. 3 and numbered according to the *E. coli* DHFR. Overall amino-acid homology between the type I and type VI enzymes was 63%, but within the first 50 amino acids, where co-enzyme, substrate and inhibitor binding take place,¹⁹ there was 80% homology. There was even greater identity (86.6%) in the residues 51-65. Similarly, comparison of the type V and type VI sequences showed 61% homology; 78% of the first 50 residues and 86.6% of residues 51-65 were identical. The degree of homology between the chromosomal

Type VI	Met	Lys	Ile	Ser	Leu	Met	Ala	Ala	Val	Ser	Glu	Asn	Gly	Val	Ile
Type I	Met	Lys	Leu	Ser	Leu	Met	Val	Ala	Ile	Ser	Lys	Asn	Gly	Val	Ile
Type V	Met	Lys	Val	Ser	Leu	Met	Ala	Ala	Lys	Ala	Lys	Asn	Gly	Val	Ile
Chr K 12	Met	—	Ile	Ser	Leu	Ile	Ala	Ala	Leu	Ala	Val	Asp	Arg	Val	Ile

Gly	Ser	Gly	Leu	Asp	Ile	Pro	Trp	His	Val	Gln	Gly	Glu	Gln	Leu
Gly	Asn	Gly	Pro	Asp	Ile	Pro	Trp	Ser	Ala	Lys	Gly	Glu	Gln	Leu
Gly	Cys	Gly	Pro	His	Ile	Pro	Trp	Ser	Ala	Lys	Gly	Glu	Gln	Leu
Gly	Met	Glu	Asn	Ala	Met	Pro	Trp	Asn	Leu	Pro	Ala	Asp	Leu	Ala

Leu	Phe	Lys	Ala	Met	Thr	Tyr	Asn	Gln	Trp	Leu	Leu	Val	Gly	Arg
Leu	Phe	Lys	Ala	Ile	Thr	Tyr	Asn	Gln	Trp	Leu	Leu	Val	Gly	Arg
Leu	Phe	Lys	Ala	Leu	Thr	Tyr	Asn	Gln	Trp	Leu	Leu	Val	Gly	Arg
Trp	Phe	Lys	Arg	Asn	Thr	Leu	Asn	Lys	Pro	Val	Ile	Met	Gly	Arg

Lys	Thr	Phe	Asp	Ser	Met	Gly	—	Lys	Leu	Pro	Asn	Arg	Lys	Tyr
Lys	Thr	Phe	Glu	Ser	Met	Gly	—	Ala	Leu	Pro	Asn	Arg	Lys	Tyr
Lys	Thr	Phe	Glu	Ser	Met	Gly	—	Ala	Leu	Pro	Asn	Arg	Lys	Tyr
His	Thr	Trp	Glu	Ser	Ile	Gly	Arg	Pro	Leu	Pro	Gly	Arg	Lys	Asn

Ala	Val	Val	Thr	Arg	Ser	Lys	Ile	Ile	Ser	Asn	Asp	Pro	Asp	Val
Ala	Val	Val	Thr	Arg	Ser	Ser	Phe	Thr	Ser	Asp	Asn	Glu	Asn	Val
Ala	Val	Val	Thr	Arg	Ser	Ala	Trp	Thr	Ala	Asp	Asn	Asp	Asn	Val
Ile	Ile	Leu	—	—	Ser	Ser	Gln	Pro	Gly	Thr	Asp	Asp	Arg	Val

Val	Tyr	Phe	Ala	Ser	Val	Glu	Ser	Ala	Leu	Ala	Tyr	Leu	Asn	Asn
Leu	Ile	Phe	Pro	Ser	Ile	Lys	Asp	Ala	Leu	Thr	Asn	Leu	Lys	Lys
Ile	Val	Phe	Pro	Ser	Ile	Glu	Glu	Ala	Met	Thr	Gly	Leu	Ala	Glu
Thr	Trp	Val	Lys	Ser	Val	Asp	Glu	Ala	Ile	Ala	Ala	Cys	Gly	Asp

Ala	Thr	Ala	His	Ile	Phe	Val	Ser	Gly	Gly	Gly	Glu	Ile	Tyr	Lys
Ile	Thr	Asp	His	Val	Ile	Val	Ser	Gly	Gly	Gly	Glu	Ile	Tyr	Lys
Leu	Thr	Asp	His	Val	Ile	Val	Ser	Gly	Gly	Gly	Glu	Ile	Tyr	Arg
Val	Pro	Glu	Ile	Met	—	Val	Ile	Gly	Gly	Gly	Arg	Val	Tyr	Glu

Ala	Leu	Ile	Asp	Glu	Ala	Asp	Val	Ile	His	Leu	Ser	Val	Ile	His
Ser	Leu	Ile	Asp	Glu	Val	Asp	Thr	Leu	His	Ile	Ser	Thr	Ile	Asp
Glu	Thr	Leu	Pro	Met	Ala	Ser	Thr	Leu	His	Ile	Ser	Thr	Ile	Asp
Gln	Phe	Leu	Pro	Lys	Ala	Gln	Lys	Leu	Tyr	Leu	Thr	His	Ile	Asp

Lys	His	Ile	Ser	Gly	Asp	Val	Phe	Phe	Pro	Pro	Val	—	Pro	Gln
Ile	Gln	Pro	Glu	Gly	Asp	Val	Tyr	Phe	Pro	Glu	Ile	—	Pro	Ser
Ile	Gln	Pro	Glu	Gly	Asp	Val	Phe	Phe	Pro	Asn	Ile	—	Pro	Asn
Ala	Glu	Val	Glu	Gly	Asp	Thr	His	Phe	Pro	Asp	Tyr	Gly	Pro	Asp

Gly	Phe	Lys	Gln	Thr	Phe	—	Glu	Gln	Ser	Phe	Ser	Ser	Asn	Ile
Asn	Phe	Arg	Pro	Val	Phe	—	Thr	Gln	Asp	Phe	Ala	Ser	Asn	Ile
Thr	Phe	Glu	Val	Val	Phe	—	Glu	Gln	His	Phe	Ser	Ser	Asn	Ile
Asp	Trp	Glu	Ser	Val	Phe	Ser	Glu	Pro	His	Asp	Ala	Asp	Ala	Gln

Asp	—	—	—	Tyr	Thr	Tyr	Gln	Ile	Trp	Ala	Lys	Gly	Stop
Asn	—	—	—	Tyr	Ser	Tyr	Gln	Ile	Trp	Gln	Lys	Gly	Stop
Asn	—	—	—	Tyr	Cys	Tyr	Gln	Ile	Trp	Gln	Lys	Gly	Stop
Asn	Ser	His	Ser	Tyr	Cys	Phe	Glu	Ile	Leu	Glu	Arg	Arg	Stop

Fig. 3. Alignment of the amino-acid sequences of the type I, V, VI and *E. coli* K12 dihydrofolate reductases. The numbering is according to the *E. coli* DHFR. Conserved residues are enclosed.

DHFR of *E. coli* K12 and the type VI DHFR was only 31%.

The residues known to be involved in folate binding are 5, 6, 7, 15, 20, 22, 28, 30, 31 and 94.²⁰ The resistant DHFRs have residues identical to those of *E. coli* K12 DHFR at positions 5, 7, 22, 31 and 94. The remaining five amino acids differ from those of the *E. coli* K12 enzyme, but the substituted residues (Met-6, Ile-20, Glu-27, Gln-28 and Leu-30) are identical in each of the resistant DHFRs. Similarly, residues associated with methotrexate binding in *E. coli* are: Ile-5, Ala-7, Asp-27, Leu-28, Phe-31, Ser-49, Ile-50, Leu-54, Arg-57, Ile-94 and Thr-113.^{19, 21} Only five of these 11 residues were conserved in the resistant DHFRs. These substitutions may explain why the binding affinities of the resistant DHFRs and the chromosomal enzyme differ.

Although the similarity in gene length, DNA and amino-acid homology between type I, type V and type VI DHFRs suggests common ancestry, the

biochemical properties of these enzymes were distinct. The type V and type VI DHFRs do, however, have certain physical characteristics in common. By Sephadex column chromatography, the mol. wt of the type V DHFR was estimated to be 5000,⁸ whereas the gene sequence data showed the mol. wt of the polypeptide to be 17 531.⁵ Similarly, the mol. wt of the type VI DHFR was estimated to be 10 000⁹ whereas the gene determined a polypeptide of over 17 000. The reason for this behaviour is unknown, but it could be due to the hydrophobicity of the enzyme molecules.²² The mol. wt of the type V DHFR was subsequently reported to be 35 000 by native polyacrylamide gel electrophoresis (PAGE)²³ indicating that, like the type I DHFRs, the type V enzyme functions as a dimer. The mol. wt of the type VI DHFR, as assessed by SDS-PAGE, was 17 500. It has not yet been determined whether it functions as a dimer, but the similarities between these three enzymes support this possibility.

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