N-terminal amino acid sequence of the novel type IIIb trimethoprim-resistant plasmid-encoded dihydrofolate reductase from *Shigella sonnei*

C. J. THOMSON,¹ N. BARG² and S. G. B. AMYES^{1*}

¹Department of Bacteriology, The Medical School, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, UK

²Vanderbilt University Medical School, Nashville, Tennessee, USA

(Received 4 October 1989; revised 7 December 1989; accepted 5 January 1990)

The type IIIb dihydrofolate reductase, a novel plasmid-encoded enzyme recently identified in *Shigella sonnei*, has been shown to have some similar biochemical properties to the type IIIa dihydrofolate reductase which was first identified in New Zealand in 1979. However, the type IIIb enzyme has a K_i for trimethoprim of 0.4 μ M, and a pI of 5.35 (as compared to 19 nM and 6.1 for the type IIIa); both these results suggest that it is a different enzyme from the prototype type IIIa. The type IIIb dihydrofolate reductase was purified by methotrexate agarose affinity chromatography, yielding a pure protein as determined by HPLC. Automatic amino acid analysis of the purified enzyme showed it to be distinct from all other known plasmid-encoded dihydrofolate reductases and quite different from the type IIIa enzyme. The purified enzyme was examined by SDS-PAGE, which revealed that the type IIIb dihydrofolate reductase was a monomeric protein of M_r 17200.

Introduction

Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] prevents the bacterial NADPH-dependent reduction of dihydrofolate to tetrahydrofolate by competitively inhibiting the enzyme dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP⁺ oxidoreductase, EC 1.5.1.3) (Burchall & Hitchings, 1965). A number of mechanisms of resistance to trimethoprim have been reported, the most important of these being the plasmid-encoded production of an additional trimethoprim-resistant dihydrofolate reductase enabling the trimethoprim blockade of the chromosomal enzyme to be bypassed (Amyes & Smith, 1974). The trimethoprim-resistant dihydrofolate reductases found in Gram-negative bacteria have been divided into a number of classes based principally on their biochemical profiles (for reviews see Amyes, 1989; Huovinen, 1987). In addition, as some of the genes responsible have also had their DNA nucleotides sequenced, the classification of these enzymes now also takes into account amino acid differences (Stone & Smith, 1979; Zolg & Hanggi, 1981; Fling & Richards, 1983; Flensburg & Steen, 1986; Fling *et al.*, 1988; Sundström *et al.*, 1988). Initially resistance

was dominated by the spread of two genes encoding dihydrofolate reductases types I and II. The type I was particularly common, probably because the gene responsible was located on a transposon, Tn7 (Barth *et al.*, 1976). However, more recently a number of new enzymes have been identified and now seven main groups have been distinguished in Gram-negative bacteria on the basis of their biochemical properties, DNA/DNA hybridization and amino acid sequences (for a review see Amyes, 1989).

Trimethoprim-sulphamethoxazole is widely used in the treatment of shigellosis (Salter, 1982). Until recently trimethoprim resistance in *Shigella* was relatively uncommon. However, increased use of trimethoprim in the treatment of shigellosis has resulted in a large increase in trimethoprim resistance. This now represents a major concern in many parts of the world. In Bangladesh the level of trimethoprim resistance rose from 5% in 1979 to 83% in 1983 (Zaman *et al.*, 1983; Shahid *et al.*, 1985) whilst in England and Wales the level of resistance rose from 1.3% to 17% in the same period (Gross *et al.*, 1984). Some studies on trimethoprim-resistant shigella have demonstrated the dominance of the type I dihydrofolate reductase whilst others have shown a significant proportion of strains which do not react with probes for genes encoding for either of the commonly occurring type I and type II enzymes (Chatkaeomrakot *et al.*, 1987). Recently, two novel plasmid-encoded dihydrofolate reduc-

The amino acid sequence reported in this paper has been registered in the protein database of the National Biomedical Research Foundation (NBRF), Washington, DC, USA, and has been assigned the accession number A33005.

tases have been identified from epidemic strains of trimethoprim-resistant *Shigella sonnei* isolated in the USA (Barg *et al.*, 1989). These novel dihydrofolate reductases were identified both by their inability to hybridize with gene probes for dihydrofolate reductases I, II and III and by their biochemical properties. However, in many respects, they appeared biochemically most similar to the type III dihydrofolate reductase. One in particular was very similar; it conferred only a moderate level of trimethoprim-resistance (MIC 128 mg l⁻¹) and its ID₅₀ of trimethoprim was 2 µM, which is identical to the value for the type III dihydrofolate reductase (Joyner *et al.*, 1984). The original type III enzyme has been shown to be a monomeric protein of M_r 16900 and the novel enzyme was similarly small. On this basis this enzyme was named the IIIb and the original type III dihydrofolate reductase the type IIIa (Barg *et al.*, 1989). However, the IIIb enzyme did differ in some biochemical properties, most notably in its isoelectric point, from the type IIIa.

To investigate the relationship between these enzymes it was essential to examine the amino acid sequence of this new dihydrofolate reductase. Here we report the use of methotrexate agarose as an affinity column matrix to purify the IIIb dihydrofolate reductase, enabling its N-amino terminal amino acid sequence and subunit structure to be determined.

Methods

Bacterial strains and plasmids. The type IIIb dihydrofolate reductase was prepared for sequence and subunit analysis from *Escherichia coli* C600(pBH600) (Barg *et al.*, 1989).

Enzyme preparation for sequence analysis. Dihydrofolate reductase was prepared from 10 litre overnight cultures of *E. coli* in Oxoid Isosensitest Broth, grown at 37 °C and shaken vigorously (Young & Amyes, 1986). Bacteria were harvested by centrifugation at 6000 g for 15 min and resuspended in buffer A (50 mM-sodium phosphate buffer pH 7.4, containing 10 mM-2-mercaptoethanol and 1 mM-EDTA). The bacteria were disrupted by sonication (2 × 30s, 8 µM, MSE Soniprep) and the lysate cleared by centrifugation at 40000 g for 1 h at 4 °C. Dihydrofolate reductase activity was assayed at 37 °C in 40 mM-sodium phosphate buffer pH 6 (Amyes & Smith, 1974); the units of activity were nmol dihydrofolate reduced min⁻¹.

Enzyme preparation for biochemical analysis. Enzyme preparation, biochemical analysis and iso-electric focusing were done as described by Amyes *et al.* (1989).

Enzyme purification and sequence analysis. Dihydrofolate reductase activity was precipitated from the crude preparation by the addition of ammonium sulphate from 50 to 80% saturation. The enzyme was resuspended in buffer and separated on a Sephadex G75 gel filtration column (2 cm² × 90 cm). Fractions showing peak enzyme activity were pooled and applied to a methotrexate-agarose column (0.375 cm² × 20 cm) made up according to the manufacturer's instructions (Sigma). This column was washed with buffer A until the A_{280} was less than 0.01 and all the unbound protein had been removed. The dihydrofolate reductase was then eluted with 2 ml 0.5 M-K₂HPO₄ containing 4 µmol dihydrofolate and washed with 75 ml 0.1 M-K₂HPO₄

containing 1 µM-dihydrofolate (Kaufman, 1974). Fractions of 5 ml were collected. The three eluted fractions showing peak activity were pooled and concentrated with Amicon Centriprep and Centricon concentrators to 30 µl. Prior to sequence analysis the preparation was checked for purity by reverse-phase HPLC analysis. The only protein peak was selected from the reverse-phase HPLC and analysed on an Applied Biosystems 477A protein sequencer (Hayes *et al.*, 1989).

Gel electrophoresis. SDS-PAGE was done on a Pharmacia Phast system according to the manufacturer's instructions. A Sigma molecular weight kit (14000–70000) was used to calibrate the gel.

Results

Biochemical properties of the type IIIb dihydrofolate reductase

The plasmid-borne dihydrofolate reductase gene from the *Shigella sonnei* strain responsible for an outbreak of dysentery at a nursing home in East Tennessee in 1985 was transferred to *E. coli* C600(pBH600). The dihydrofolate reductase from this transconjugant was then examined in detail.

The M_r of the dihydrofolate reductase produced by the transconjugant was 17000 when measured by Sephadex gel filtration. When the measurement was repeated in the presence of 4 µM-trimethoprim to remove any chromosomal dihydrofolate reductase the position of the peak was unchanged. The enzyme was sensitive to the presence of trimethoprim, requiring 2 µM of the drug to give 50% reduction in activity. It was also sensitive to methotrexate, with 0.02 µM-methotrexate reducing the enzyme's activity by half. The enzyme was, however, heat stable, maintaining more than 50% of its activity after 12 min at 45 °C.

The activity of the enzyme was measured at limiting substrate concentrations in the presence and absence of trimethoprim. Analysis by the method of Lineweaver and Burk showed that the type IIIb dihydrofolate reductase had a K_m for dihydrofolate of 9.5 µM and that it was competitively inhibited by trimethoprim with a K_i of 0.4 µM.

Biochemically the enzyme had several properties which were very similar to the type IIIa dihydrofolate reductase isolated in New Zealand in 1979. However, iso-electric focusing of the type IIIb enzyme showed that it focused clearly with a pI of 5.34 which was distinct from that of the New Zealand type IIIa dihydrofolate reductase (Table 1).

Enzyme purification

The activity of the enzyme preparation was measured throughout the purification procedure. Prior to gel filtration the resuspended pellet from ammonium sul-

Fig. 1. Elution profile of pBH600-encoded enzyme during Sephadex G-75 gel filtration. ●, Dihydrofolate reductase (DHFR) activity obtained after Sephadex exclusion chromatography of a 50–80% ammonium sulphate precipitate from *E. coli* C600(pBH600). The positions of the elution peaks of the standard marker proteins are indicated by arrows.

phate precipitation contained 78124 units of dihydrofolate reductase activity. Separation on Sephadex gel filtration gave a clearly defined peak of enzyme activity (Fig. 1). Fractions 62–72 were pooled; they contained a total of 28874 units. The pooled fractions were run onto the methotrexate column. No dihydrofolate reductase activity was detected in the protein which initially passed through the column, indicating that the enzyme had been completely bound to the methotrexate agarose. The column was washed continuously until the A_{280} of the eluate had returned to a value of less than 0.01, i.e. no further protein was passing through. The dihydrofolate reductase activity was eluted by the addition of 2 ml 0.5 M- K_2HPO_4 containing 4 μ mol dihydrofolate. Fractions of 5 ml were collected and assayed for enzyme activity. The three fractions showing peak activity were pooled and contained a total of 19933 units. This 15 ml sample was concentrated with Amicon Centriprep and Centricon concentrators to 30 μ l, which retained 16499 units. Before sequence analysis this sample was tested for purity by reverse-phase HPLC and this revealed the presence of a single protein peak.

Fig. 2. Determined N-terminal amino acid sequence (47 amino acids) of the type IIIb plasmid-encoded dihydrofolate reductase. Identical amino acids are shown by shading. Homologous amino acids, (D E) (R K H) (Q N) (F Y W) (I V L M), are underlined. The amino acids involved in the active site are shown by the symbols at the top of the diagram: ▲, binding positions of trimethoprim; △, additional binding positions of methotrexate; ■, binding positions of NADPH. Positions are taken from Rouch *et al.* (1989) and numbering is based on the type IIIb sequence.

Table 1. Comparison of the biochemical properties of the type IIIa and IIIb dihydrofolate reductases

Enzyme	Tp ID ₅₀ (μ M)	Mtx ID ₅₀ (μ M)	TD ₅₀ (min)	DHF K_m (μ M)	Tp K_i (μ M)	M_r	pI	Tp MIC (μ g ml ⁻¹)
IIIa	2.0	0.02	>12	0.4	0.019	16900	6.10	64
IIIb	2.0	0.02	>12	9.5	0.4	17000	5.34	128

Abbreviations: Tp, trimethoprim; Mtx, methotrexate; DHF, dihydrofolate.

Fig. 3. PAGE of purified dihydrofolate reductase encoded by plasmid pBH600. The bands were visualized by staining with Coomassie blue. A, dihydrofolate reductase from *E. coli* C600(pBH600); B, Sigma standard marker proteins (M_r 14000–70000).

Sequence analysis of the type IIIb dihydrofolate reductase

Fifteen microlitres of the purified sample was analysed on an Applied Biosystems 477A automatic sequencer. This gave a clear sequence for the first 47 amino acids (Fig. 2). The M_r of the enzyme suggests a protein of 162 amino acids, thus approximately 30% of the protein has been examined. The sequence obtained starts with a threonyl residue, indicating that the f-methionyl residue had been removed intracellularly. Comparing the sequence with that of the other known plasmid-encoded dihydrofolate reductases (types I–V and S1) showed this enzyme to be clearly distinct. Although the type IIIb enzyme is biochemically similar to the type IIIa enzyme isolated in New Zealand in 1979, its homology with this enzyme is not significantly greater than that with any of the other plasmid-encoded dihydrofolate reductases.

Subunit structure of the type IIIb dihydrofolate reductase

The subunit structure of the type IIIb dihydrofolate reductase was examined by running the purified enzyme on a Pharmacia Phast system SDS gel. The sample was run on a 10–15% Pharmacia Phast gradient gel according to the manufacturer's instructions. After running on the SDS gel the type IIIb enzyme produced a single band of protein when stained (Fig. 3); this corresponded to an M_r of 17200. As the M_r of the native type IIIb enzyme determined by Sephadex gel filtration is 17000, the enzyme is presumably a monomeric protein.

Discussion

The major groups of plasmid-encoded dihydrofolate reductases have, in the past, been distinguished from one another primarily by their biochemical profiles (Amyes, 1986). Recently, however, the number of different enzyme types has increased (for a review see Amyes, 1989). In some cases the different dihydrofolate reductases have quite similar biochemical properties (e.g. the types I and V) and so it has become necessary to look at the structure of these enzymes in more detail. This has been achieved by the examination of either the DNA nucleotide or amino acid sequences of the enzymes.

If determination of the enzyme's amino acid sequence is to become a crucial feature in the distinction of plasmid-encoded dihydrofolate reductases, an alternative to DNA nucleotide sequencing is required since it is too lengthy a process for the routine study of antibiotic resistance. Therefore a more rapid method is needed and we thus report the use of such an alternative, namely rapid automatic amino acid sequencing to give a partial sequence sufficient to determine evolutionary relationships amongst trimethoprim resistance genes. Two properties make dihydrofolate reductases suitable for rapid automatic amino acid sequencing. Firstly, most of the active site is situated at the N-terminal end of the protein so that a single run through a sequencer provides enough information to evaluate the relationships (Rouch *et al.*, 1989; Novak *et al.*, 1983). Secondly, in many cases the enzyme is easily purified with the use of methotrexate agarose, and a native protein, sufficient for sequencing, can be obtained in a few steps.

Biochemically, the type IIIb dihydrofolate reductase is more similar to the type IIIa enzyme isolated in New Zealand in 1979 than to any other plasmid-encoded dihydrofolate reductase. However, it does differ from the type IIIa enzyme in pI value and in Michaelis–Menten kinetics.

Comparison of the amino acid sequence for the first 47 amino acids with the sequence of the type IIIa plasmid-

encoded dihydrofolate reductase shows the type IIIb enzyme to be clearly distinct (Fig. 2). Only fifteen direct matches are found between the two enzymes; two further amino acids in the type IIIb enzyme were homologous to the amino acids at the same position in the type IIIa. The type IIIb enzyme showed slightly less direct matching with the *E. coli* chromosomal dihydrofolate reductase, with only 13 direct matches and three further homologous amino acids. This in contrast to the IIIa enzyme, which shows a high degree of homology with the *E. coli* K12 chromosomal dihydrofolate reductase. The type IIIb sequence was also compared with the sequences of the other plasmid-encoded dihydrofolate reductases types I, II, V and S1 (Fig. 2). The type IIIb enzyme was distinct from these, showing 10 direct matches with the type Ia and five, six and six direct matches with the types IIa, IIb and IIc respectively. The type IIIb showed 12 direct matches with the type V and 13 with the type S1. It showed 13 direct matches with the type IV enzyme (unpublished results). The sequence data show that although the IIIa and IIIb dihydrofolate reductases are biochemically similar these enzymes are not closely related in an evolutionary sense.

Like the type IIIa enzyme, the type IIIb dihydrofolate reductase is monomeric. It also has a similar M_r . Therefore, there does appear to be some evolutionary advantage in the selection of enzymes with this biochemical profile.

We would like to thank the Wellcome Trust for grant number 16376/1.5 which supported this work. We thank the WELMET Protein Characterisation Facility, University of Edinburgh, for the HPLC analysis and sequencing. WELMET is supported by the Wellcome Trust, Edinburgh and Heriot-Watt Universities and the Salvesens Trust. We also thank Dr Hilary-Kay Young of Dundee University for the use of her Phast system.

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