Purification and characterization of dihydrofolate reductase from *Mycobacterium phlei*

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The dihydrofolate reductase from *Mycobacterium phlei* was purified and characterized; it has an M_r of 15000 and a pI of 4.8. It is competitively inhibited by both methotrexate and trimethoprim, although the affinity is less than for other bacterial dihydrofolate reductases.

INTRODUCTION

Dihydrofolate reductase (EC 1.5.1.3) catalyses the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. Failure to reduce dihydrofolate causes depletion of active folate, with consequent cessation of DNA synthesis and, ultimately, cell death. Dihydrofolate reductase is of considerable pharmacological interest as the site of action of drugs such as trimethoprim and methotrexate (Hitchings & Burchall, 1965; Blakley, 1969).

Although dihydrofolate reductases from *Escherichia coli* and several other bacterial species have been studied in depth, nothing is known about this enzyme in mycobacteria. These organisms are important because of the continuing prevalence of mycobacterial diseases such as tuberculosis and leprosy, and because of the limited range of antibiotics currently available to treat these infections. As a first step, we have purified and characterized the dihydrofolate reductase of a saprophytic organism, *Mycobacterium phlei*.

EXPERIMENTAL

Materials

NAPH, dihydrofolic acid (dihydropteroyl-L-glutamic acid), trimethoprim, methotrexate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were obtained from Sigma Chemical Co. Sephadex G-75 and Blue Sepharose were obtained from Pharmacia. Other chemicals were high-purity preparations from commercial sources. The standard buffer used was 10 mM-potassium phosphate, pH 7.0, containing 1 mM-EDTA and 10 mM-2-mercaptoethanol. Protein concentrations were measured by using a Bio-Rad Laboratories protein assay kit.

Enzyme assays

Dihydrofolate reductase activity was determined at 30 °C by monitoring the decrease of NADPH and dihydrofolate at 340 nm in a Beckman model 24 spectrophotometer. For routine assays we used 140 μ M-NADPH and 70 μ M-dihydrofolate in the standard buffer; the reaction was started by the addition of dihydrofolate and the reaction velocity measured 60 s later. The activity was corrected for any dihydrofolate-independent NADPH oxidase activity. One unit of enzyme is defined as the amount that reduces 1 μ mol of dihydrofolate/min,

a molar absorption coefficient of 12300 $\text{M}^{-1} \cdot \text{cm}^{-1}$ being used for the conversion of dihydrofolate and NADPH into tetrahydrofolate and NADP⁺.

Enzyme preparation

M. phlei was grown with stirring in nutrient broth containing 0.02% Tween 80 at 37 °C for 36 h, in 30-litre batches. The cells were recovered by filtration, suspended in phosphate buffer and centrifuged at 10000 g for 10 min at 4 °C. The washed cells were resuspended in a minimum volume of standard buffer and broken by ultrasonication in an MSE ultrasonicator for 16 $\times$ 30 s. The sonicated suspension was clarified by centrifugation, and the enzyme was precipitated by (NH₄)₂SO₄ (30–80% saturation). After the precipitate had been redissolved in standard buffer, it was fractionated on a Sephadex G-75 column (54 cm $\times$ 2.5 cm) previously equilibrated with standard buffer. The active fractions were pooled, and bovine serum albumin (1 mg/ml) was added to stabilize the enzyme. The solution was applied to a Blue Sepharose column (20 cm $\times$ 1.5 cm), which was then washed with the standard buffer until the A_{280} of the effluent was less than 0.02. The enzyme was eluted with 1 mM-dihydrofolate in the same buffer. Although no attempt was made to remove the dihydrofolate, all enzyme preparations were routinely tested for the presence of NADPH oxidase activity in the absence of added dihydrofolate; the purified enzyme showed no such activity.

Inhibition studies

The standard assay conditions were used with various concentrations of either trimethoprim or methotrexate. The assay mixture was normally left to stand for 5 min before the reaction was started by adding dihydrofolate; however, there was no indication that this period of preincubation had any significant effect.

Enzyme characterization

The M_r of the purified enzyme was determined by SDS/polyacrylamide-gel electrophoresis (Laemmli & Favre, 1973). Isoelectric focusing was carried out in an LKB Multiphor apparatus fitted with a cooling system. Pre-prepared Ampholine polyacrylamide-gel plates (pH range 3.5–9.5) were purchased from LKB. Bands of dihydrofolate reductase activity were located on the gel by using the zymographic stain described by Tennhammar-Ekman & Skold (1979) and Broad & Smith (1982). The pH-dependence of enzyme activity was determined by using 0.1 M-sodium citrate, 0.1 M-potassium phosphate

Table 1. Purification of dihydrofolate reductase from *M. phlei*

Dihydrofolate reductase was purified from a 30-litre culture as described in the Experimental section. It was not possible to determine the activity of the enzyme in the crude lysate because of the presence of non-specific reacting material.

Step	Volume (ml)	Total enzyme (units)	Total protein (mg)	Specific activity (units/mg)
Crude lysate	56	—	1232	—
(NH ₄) ₂ SO ₄ (30–80% satn.)	10	7.0	550	0.013
Sephadex G-75	24	8.16	57.6	0.14
Blue Sepharose	7.5	7.5	0.15	50

Fig. 1. pH-dependence of the activity of *M. phlei* dihydrofolate reductase

The enzyme activity was measured by the standard assay described in the Experimental section. The buffers used were 0.1 M-sodium citrate (▲), 0.1 M-potassium phosphate (●) and 25 mM-sodium borate (○).

and 25 mM-sodium borate buffers. Tris/HCl and sodium acetate buffers were found to cause an unacceptably high rate of enzyme-independent decline of absorbance.

RESULTS AND DISCUSSION

The results of a typical purification are shown in Table 1. No value is quoted for the enzyme activity in the crude lysate since the presence of high levels of NADPH oxidase activity makes it impossible to assay the enzyme. This contaminating activity sometimes persisted through the (NH₄)₂SO₄ fractionation as well. A similar problem was encountered by Amyes & Smith (1976) in the purification of a plasmid-mediated dihydrofolate reductase from *E. coli*. A further problem encountered was instability of the partially purified enzyme, after elution from Sephadex. The addition of glycerol or NADPH, reported previously to stabilize the enzyme from the *E. coli* plasmid R483 (Novak *et al.*, 1983), had no effect on the *M. phlei* dihydrofolate reductase. However, it was found that the use of bovine serum albumin (1 mg/ml) was effective in stabilizing the enzyme, since no loss of activity was seen after 3 weeks at –20 °C or after 3.5 h at 45 °C.

A key step in the purification is the use of Blue Sepharose, which will bind many enzymes that use

nucleotide cofactors; in the case of dihydrofolate reductase, specific elution from the column is obtained by the use of dihydrofolate as the eluting agent. This procedure resulted in a very high degree of purification with good recovery of enzyme, although only small amounts of protein could be obtained from each batch. The method is likely to be applicable to dihydrofolate reductases from other mycobacteria (or other sources) with only minor modifications; we have used the same procedure successfully with *Mycobacterium smegmatis* (results not shown).

When the purified enzyme was subjected to SDS/polyacrylamide-gel electrophoresis, a single protein band was observed, corresponding to an *M_r* of 15000; this is less than the values of 21000 and 17900 observed for the dihydrofolate reductases from *E. coli* and *Lactobacillus casei* respectively (Chambers & Dunlap, 1979; Dann *et al.*, 1976). Isoelectric focusing showed the *M. phlei* enzyme to have a pI of 4.8, compared with the two bands at 4.4 and 4.0 obtained with the enzyme from *E. coli* (Broad & Smith, 1982). In the absence of dihydrofolate, no bands were seen, indicating that no non-specific reactions had occurred. The pH-dependence of the activity of the enzyme (Fig. 1) is similar to that of the enzymes from *E. coli*, *Staphylococcus aureus* and *Proteus vulgaris* (Burchall & Hitchings, 1965), with a maximum at pH 7.0.

Apparent *K_m* values were obtained from double-reciprocal plots, giving values of 8.6 μM for dihydrofolate and 35 μM for NADPH. Ackermann–Potter plots (Ackermann & Potter, 1949) of the inhibition by trimethoprim or methotrexate gave straight lines passing through the origin (results not shown), indicating that the inhibition is reversible. Double-reciprocal plots also showed competitive inhibition. Dixon plots (Dixon & Webb, 1979) for both inhibitors are shown in Fig. 2; the lines were fitted by linear regression and the intercepts of the lines were determined algebraically. The mean *K_i* values derived from these intercepts were 0.9 μM for trimethoprim and 1.8 nM for methotrexate. These values are several orders of magnitude higher than those observed for the enzymes from *E. coli* (Baccanari & Joyner, 1981) and *Neisseria gonorrhoeae* (Baccanari & Tansik, 1984), indicating a relatively weak binding of these competitive inhibitors to the *M. phlei* enzyme. The binding was, however, more rapid than that observed with the *E. coli* enzyme by Baccanari & Tansik (1984): progress curves quickly attained steady state (less than 60 s for trimethoprim; 2–3 min for methotrexate) irrespective of whether the reaction was started with substrate or enzyme addition.

Fig. 2. Dixon plots of the inhibition of *M. phlei* dihydrofolate reductase by (a) trimethoprim and (b) methotrexate

The enzyme was preincubated for 5 min with NADPH and different concentrations of either trimethoprim or methotrexate. The reaction was started by the addition of dihydrofolate (FAH_2) at the concentrations shown on the Figure.

Further studies of other mycobacterial dihydrofolate reductases, especially that of the leprosy bacillus *Mycobacterium leprae*, will depend on the outcome of gene cloning work, and in particular on the identification of the gene in recombinant DNA libraries. The results reported here establish a baseline for such work and confirm that, although these enzymes are similar in many respects to that of *E. coli*, it should be possible to distinguish a cloned mycobacterial dihydrofolate reductase from the resident *E. coli* enzyme.

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