Biochem. J. (1989) 261, 265–268 (Printed in Great Britain)

High-level expression of human dihydropteridine reductase (EC 1.6.99.7), without *N*-terminal amino acid protection, in *Escherichia coli*

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The cDNA coding for human dihydropteridine reductase [Dahl, Hutchinson, McAdam, Wake, Morgan & Cotton (1987) Nucleic Acids Res. **15**, 1921–1936] was inserted downstream of tandem bacteriophage λ P_R and P_L promoters in *Escherichia coli* vector pCE30. Since pCE30 also expresses the λ *cI857*^{ts} gene, transcription may be controlled by variation of temperature. The recombinant plasmid in an *E. coli* K12 strain grown at 30 °C, then at 45 °C, directed the synthesis of dihydropteridine reductase to very high levels. The protein was soluble, at least as active as the authentic human enzyme, and lacked the *N*-terminal amino acid protection.

INTRODUCTION

Human dihydropteridine reductase (hDHPR) is a ubiquitous enzyme which recycles the quinonoid dihydrobiopterin cofactor for phenylalanine hydroxylase (EC 1.14.16.2) and tryptophan hydroxylase (EC 1.14.16.4) (Kaufman & Fisher, 1974; Armarego *et al.*, 1984). It may also be involved in recycling the same cofactor for other oxygenases, e.g. glyceryl etherase (Tietz *et al.*, 1964), and for electron transport (Rembold & Buff, 1972). The reductase is essential for normal mammalian development and function. Deficiency in this enzyme in humans leads to hyperphenylalaninaemia and neurological disturbances due to lack of cofactor (tetrahydrobiopterin) regeneration. It is ultimately lethal if untreated (Danks *et al.*, 1978). For a fuller understanding of the metabolic diseases with which this enzyme is associated, it is important to know as much as possible about its structure and function. Although considerable progress has already been made, the determination of the primary amino acid sequence has been hampered by the shortage of large amounts of the protein and the finding that the *N*-terminal amino acid residues of the two identical subunits are acylated (Firgaira *et al.*, 1981). Recourse to molecular genetics techniques has been more successful, and recently the reverse transcript of the hDHPR mRNA has been isolated, and the complete cDNA sequence has been determined (Dahl *et al.*, 1987; Lockyer *et al.*, 1987). The gene contains 732 bp which encode for 244 amino acid residues. The amino acid sequences deduced by these two groups differ only in residue 51, which is a serine (from an AGC codon) in one and a threonine (from an ACG codon) in the other. The difference could be due to a normal gene variation since the hydrophilicity of the amino acid residue at position 51 is conserved.

The availability of a plasmid containing the sequenced dDHPR cDNA has opened the way for the preparation of large amounts of the protein by expression of the gene, e.g. in a bacterium. The present communication describes the high-level synthesis of hDHPR, without the *N*-terminal amino acid protection, in *E. coli*.

MATERIALS AND METHODS

Restriction enzymes, T4 DNA ligase, horseradish peroxidase and DNA molecular mass marker III were from Boehringer-Mannheim; lysozyme and thymine were from Sigma, and ultrapure DNA grade agarose was from Bio-Rad, Richmond, CA, U.S.A. Luria Bertani (LB) culture broth and agar plates each containing thymine (50 mg/l) and ampicillin (50 μg/ml; Beecham Veterinary Products, Vic 3175, Australia) when required, were as described (Maniatis *et al.*, 1982). *E. coli* K12, strain AN1459 [F$^-$, *ilv*, *thr*, *leu*, *hsd*R, *rec*A, *srl*:: *Tn10*], provided by Professor F. Gibson (Australian National University) was used throughout. Details about the construction of plasmid vector pCE30 are available on request from Dr. N. E. Dixon, Research School of Chemistry, Australian National University. An M13-Bluescribe plasmid derivative containing the complete hDHPR cDNA (Dahl *et al.*, 1987) was used as source of the gene.

Restriction endonuclease digestions (in buffers recommended by the suppliers), electrophoresis of DNA in agarose gels containing ethidium bromide (0.5 μg/ml), ligation of DNA fragments with T4 DNA ligase and transformation of $CaCl_2$-treated AN1459 cells, were essentially as described (Maniatis *et al.*, 1982). Small-scale plasmid preparations for analytical purposes were carried out by a modification of an alkaline-SDS lysis procedure (Silhavy *et al.*, 1984). For larger scale preparations, cells were lysed with lysozyme and Triton X-100 (Katz *et al.*, 1973). Plasmid DNA was recovered from the

Abbreviations used: (h)DHPR, (human) dihydropteridine reductase; LB broth, Luria Bertani broth.

cleared lysate by centrifugation to equilibrium in CsCl gradients containing ethidium bromide (Maniatis *et al.*, 1982). Fragments of DNA were isolated from agarose gels by electroelution on to NA45 membranes (Schleicher and Schuell, Dassel, East Germany), from which they were recovered by elution at 70 °C into 1 M-NaCl/50 mM-arginine, and precipitation with ethanol. Waterbath temperatures were continuously monitored with a mercury thermometer and were within ±0.5 °C.

Insertion of the hDHPR gene into pCE30

A 1013 bp DNA fragment of the M13-Bluescribe plasmid bearing the hDHPR cDNA was isolated from a 2% agarose gel following digestion with restriction endonucleases *Bam*HI and *Hin*dII. A portion (approx. 0.6 μg) was mixed with pCE30 (4.0 kb) that had been digested with *Bam*HI and *Sma*I and similarly isolated (approx. 1 μg). The mixture (50 μl) was treated for 24 h at 12 °C with approx. 2 units of T4 DNA ligase, then used to transform strain AN1459. Transformants were selected on LB plates containing ampicillin. They were screened for the presence of a plasmid of ~ 5 kb by small-scale plasmid preparations, and for enhanced DHPR activity following treatment at elevated temperature. One transformant (WLA8) containing plasmid pWLA8 (Fig. 1) was retained for further study.

Expression of hDHPR in *E. coli*

Cultures (30 or 60 ml) of WLA8, AN1459 and AN1459 containing pCE30 were grown in LB broth (with ampicillin when required) at 30 °C to mid-log phase (A_{595} ~ 0.5), then at 45 °C (16–20 h). The cells were harvested by centrifugation at 4350 *g* (6000 rev./min in a Sorvall SS34 rotor for 20 min at 4 °C) and lysed in one of two ways. (1) A thoroughly mixed suspension of cells in ice-cold lysis buffer (900 μl; 50 mM-Tris/HCl, pH 7.4/1 mM-EDTA/0.1 M-KCl), lysozyme (100 μl; 10 mg/ml) and phenylmethanesulphonyl fluoride (2 μl of a 10 mg/ml solution in propan-2-ol as protease inhibitor) was kept at 0 °C for 30 min, dropped into liquid N_2 and then allowed to thaw at 4 °C (2–6 h, or overnight). The thick mixture was centrifuged at 356000 *g* (100000 rev./min with a Beckman TLA 100.2 rotor) for 30 min at 4 °C and the supernatant immediately collected (~ 1 ml). (2) The cells were thoroughly mixed in the above lysis buffer (1 ml) containing phenylmethanesulphonyl fluoride (2 μl), sonicated for 3 min in an ice bath and centrifuged, and lysate was collected as in (1). The clear supernatants were assayed for reductase activity (see below) and stored at −20 °C. The reductase activities of supernatants obtained by either method were identical, and were unaltered by repeated freezing and thawing or storage at −20 °C. The protein concentrations were determined using the micro Bio-Rad method (Bradford, 1976). SDS/gel electrophoresis was performed on minigels (PhastGel gradient 8–25, with SDS buffer strips) using a Phast-System apparatus (Pharmacia AB, Uppsala, Sweden) at 15 °C and stained with Coomassie Blue as directed by the manufacturers. To study the time-course of hDHPR production by WLA8 at 45 °C, incubations of separate 30 ml cultures were stopped at the required time intervals and treated as in (2) (Table 1). The AN1459 strains with and without pCE30 were always grown alongside WLA8 and analysed in the same manner.

Enzyme assay

hDHPR activity was determined essentially as before (Armarego *et al.*, 1983), i.e. by placing the stock solution [850 μl of: 8 ml M-Tris/HCl buffer, pH 7.4/8 ml of horseradish peroxidase (0.2 mg/ml)/0.125 ml of 12% hydrogen peroxide/52 ml of water] in two thermostatted cuvettes of a Varian 219 u.v. spectrophotometer set at 340 nm and 25 °C, followed by 2 mM-NADH (50 μl; 100 μM final solution) and 6-methyl-5,6,7,8-tetrahydropterin hydrochloride in 4 mM-HCl (100 μl, 100 μM final solution) in each cuvette. The mixtures were allowed to equilibrate and the reaction was started by injecting the clear cell-free supernatants (2 μl) into one cell. The rate of change of absorbance (with full pen deflection at 0.05 absorbance units) was scanned on the recorder. One unit of activity was taken as being the amount of enzyme that caused the oxidation of 1 μmol of NADH per min under the above conditions (ϵ_{340} for NADH is 6200 $M^{-1} \cdot cm^{-1}$).

RESULTS AND DISCUSSION

Clones from a human cDNA library, constructed from λgt11 and expressed in *E. coli* RY1090, had been selected from recombinant plaques which were screened with an affinity-purified antibody to hDHPR. The cDNA insert was subcloned into the *Eco*RI restriction endonuclease site of M13-Bluescribe to enable determination of the complete sequence of the gene (Dahl *et al.*, 1987). This construction places the whole of the hDHPR gene on a 1013 bp DNA fragment bounded by *Bam*HI (upstream of the gene in the vector polylinker region) and *Hin*dII

Table 1. Expression of dihydropteridine reductase in *E. coli* K12 (AN1459)

	AN1459 (plasmid-free)*	AN1459 (+pCE30)†	WLA8†					
Time at 45 °C (h)	14	20	0	0.5	1	3	7	14
Specific activity (units/mg)‡	0.1§	0.03	0.09	0.99	2.0	4.0	7.3	12
Ratio	3.3	1.0	3.0	33.0	66.6	133.3	243.3	400

* Grown at 30 °C to mid-log phase then at 45 °C in LB broth.
† As in * but grown in LB broth containing ampicillin (50 μg/ml).
‡ See the Materials and methods section.
§ Bacterial DHPR has a specific activity of 8.2 units/mg (Vasudevan *et al.*, 1988).

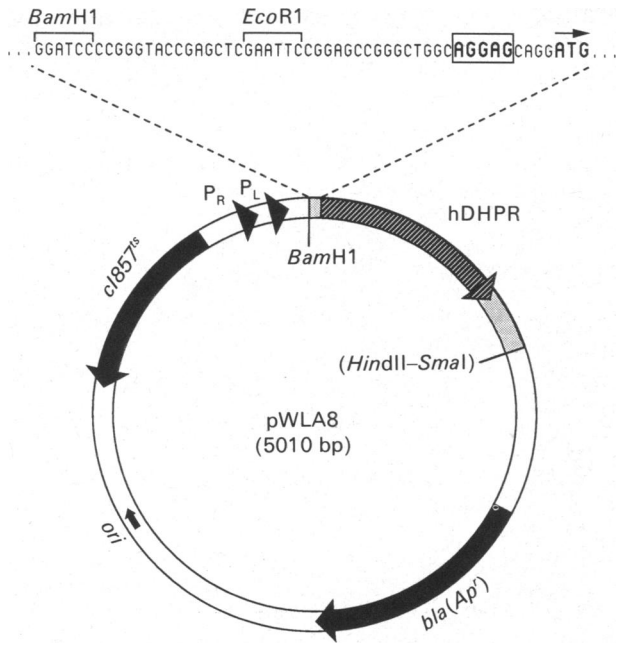

Fig. 1. Diagrammatic representation of plasmid pWLA8 that promotes high-level expression of dihydropteridine reductase (not to scale)

AGGAG is the putative eukaryotic ribosomal binding site.

(downstream of the gene in the cDNA insert) restriction sites. To express the hDHPR gene in *E. coli* we chose to use the plasmid pCE30 (see the Materials and methods section). This pBR322-derived vector (4002 bp) bears tandem bacteriophage lambda promoters P_R and P_L upstream of several unique restriction sites, the *cI857* gene encoding a thermolabile lambda repressor, and the *bla* gene that confers resistance to ampicillin. When cells containing a pCE30 derivative grown at 30 °C are transferred to a bath set at 42 °C, the repressor is immediately inactivated and transcription from the powerful promoters is enabled.

Insertion of the *Bam*HI–*Hin*dII fragment bearing the hDHPR gene between the *Bam*HI and *Sma*I sites of pCE30 ensured that the gene was inserted in one orientation, and in the correct sense for transcription from the lambda promoter. Separate introduction of a putative prokaryotic ribosome-binding site did not appear to be necessary, since inspection of the DNA sequence of the insert between the *Bam*HI site and the reductase open reading frame revealed the sequence 5′...AGGAG... four nucleotides upstream of the ATG initiaton codon (Fig. 1). Nine of the sixteen randomly selected ampicillin-resistant transformants of the engineered plasmid in *E. coli* strain AN1459 contained the 5 kb plasmid. Four strains were studied further, and behaved similarly. The work reported here was carried out with one of these (designated WLA8) which contained the required 5010 bp plasmid pWLA8 (Fig. 1).

WLA8 was grown in LB broth containing ampicillin at 30 °C to mid-log phase, then for 17 h at 42 °C. Total cell protein was analysed by SDS/polyacrylamide-gel electrophoresis. The gel showed clear production of a 27 kDa protein absent from WLA8 grown at 30 °C, which comprised approx. 10% of total cell protein (determined by densitometric scanning; results not shown). Nevertheless, it was found that the dihydropteridine reductase activity of the soluble extract per mg of total protein was only slightly elevated (3–5-fold) when compared with that of the AN1459 plasmid-free strain. We have previously isolated and characterized an *E. coli* protein which has dihydropteridine reductase activity (as well as a pterin-independent NADH oxidase activity) and shown that it was a dimeric flavoprotein with subunit M_r of 27000 (Vasudevan *et al.*, 1988). This accounts for the reductase activity of the plasmid-free strain. Further experimentation revealed that if WLA8 was grown at 30 °C to an absorbance of approx. 0.5 and then grown at 45 °C overnight, a considerable increase in soluble dihydropteridine reductase activity resulted. These growth conditions were repeated fifteen times in 30 ml broths and eight times in 60 ml broths, with equal and consistent success. A growth time-course study of WLA8 was made and the data, together with data from AN1459 containing pCE30 and from the plasmid-free strain are in Table 1. Maximum production of reductase activity was observed after 14 h or an overnight growth at the higher temperature, and the presence of pCE30 without the cDNA insert appeared to repress reductase activity in the AN1459 strain.

Derivatives of pCE30 have been used in one of our laboratories to achieve high-level overproduction of many *E. coli* proteins, in each case by heating mid-log phase cultures at 42 °C for from 1–4 h (W. L. F. Armarego, R. G. H. Cotton, H.-H. M. Dahl & N. E. Dixon, unpublished work). The implication of the unusual effect of temperature on overproduction of soluble hDHPR is that the polypeptide folds correctly to give sustained amounts of active, soluble enzyme at 45 °C, but not at 42 °C. The ratio of the specific activity of extracts of WLA8 grown for 14 h at 45 °C to that of the pure protein (800 units/mg, see below) indicates that approx. 1.5% of soluble protein is active hDHPR. Further detailed work is required to define this phenomenon more clearly, but it is tempting to speculate that temperature might carefully be raised in other systems, where foreign proteins expressed in *E. coli* are either insoluble or inactive. Although *E. coli* does not continue to grow and divide at 45 °C, the data in Table 1 suggest that its transcription and its protein synthetic machinery may continue to function for prolonged periods.

SDS/polyacrylamide-gel electrophoresis (Fig. 2) showed that the overexpressed protein has a subunit molecular mass similar to that of the human reductase. This was confirmed by its purification from the cell-free extract by affinity chromatography through a naphthoquinone–Sepharose column (c.f. Cotton & Jennings, 1978). The reductase activity was retained by the column and was released with 10 mM-dithiothreitol to give a protein that was more than 90% pure as judged by densitometric scans of Coomassie Blue-stained gels (results not shown). A small amount of reductase activity flowed through the affinity column and was most probably due to the *E. coli* enzyme, known not to bind to naphthoquinone–Sepharose columns (Vasudevan *et al.*, 1988). The purified protein had an M_r of ~ 26000 as the human enzyme, and an apparent specific activity of 800 units/mg. This compares with 224 units/mg for the authentic dihydropteridine reductase from human brain purified and measured in this work, and 207 units/mg

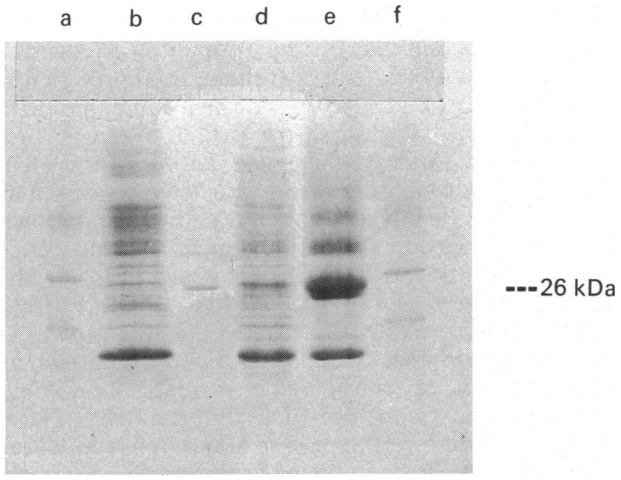

Fig. 2. PhastGel of cell extracts run with SDS buffer strips at 15 °C and stained with Coomassie Blue

Tracks a and f are of the low-molecular-mass standards (Pharmacia AB, Uppsala, Sweden), track b is of AN1459 containing plasmid pCE30, track c is of authentic human brain dihydropteridine reductase, track d is of AN1459 (plasmid-free) and track e is of WLA8.

reported previously (Armarego *et al.*, 1986). Final confirmation of the identity of the reductase was obtained by concentrating the naphthoquinone eluate and subjecting it to gas-phase sequencing (Applied Biosystems 477A). The first twenty residues starting at the *N*-terminus with Met-Ala-Ala-Ala-Ala-Ala-Ala-Gly-Glu... were clearly identified and compared exactly with the residues deduced from the cDNA that encodes the protein. Careful examination of the charts showed that none of the *E. coli* reductase, which has the *N*-terminal sequence Met-Asp-Ile-Ile-Cys-Val-Ala-Leu-Lys-Cys... (Vasudevan *et al.*, 1988) was contaminating the protein.

The expressed dihydropteridine reductase in WLA8 is the gene product of the cloned DNA encoding it, i.e. without post-translational modification. It differs from the authentic human reductase in that the *N*-terminal amino acid residue is not protected, but it is not yet known whether the human enzyme had undergone post-translational changes prior to *N*-terminal amino acid protection. The protein is clearly enzymically active without *N*-terminal protection; if anything it is more active.

Received 15 December 1988/14 March 1989; accepted 18 April 1989

We thank Dr. D. C. Shaw for the *N*-terminal protein sequencing, and Mrs. P. E. M. Lilley and Mr. Bela Paal for expert technical assistance.

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