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Dihydropteridine reductase from *Escherichia coli**

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A dihydropteridine reductase from *Escherichia coli* was purified to apparent homogeneity. It is a dimeric enzyme with identical subunits (M_r 27000) and a free *N*-terminal group. It can use NADH ($V_{max.}/K_m$ 3.36 s^{-1}) and NADPH ($V_{max.}/K_m$ 1.07 s^{-1}) when 6-methyldihydro-(6*H*)-pterin is the second substrate, as well as quinonoid dihydro-(6*H*)-biopterin ($V_{max.}/K_m$ 0.69 s^{-1}), dihydro-(6*H*)-neopterin ($V_{max.}/K_m$ 0.58 s^{-1}), dihydro-(6*H*)-monapterin 0.66 s^{-1}), 6-methyldihydro-(6*H*)-pterin and *cis*-6,7-dimethyldihydro-(6*H*)-pterin ($V_{max.}/K_m$ 0.66 s^{-1}) when NADH is the second substrate. The pure reductase has a yellow colour and contains bound FAD. The enzyme also has pterin-independent NADH and NADPH oxidoreductase activities when potassium ferricyanide is the electron acceptor.

INTRODUCTION

Dihydropteridine reductase (DHPR; EC 1.6.99.7) is widely distributed in the animal kingdom (Armarego *et al.*, 1984; Hasler & Niederweisser, 1986). It is found in all mammalian tissues that contain the aromatic amino acid hydroxylases and it recycles the quinonoid dihydrobiopterin cofactor by reducing it to 5,6,7,8-tetrahydrobiopterin, which is the natural substrate for these hydroxylases (Kaufman, 1962) (Scheme 1).

A deficiency in phenylalanine hydroxylase results in the inherited metabolic disease known as classical phenylketonuria (Tourian & Sidbury, 1983). Less common variants of this disease are known in which the biochemical defect is in the biosynthesis of the cofactor or the cofactor-recycling enzyme DHPR (Hasler & Niederwiesser, 1986; Kaufman, 1962, 1964; Tourian & Sidbury, 1983).

A similar reductase has also been isolated from a *Pseudomonas* species (A.T.C.C. 11299a) (Williams *et al.*, 1976). It was partially purified, and its enzymic properties were studied in detail, because this bacterium possessed phenylalanine hydroxylase and could be used as a model for the genetic disease. It was also present in some mammalian cells that do not contain any of the aromatic amino acid hydroxylases (Firgaira *et al.*, 1981*b*). Several reasons have been suggested for the presence of DHPR in these cells, including the recycling of the pterin cofactor for other mono-oxygenases, e.g. for glyceryl etherases (Snyder *et al.*, 1973), the involvement in mitochondrial electron transport (Rembold & Buff, 1972) and the reduction of quinonoid dihydrofolate to tetrahydrofolate (Chauvin *et al.*, 1979).

In looking for a bacterial model of the latter mammalian cells, which do not contain aromatic amino acid hydroxylases, we chose to study the wild-type of the bacterium *Escherichia coli* K12, because we knew that this bacterium did not contain phenylalanine hydroxylase and we were unable to detect any tyrosine hydroxylase or tryptophan hydroxylase activities in its cell-free extracts. We have, on the other hand, found DHPR activity in the cell-free extracts and have now isolated the enzyme and purified it to apparent homogeneity and report its properties.

MATERIALS AND METHODS

The bacterial strains used in the present study are derivatives of *E. coli* K12, strain H712 (F⁻, *guaB22 xyl-7 rpsL125*) and D3-157 (F⁻, as H712, *fol200 xyl*⁺) which were generously given by Dr. Sara Singer of Burroughs Wellcome Research Laboratories, Research Triangle Park, NC, U.S.A. (Singer *et al.*, 1985). The 5,6,7,8-tetrahydropterins used in the assays were prepared by standard procedures (Armarego & Schou, 1978).

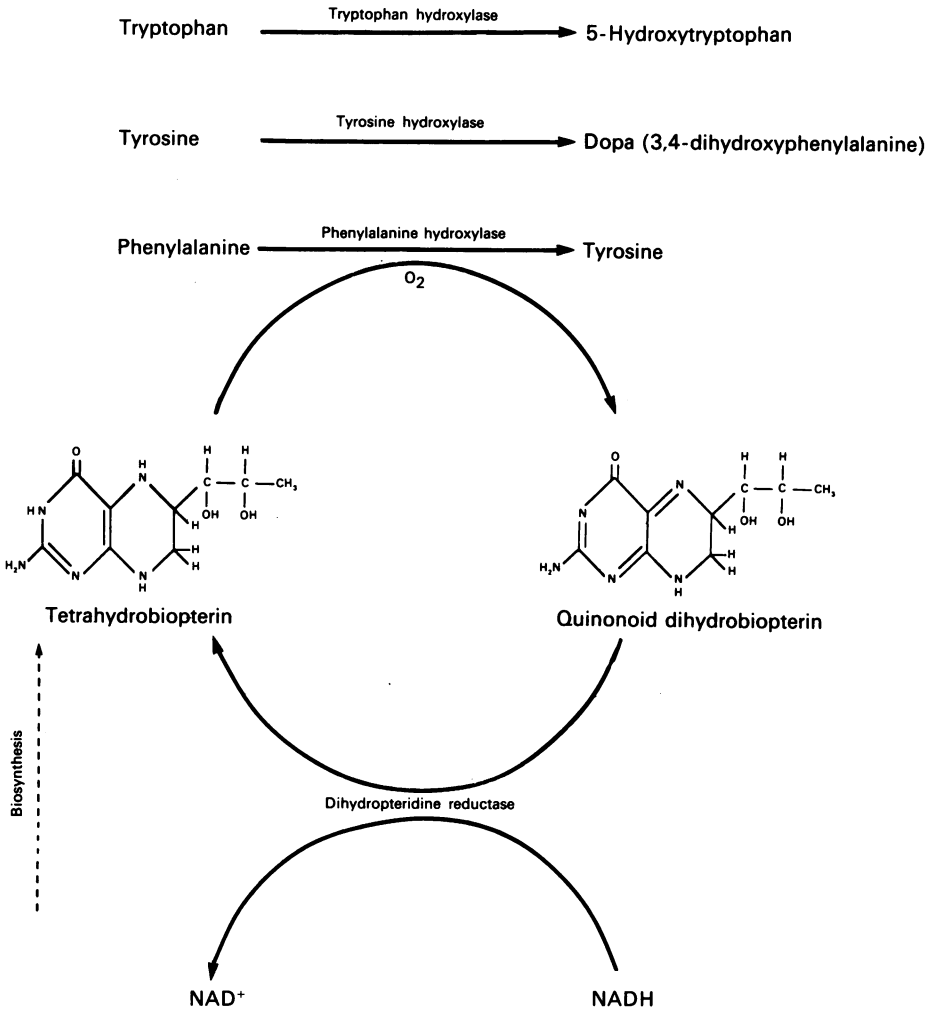
Growth of cells and preparation of cell-free extracts

Strains H712 and D3-157 were routinely cultured on LB (Luria–Bertani) liquid medium, pH 7.5, supplemented with 0.2% glucose and 50 μg of thymine/ml. Large quantities of strain H712 for isolation of the reductase were prepared by using the following procedure. Two 2-litre culture flasks, each containing 1 litre of LB liquid medium with necessary supplements were inoculated from single colonies on LB agar plates. The cultures were grown with aeration at 37° for 15 h in a gyratory environmental shaker. The cell suspension was used to inoculate a fermenter, which contained 38 litres of the appropriate growth medium. The culture was incubated at 37° with stirring. The cells were harvested at the end of the exponential phase of growth (~ 5 h) and sedimented in a Scharpel centrifuge.

The cells (130 g) were washed twice with 50 mM-Tris/HCl, pH 7.4, containing 1 mM-EDTA, 200 mM-KCl and 2 mM-DTT (Buffer A), suspended in the same buffer (260 ml) and portioned out (20 ml) into 60 Ti Beckman centrifuge tubes. Chicken egg-white lysozyme (Sigma; 20 mg/ml of Buffer A) was added to the suspension to a final concentration of 1 mg/ml and shaken vigorously.

Abbreviations used: DHPR, dihydropteridine reductase; LB, Luria–Bertani; DTT, dithiothreitol; f.p.l.c., fast protein liquid chromatography; PAGE, polyacrylamide-gel electrophoresis; PTH, phenylthiohydantoin; i.e.f., isoelectric focusing.

* This paper is dedicated to Professor Dr. Dr. Wolfgang Pfleiderer on the occasion of his 60th birthday and in gratitude for his valued advice, friendship and hospitality over many years.



Scheme 1. Role of DHPR in the hydroxylation of aromatic amino acids

The suspension was incubated at 0° for 30 min, frozen in liquid N_2 and thawed at 0° to produce a gelatinous suspension. This was centrifuged at 150000 *g* for 1 h to give the cell-free extract.

Enzyme purification

All operations were at 4 °C unless otherwise stated. The cell-free extract (300 ml) was fractionated with solid $(NH_4)_2SO_4$, and the 35–50%-satd.-$(NH_4)_2SO_4$ precipitate contained most of the reductase activity. It was collected by centrifugation (150000 *g*) and resuspended in 50 mM-Tris/HCl (pH 7.4, 80 ml; Buffer B). This was dialysed against buffer B (3 × 2 litres) overnight. The dialysis residue (100 ml) was applied to a DEAE-cellulose DE 52 column (2.0 cm × 15 cm; Whatman) which was pre-equilibrated with buffer B, and eluted with this buffer at a flow rate of 1 ml/min until the u.v. absorbance of the effluent at 280 nm was less than 0.1. A linear gradient between 250 ml of buffer B and 250 ml of buffer B containing 250 mM-NaCl was used, and the activity was eluted at about 100 mM-NaCl. Fractions of the eluent (8 ml) were collected in tubes containing DTT and NADH to give final concentrations of 2 mM and 20 μM respectively. The active fractions were pooled and concentrated to 8 ml in an Amicon ultrafiltration stirrer cell using a PM-10 membrane at a pN_2 of 310 kPa (45 lbf · in^{-2}). The concentrate was centrifuged (3000 *g*) to remove some precipitated protein, and the supernatant was stored in glass vials (1 ml aliquots) at −70 °C.

F.p.l.c.

Mono-Q anion-exchange chromatography. A sample (1 ml) of the above was thawed and dialysed against 50 mM-Bistris/HCl, pH 6.5, containing 2 mM-DTT and 0.02 mM-NADH (Buffer C, 2 × 500 ml) using a Spectrapore (Spectrum Medical Industries, Los Angeles, CA, U.S.A.) dialysis tube. The dialysis residue (1 ml) was applied to a Mono-Q HR 5/5 column (Pharmacia; a complete f.p.l.c. system being used). After a 5 ml wash with buffer C at 1 ml/min, a linear gradient of 0–200 mM-NaCl in the same buffer (30 ml) was used. The enzyme was eluted at 100 mM-NaCl, and the active fractions (3 ml) were stored at −70 °C.

Mono-P chromatography. The above active fraction (3 ml) was dialysed against 25 mM-piperazine/HCl (pH 6.3; 2 × 500 ml). The sample (1 ml) was applied to a Mono-P HR 5/20 column (Pharmacia), which was treated and pre-equilibrated as specified by the manufacturers. The eluent buffer was 10% (v/v) Polybuffer 74/HCl (Pharmacia, pH 4.5) and was used to form a pH gradient between 5.5 and 4.5. The reductase activity

was eluted at a pH of 5.30. The effluent was adjusted to pH 7.4 with buffer B, containing 2 mM-DTT and 20 μM-NADH, and dialysed against the same buffer (2 × 500 ml).

Electrophoretic methods

SDS/PAGE was performed essentially as outlined previously (Weber & Osborn, 1969) on 0.5 mm-thick 12% (w/v) polyacrylamide gels. Non-denaturing PAGE (Ornstein, 1964; Davis, 1964) and two-dimensional PAGE (O'Farrell, 1975) were carried out as described in the cited references. The gels were stained for protein with Coomassie Brilliant Blue G250 (0.25%) in methanol/acetic acid/water (50:9:41, by vol.), and destained in propan-2-ol/acetic acid/water (1:1:8, by vol.). Enzyme activity staining of the gels using MTT tetrazolium (Sigma) was as described by Cotton & Jennings (1978) (note that the free acid of dichlorophenol-indophenol should be used to generate the quinonoid dihydropterin substrate and not the sodium salt).

Protein determination

Protein concentration was determined by the method of Bradford (1976), using the Bio-Rad protein assay kit and bovine serum albumin as standard.

Enzyme-activity measurement

Assay (*a*). Solutions for DHPR activity contained Tris/HCl, pH 7.4 (0.1 M), peroxidase (11 μg, Boehringer), H_2O_2 (5 μM), 6-methyl-5,6,7,8-tetrahydropterin (0.1 mM in 4 mM-HCl) and NADH (or NADPH) (100 μM) in a total volume of 1 ml at 25 °C. The reaction was initiated by injecting the enzyme solution (5–10 μl, 0.25–0.5 μg) into one cuvette only. The initial rates were obtained from the rate of decrease of absorbance at 340 nm (ϵ_{340} for NADH or NADPH is 6200 $M^{-1} \cdot cm^{-1}$).

Assay (*b*). Solutions for the pterin-independent oxidoreductase activity contained Tris/HCl, pH 7.4 (0.1 M), $K_3Fe(CN)_6$ (0.30 mM) and NADH (or NADPH) (100 μM) in a total volume of 1 ml at 25 °C. The reaction was initiated by injecting the enzyme solution, and the initial rates were determined as above.

One unit of enzyme activity is defined as the amount of enzyme that causes the oxidation of 1 μmol of NADH/min under the above conditions. The measurements were performed on a Unicam SP.1800 double-beam spectrometer, and the results were analysed as outlined previously (Armarego *et al.*, 1983).

Analysis of naturally occurring pterins

Strain H172 (20 g) was suspended in 0.1 M-H_3PO_4 (40 ml) and smashed in a Sorvall Ribi cell smasher [13.8 MPa (2000 $lbf \cdot in^{-2}$)]. The cell-free extract was obtained by centrifugation (2000 *g*, 20 min) and concentrated (to 4 ml) by freeze-drying. The sample was oxidized in a mixture of trichloroacetic acid (1 ml, 2 M) and iodine (0.2 ml of a 0.1 M-HCl solution containing 9 mg of I_2 and 1.8 mg of KI/ml) for 1 h at 25 °C in subdued light. The suspension was centrifuged (2000 *g*, 10 min), and the supernatant was concentrated to 2 ml by freeze-drying. The sample was applied to Dowex 50W X4 (0.2 ml, 100–200 mesh) resin in a Pasteur pipette. After washing with water (5 ml) the pteridines were eluted with 0.25 M-NH_3 (6 ml) directly on to a Dowex 1 column (0.2 ml, 50–100 mesh) also in a Pasteur pipette. This was washed with water (3 ml, with suction), and the pterins were eluted with 1 M-acetic acid (0.6 ml). The effluent was analysed for pterins on an ODS column (0.46 cm × 25 cm) fitted to a Varian 5000 h.p.l.c. system with a Fluorichrome detector (cf. Fukushima & Nixon, 1980). Monapterin, biopterin, neopterin and pterin were used as standards for identification.

M_r determination

A Superose 12 analytical column with the f.p.l.c. system (Pharmacia) and Buffer B as eluent were used to determine the native M_r of the enzyme. The column was calibrated with a mixture (0.2 ml) of phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000) trypsin inhibitor (M_r 20100) and α-lactalbumin (M_r 14400) at a flow rate of 0.5 ml/min. The M_r of the native DHPR was estimated from the calibration plot. SDS/PAGE run concurrently with low-M_r standards (Pharmacia) was carried out to estimate the M_r of the denatured enzyme.

Identification of the prosthetic group

The purified reductase (400 μg) in Buffer B (1 ml) was chromatographed on a pre-equilibrated Sephadex G-25 (Superfine) column (0.9 cm × 10 cm) with 50 mM-Hepes buffer, pH 7.4. The active fraction (3 ml) was collected, denatured by addition of trifluoroacetic acid (90 μl), centrifuged (2000 *g*, 30 min), and the supernatant filtered through a Centricon YM 10 (Amicon; M_r cut-off 10000) by centrifugation (4000 *g*, 30 min). The u.v.-absorption spectrum of the filtrate (protein-free) was determined on a Cary 219 spectrometer, and the fluorescent spectrum was measured on a Perkin–Elmer 3000 fluorescence spectrometer with the excitation wavelength set at 450 nm. FAD solution (2.0 μM) was used for comparison. The solution from the spectroscopic measurements was freeze-dried, dissolved in 20 μl of water and a sample was run on a silica-gel 60 (Merck) plate together with authentic FAD, FMN and riboflavin. The R_F values of the isolated fluorescent cofactor, authentic FAD and authentic FMN relative to riboflavin were 0.16, 0.16 and 0.31 respectively (cf. Spitzer & Weiss, 1985).

Stoichiometry of NADH oxidoreductase activity

Assay (*b*) (see above) was used to investigate the stoichiometry. The enzyme (0.5 μg) was added to one of the cuvettes to start the reaction. The rate of decrease of absorbance at 340 nm (due to NADH oxidation) and the rate of decrease of absorbance at 420 nm (due to reduction of ferricyanide, ϵ 1050 $cm^{-1} \cdot M^{-1}$) were recorded on a Cary 219 double-beam spectrometer driven by an Apple II-PLUS computer using a multiscan kinetic program. The absorbance readings were taken at 22 s intervals, and the readings at 340 nm were taken 11 s after the readings at 420 nm. Data were collected for 15 min. The infinity absorbance values for the two wavelengths were calculated by using a program (Curfit) provided by Dr. David Randles (Department of Community Services and Health, Phillip, Canberra, A.C.T., Australia). The stoichiometry for the reaction was calculated from the total absorbance changes for NADH and $K_3Fe(CN)_6$, which were 1.00 and 1.96 respectively.

N-Terminal amino acid analyses

The purified reductase (0.4 ml, 50 μg) was dialysed against 0.5% NH_4HCO_3 (3 × 500 ml) and then with Millipore-MQ water (500 ml). One half of the dialysis residue was applied directly on to a Beckman model 890M2 spinning-cup sequencer, with automatic conversion. The amino acid PTH derivatives were analysed on a du Pont PTH column, using a Hewlett–Packard 1084B h.p.l.c. system with an Altex 165 variable-wavelength detector. The second half of the dialysis residue was made 70% in formic acid, treated with CNBr (200 μg) and analysed without separation of peptides. The reaction mixture was applied to the spinning cup and washed with ethyl acetate before the sequencing was commenced.

RESULTS AND DISCUSSION

Purification of the reductase

The *E. coli* DHPR, unlike the human liver (Cotton & Jennings, 1978) and the brain enzyme (Armarego & Waring, 1983), did not bind to the naphthoquinone affinity adsorbent, and we were unable to purify it in this way. The purification of the only other bacterial DHPR that had been reported (Williams *et al.*, 1976) was from a *Pseudomonas*, species, so we used that procedure to purify our reductase. The fractions that were eluted from the DE-52 column were assayed for reductase activity by using $K_3Fe(CN)_6$ as oxidant to generate the pterin cofactor, quinonoid 6-methyl-7,8-dihydro-(6*H*)-pterin. Two bands possessing NADH oxidoreductase activity were isolated. At first we thought that we had isolated isoenzymes, particularly since the band that was eluted at 100 mM-NaCl (band I in Fig. 1) had 50% of the reductase activity with NADPH as cofactor compared with that with NADH as cofactor. The second band (band II in Fig. 1) that was eluted at 200 mM-NaCl, on the other hand, did not oxidize NADPH. Upon further studies of the activities using peroxidase/H_2O_2 as oxidant to generate the pterin cofactor, only band I possessed pterin-dependent activity; band II was inactive. Clearly both bands had NADH oxidoreductase activity (with ferricyanide as electron acceptor), but only one band had DHPR activity. The gel-filtration-chromatography (> 24 h) procedure used for purifying the *Pseudomonas* reductase was not suitable for our enzyme, because ours was not very stable, even in the presence of 20 μM-NADH and 2 mM-DTT at 4 °C. Rapid gel filtration (60 min) at 15 °C using a Pharmacia f.p.l.c. system with a Superose 6 column followed by a Superose 12 column and final purification with Mono-Q anion-exchange chromatography gave a reductase-active (pterin-dependent and -independent) protein mixture that showed two bands of equal intensity on SDS/PAGE with M_r values of 18000 and 27000. Non-denaturing PAGE of this mixture gave one band when stained for protein, and this band also stained for DHPR activity. When the band corresponding to the protein and activity stain was cut out of the gel and extracted into buffer B, it was found to possess both pterin-dependent and pterin-independent activities.

At this stage it appeared that the protein could be a dimer with subunits of unequal M_r values and different NADH oxidase activities. However, further evidence that a single protein had both the pterin-dependent and -independent reductase activities came from looking at the ratios of activities during purification, which remain unchanged, and the percentage loss in the activities when assayed in the presence of methotrexate (see below). These results suggest that the active sites for the two activities are similar or close to each other.

When the above partially purified protein was subjected to non-denaturing i.e.f. and stained for protein, two bands were apparent close to the anode. The second dimension (SDS/PAGE) of the i.e.f. disc gel clearly showed that there were two different proteins (results not shown). The protein was finally purified to apparent

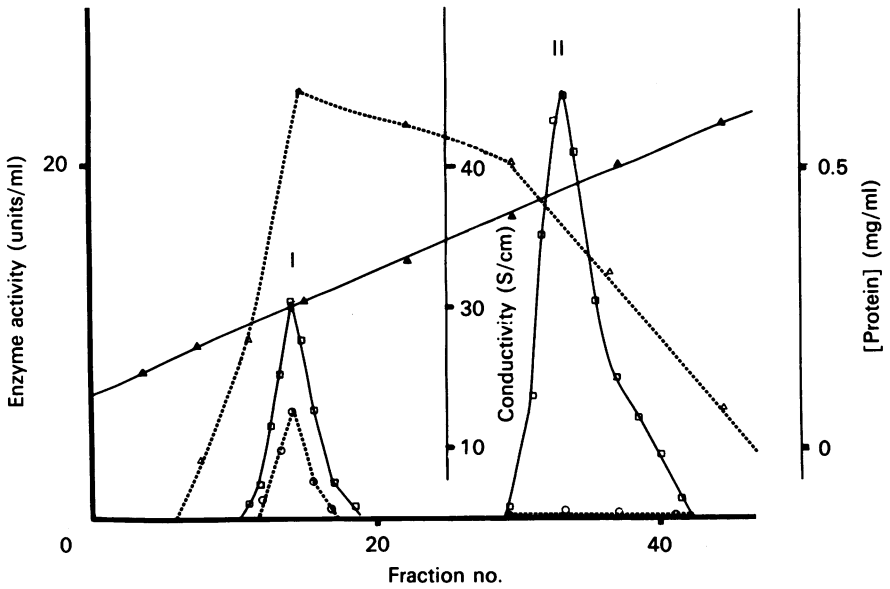


Fig. 1. DE-52 anion-exchange chromatography of *E. coli* dihydropteridine reductase

Key to symbols: ▲, conductivity; △, protein concentration; ○, dihydropteridine reductase activity [assay (*a*) in the Materials and methods section]; □, NADH oxidoreductase activity [assay (*b*) in the Materials and methods section].

Table 1. Purification of DHPR from *E. coli*

Purification step*	Volume (ml)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Cell-free extract	300	15800	0.04	1	—
$(NH_4)_2SO_4$ fraction	80	4300	0.08	2	55
DE-52 anion exchange	8	160	0.50	12	14
Mono-Q anion exchange†	6	5.2	8.64	216	8
Mono-P chromatofocusing† (pH 5.5–pH 4.5)	3	1.5	20.7	517	5

* The ratio of pterin-dependent and pterin-independent oxidation of NADH remained constant throughout the purification.
† Carried out on a Pharmacia f.p.l.c. system.

homogeneity by Mono-P chromatofocusing between pH 4.5 and 5.5, where two peaks appeared at pH 5.2 and 5.3. The fraction that was eluted at pH 5.2 exhibited no enzymic activity, whereas that eluted at pH 5.3 possessed both enzymic activities and appeared as a single band on SDS/PAGE (M_r 27000). With the knowledge that the protein can be purified to apparent homogeneity using chromatofocusing, we optimized the purification procedure in four steps (Table 1). The buffer exchange for the second anion-exchange column (Mono-Q) was crucial, because it gave a far better separation of the proteins. Since the pI of the contaminating protein (M_r 18000) is close to that of the reductase, we used a narrow pH range for chromatofocusing in the final step of purification. Samples taken at different stages of purification were analysed by SDS/PAGE (Fig. 2).

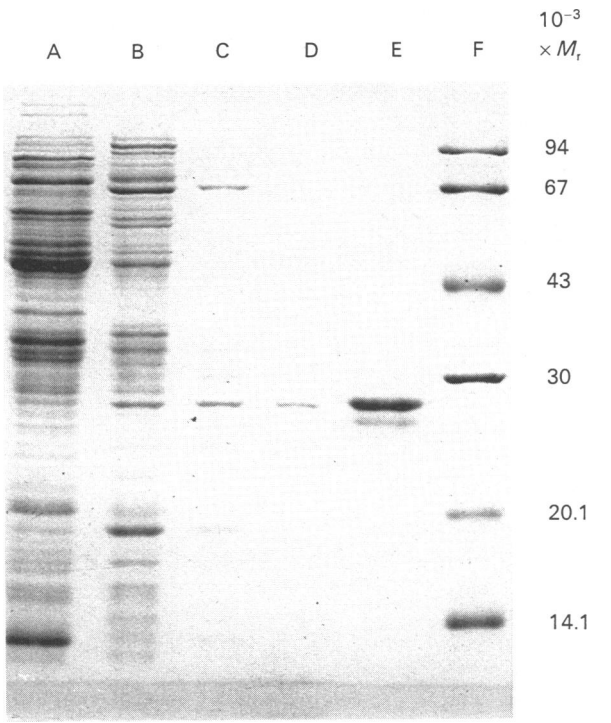


Fig. 2. SDS/PAGE of samples of DHPR taken at different stages of purification

A, cell-free extract; B, after DE-52 anion-exchange chromatography; C, after Mono-Q anion-exchange chromatography; D, after Mono-P chromatofocusing; E, pure human brain DHPR [the lower band is due to anomalous behaviour (Firgaira *et al.*, 1981*a*)]; F, Pharmacia low-M_r protein standards.

M_r of the reductase

The native protein has an M_r value of 54000 ± 2000 as determined by gel-filtration chromatography. This value is comparable with values obtained from reductases from mammalian sources (Armarego *et al.*, 1984). The *E. coli* reductase also consists of two identical subunits (see below) with an M_r value of 27000, as demonstrated by SDS/PAGE (Fig. 2). The M_r value reported for the *Pseudomonas* enzyme is 44000.

N-Terminal sequence of the reductase

The *N*-terminal amino acid of the *E. coli* reductase is not protected. This contrasts with the *N*-terminal residues of the ox liver, sheep liver (Chauvin *et al.*, 1979) and human liver and brain (Armarego *et al.*, 1984; W. L. F. Armarego & S. G. Vasudevan, unpublished work) enzymes, which are blocked by acylation. The reductase was subjected to amino-acid-sequencing analysis and a short sequence (Met-Asp-Ile-Ile-Cys-Val-Ala-Leu-Lys-Cys-Ile-Val-Xaa-Xaa-Ala-Phe-Asp-Ala-Met-Lys-) was readily obtained. There was no significant homology of its sequence with other enzymes in the National Biomedical Research Foundation Protein Data Bank. The possibility that the dimeric protein was made up of a subunit that had a free *N*-terminal amino acid and another subunit that had a protected *N*-terminal residue was discarded, because sequencing of the CNBr reaction mixture (without separation of polypeptides) gave recoveries of additional amino acids that were comparable with those obtained for the native protein; the number of extra sequences was consistent with the methionine content of the protein expressed as mol of methionine (5–6) per subunit of M_r ~27000. The data clearly indicated that the protein was essentially pure and that it was composed of two identical subunits with a free *N*-terminal amino acid.

pI

The pI of the *E. coli* reductase was found to be 5.3 by isoelectric focusing on a non-denaturing gel, and this value was confirmed by narrow-pH-range chromatofocusing. It compares favourably with values obtained for the sheep liver (5.4; Webber *et al.*, 1978) and the ox liver 5.7; Aksnes *et al.*, 1979). The values for the human

Table 2. Requirements of pterin-dependent and pterin-independent enzyme activities in Tris/HCl, pH 7.3, at 25 °C

Omission	NADH oxidized (μmol/min per ml of enzyme)	
	Pterin-dependent*	Pterin-independent†
None	4.58	5.16
Enzyme	0.42	0.38
6-Methyldihydro-(6*H*)-pterin	0.23	4.52
NADH	0	0
Peroxidase/H_2O_2	0.48	—
$K_3Fe(CN)_6$	—	0.45

* Assay (*a*) in the Materials and methods section.
† Assay (*b*) in the Materials and methods section.

liver (Firgaira *et al.*, 1981*a*) and the rat liver (Webber *et al.*, 1978) are 7.0 and 6.4 respectively.

Activity of substrates

The spectrophotometric assay for DHPR activity *in vitro*, as measured by the rate of NADH oxidation, is dependent on the rapid generation *in situ* of the substrate, quinonoid 6-methyl-7,8-dihydro-(6*H*)-pterin, from the corresponding 5,6,7,8-tetrahydropterin. $K_3Fe(CN)_6$ is a good electron acceptor and is also capable of similarly generating the quinonoid dihydropterin *in situ*. The apparently homogeneous protein that we isolated from *E. coli* exhibited NADH oxidoreductase activity when assayed with peroxidase/H_2O_2 in the presence of the tetrahydropterin and when assayed with $K_3Fe(CN)_6$ alone (i.e. in the absence of the pterin; see Table 2). Hence the apparently pure protein has two enzymic activities; its kinetic parameters for several pterins with NADH and NADPH are listed in Table 3.

The K_m values for several of the substrates in Table 3 are comparable with those reported for the *Pseudomonas* enzyme (Williams *et al.*, 1976). However, whereas the *Pseudomonas* enzyme was essentially inactive towards NADPH in the pterin-dependent reactions, the *E. coli* enzyme was active. With the latter enzyme, NADH was oxidized twice as rapidly as NADPH under identical assay conditions. The data in Table 3 show that, although the apparent $V_{max.}$ values for NADH and NADPH are similar, the K_m value is more than 3-fold lower for NADH. This may possibly indicate that NADH binds more tightly to the enzyme.

Careful analysis of the pteridines from *E. coli* extracts using h.p.l.c. methods (see the Materials and methods section), revealed the presence of mainly one pterin, namely monapterin. This pterin may possibly be the natural substrate for the pterin-dependent reductase of the *E. coli* enzyme. However, although monapterin is the main pterin, some pterin and traces of biopterin were detected by h.p.l.c. The K_m value for quinonoid dihydromonapterin is 1.3 mM (Table 3) and compares favourably with the value reported for the *Pseudomonas* enzyme (Guroff & Rhoads, 1969), where quinonoid dihydromonapterin had also been implicated as the probable natural substrate, because it was also the major pterin that was identified. The K_m values for quinonoid dihydrobiopterin with the reductase from both bacterial sources are lower (see Table 3).

Effect of methotrexate

Methotrexate, the potent inhibitor of dihydrofolate reductase used in cancer chemotherapy (Sather *et al.*, 1979; Carter *et al.*, 1981), also inhibits DHPR from mammalian sources, albeit less strongly (Armarego *et al.*, 1984). Methotrexate was shown to inhibit the reductase from *E. coli* (Fig. 3). Both the pterin-dependent and pterin-independent reductase activities are decreased (see Fig. 3). An apparent K_i of 0.26 mM at 25 °C was calculated from the rate data for the pterin-dependent-reductase using the Cornish–Bowden (Cornish-Bowden & Endrenyi, 1981) program. Double-reciprocal plots of the data (not shown) gave a pattern of lines consistent with competitive inhibition with respect to quinonoid 6-methyl-7,8-dihydro-(6*H*)-pterin, as was observed for the *Pseudomonas* enzyme (Williams *et al.*, 1976).

The prosthetic group

The concentrated *E. coli* reductase is yellow in colour. Several NADH oxidoreductases which can use $K_3Fe(CN)_6$ as an artificial electron acceptor are flavoproteins (Dixon & Webb, 1979) and have tightly bound flavin cofactors. We examined the pure reductase for the presence of flavins. The pure protein has u.v. absorbance maximum at 449 nm, with a shoulder at about 475 nm, which is indicative of FAD (Fig. 4). When NADH was

Table 3. Kinetic data for *E. coli* DHPR (Tris/HCl, pH 7.4, 25 °C)

Abbreviations used: q-BH_2, q-7,8-(6*H*)-dihydrobiopterin; q-6Me-DHP, q-6-methyl-7,8-(6*H*)-dihydropterin; q-6,7-Me_2-DHP, q-6,7-dimethyl-7,8-(6*H*)-dihydropterin. The second substrates were all at a concentration of 100 μM.

Substrate	Apparent K_m (μM)	Apparent $V_{max.}$*	$V_{max.}/K_m$ (s^{-1})	Second substrate
NADH	91 (90†)	5.1 (45†)	3.36	q-6Me-DHP
NADPH	290	5.2	1.07	q-6Me-DHP
q-BH_2	226 (160†)	2.6 (8.9†)	0.69	NADH
q-dihydroneopterin	1050	10.1	0.58	NADH
q-dihydromonapterin	1320 (1450†)	10.1 (−†)	0.66	NADH
q-6-Me-DHP	128	4.1	0.66	NADH
q-6,7-Me_2-DHP	525 (430†)	8.6 (21.6†)	0.66	NADH

* μmol of NADH (or NADPH) oxidized/min per 0.5 μg of protein.
† Data for *Pseudomonas* sp. DHPR (Williams *et al.*, 1976).

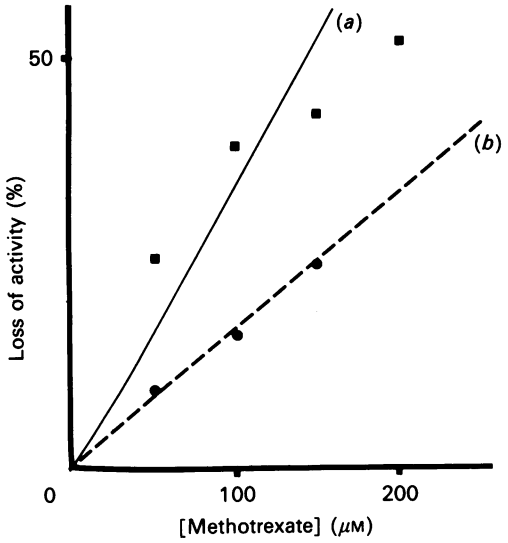


Fig. 3. Effect of methotrexate on *E. coli* DHPR

(*a*) Percentage loss of pterin-independent NADH oxidoreductase activity [assay (*b*) in the Materials and methods section]. (*b*) Percentage loss of DHPR activity [assay (*a*) in the Materials and methods section].

added to the enzyme the peak at 449 nm and the shoulder at 475 nm disappeared. After about 5–10 min in air the original spectrum of the protein was restored (Fig. 4). This suggests that the flavin in the resting enzyme is in the oxidized form. The prosthetic group was isolated (see the Materials and methods section), and an absorption spectrum which is typical of FAD was obtained (Dixon & Webb, 1979) (Fig. 5). The fluorescence emission spectrum of the isolated prosthetic group had a

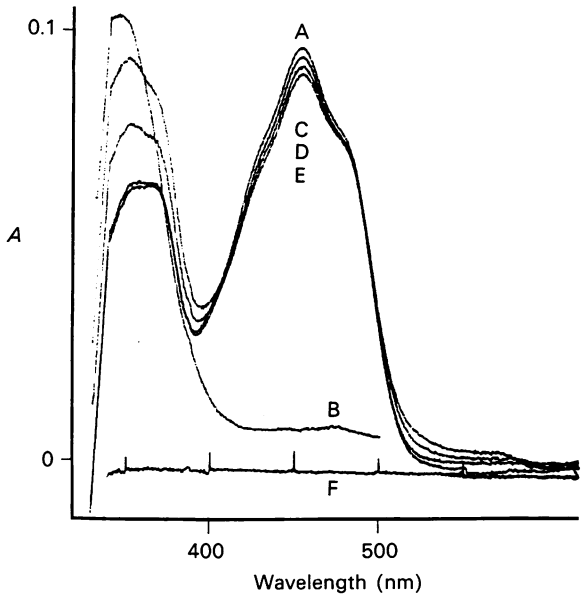


Fig. 4. U.v. absorption of purified *E. coli* DHPR

A, spectrum of protein (15 μM) in Hepes (50 mM) buffer at pH 7.4 and 25 °C; B, as in A, ~ 1 min after adding 100 μM-NADH; C, D and E, spectra after standing in air for 5–10 min after addition of NADH (100 μM); F, baseline.

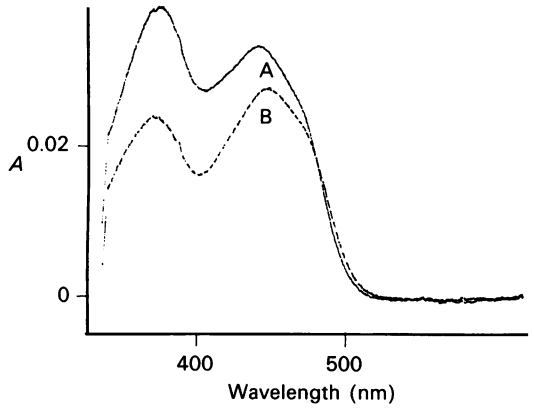


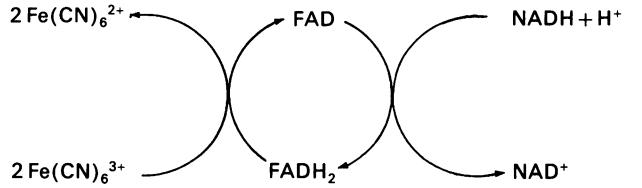
Fig. 5. U.v. absorption spectrum of the prosthetic group isolated from *E. coli* DHPR

A, Spectrum of the prosthetic group isolated from 400 μg of protein in Hepes buffer at pH 7.4 and 25 °C; B, spectrum of authentic FAD^+ (20 μM) in Hepes (50 mM) buffer at pH 7.4 and 25 °C.

maximum at 530 nm (excitation wavelength is 450 nm) and is the same as that observed for free flavins (Morton, 1975). However, the spectroscopic data do not distinguish between FAD^+ and FMN. Unequivocal evidence that the flavin was FAD^+ was obtained by t.l.c. (see the Materials and methods section). A possible mechanism for the involvement of $K_3Fe(CN)_6$ in the pterin-independent NADH oxidoreductase reaction is shown in Scheme 2.

The FAD^+ is reduced to $FADH_2$ by NADH. This in turn is oxidized to FAD^+ by the ferricyanide. Theoretically the oxidation of one molecule of NADH requires two molecules of ferricyanide, and the stoichiometry of this reaction was confirmed (see the Materials and methods section). The rate of oxidation of NADH (from the rate of change of absorbance at 340 nm) and the rate of reduction of ferricyanide (from the rate of change of absorbance at 420 nm) are the same and follow first-order kinetics for more than 80% of the reactions, from which rate constants of $8.8 \times 10^{-3}\ s^{-1}$ and $8.6 \times 10^{-3}\ s^{-1}$ (at 25 °C) respectively can be calculated. This suggests that the enzymic reactions are tightly coupled, although the rate constants cannot be identified for NADH or $K_3Fe(CN)_6$, but are a measure of the slowest, rate-limiting, step.

Attempts were made to see if phenol, salicylate, 4-hydroxybenzoate, 4-hydroxyphenylacetate, orcinol and 2,6-dihydroxypyridine caused the oxidation of NADH in the presence of the enzyme, but no activity was observed. There have been no reports of the presence of a bound prosthetic group in the DHPRs studied so far (Armarego



Scheme 2. Proposed mechanism of pterin-independent NADH oxidase activity

et al., 1984). The possibility that the *Pseudomonas* reductase may have a bound prosthetic group cannot be ruled out, in view of the similarities with our *E. coli* reductase.

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