Isolation and characterization of dihydropteridine reductase from human liver

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Dihydropteridine reductase (EC 1.6.99.7) was purified from human liver obtained at autopsy by a three-step chromatographic procedure with the use of (1) a naphthoquinone affinity adsorbent, (2) DEAE-Sephadex and (3) CM-Sephadex. The enzyme was typically purified 1000-fold with a yield of 25%. It gave a single band on non-denaturing and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and showed one spot on two-dimensional gel electrophoresis. The molecular weight of the enzyme was determined to be 50 000 by sedimentation-equilibrium analysis and 47 500 by gel filtration. On sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, a single subunit with mol.wt. 26 000 was observed. A complex of dihydropteridine reductase with NADH was observed on gel electrophoresis. The isoelectric point of the enzyme was estimated to be pH 7.0. Amino acid analysis showed a residue composition similar to that seen for the sheep and bovine liver enzymes. The enzyme showed anomalous migration in polyacrylamide-gel electrophoresis. A Ferguson plot indicated that this behaviour is due to a low net charge/size ratio of the enzyme under the electrophoretic conditions used. The kinetic properties of the enzyme with tetrahydrobiopterin, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, NADH and NADPH are compared, and the effects of pH, temperature and a number of different compounds on catalytic activity are presented.

Dihydropteridine reductase (NADH:quinonoid 6,7-dihydropteridine oxidoreductase, EC 1.6.99.7) is an essential enzyme component of the complex systems catalysing the hydroxylation of phenylalanine, tyrosine and tryptophan (Kaufman & Fisher, 1974).

As shown in Scheme 1, hydroxylation of these amino acids is coupled to the oxidation of tetrahydrobiopterin (pterin cofactor) to quinonoid di-hydrobiopterin, and is catalysed by the respective amino acid hydroxylase enzyme. A second enzyme, dihydropteridine reductase, is common to the three systems and is required for the reduction of the unstable quinonoid pterin to the active tetrahydro form through the oxidation of NADH. This recycling of the cofactor allows tetrahydrobiopterin to function catalytically in these reactions.

Dihydropteridine reductase has been isolated and
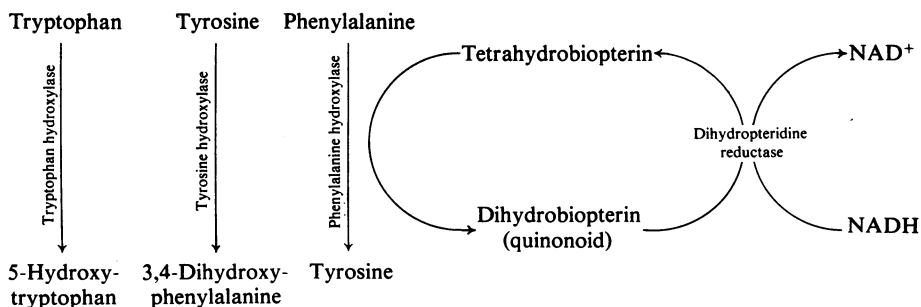
Scheme 1. *Involvement of dihydropteridine reductase in the hydroxylation of the aromatic amino acids*

characterized from sheep liver (Craine et al., 1972; Cheema et al., 1973; Webber et al., 1978) and brain (Cheema et al., 1973), bovine liver (Hasegawa, 1977; Aksnes et al., 1979), adrenal medulla (Cheema et al., 1973; Aksnes et al., 1979), kidney (Chauvin et al., 1979) and brain (Snady & Musacchio, 1978), rat liver (Webber et al., 1978) and a Pseudomonas species (Williams et al., 1976). In the main, these studies have indicated a similarity of properties and structure for dihydropteridine reductase from all these sources. These enzymes were shown to have molecular weights ranging from 42000 to 52000, composed of two subunits of half that weight.

The functional significance of dihydropteridine reductase has been demonstrated by the description of children with malignant hyperphenylalaninaemia due to an inherited deficiency of this enzyme (for review see Danks et al., 1978). A molecular understanding of this disorder depends on the analysis of the structure of normal and mutant human enzyme.

Until recently, however, little was known about the structure of human dihydropteridine reductase. Craine et al. (1972) reported some kinetic properties of a 25-fold-purified enzyme from human liver. Later Cotton & Jennings (1978) described an affinity adsorbent for this enzyme and purified it 400-fold from human liver; the preparation was shown to be approx. 50% pure and they reported a molecular weight of 100000 for the native enzyme. This finding was of interest, as it indicated that perhaps a major difference existed between human dihydropteridine reductase and other mammalian dihydropteridine reductases previously examined.

In the present paper we describe an efficient procedure for the purification to homogeneity of dihydropteridine reductase from normal human liver. Studies of the enzyme's native and subunit molecular weight were performed, and these and other physicochemical characteristics of the enzyme are presented.

Materials and methods

Tissue obtained at autopsy

Human livers were obtained 4–24 h post mortem from children suffering from various causes. The tissue was used immediately or stored at $-20°C$. During the development of the purification procedure, six different livers were used.

Chemicals

All chemicals used were of analytical grade where possible. NADH, NADPH, NAD$^+$ and dithiothreitol were from Calbiochem (San Diego, CA, U.S.A.); 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine hydrochloride and 5,5'-dithio-bis-(2-nitrobenzoic acid) were from Aldrich (Milwaukee, WI, U.S.A.). Tetrahydrobiopterin was generously supplied by Dr. W. L. F. Armarego (Australian National University, Canberra, A.C.T., Australia). AH-Sepharose, DEAE-Sephadex A-50, CM-Sephadex C-50 and Sephadex G-100 were from Pharmacia Fine Chemicals (Uppsala, Sweden); proteins used to calibrate the column in gel filtration were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); chymotrypsinogen was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Sodium dodecyl sulphate and ammonium persulphate were purchased from BDH Chemicals (Poole, Dorset, U.K.); acrylamide, NN'-methylenebisacrylamide and 2-mercaptoethanol were from Merck (Darmstadt, Germany); NNN'N'-tetramethylethylenediamine and dimethyl suberimidate were obtained from Sigma. N-Ethylmaleimide and p-chloromercuribenzoic acid were from Nutritional Biochemical Corp. (Cleveland, OH, U.S.A.); iodoacetamide, HgCl$_2$, MgCl$_2$, CoCl$_2$, 2,2'-bipyridyl and EDTA were from BDH; MnCl$_2$ was from Sigma; butane-2,3-dione monoxime was from Eastman Organic Chemicals (Rochester, NY, U.S.A.); o-phenanthroline was from Hopkin and Williams (Chadwell Heath, Essex, U.K.).

Dihydropteridine reductase assay

Dihydropteridine reductase activity (pterindependent oxidation of NADH) was recorded on a Varian 625 double-beam spectrophotometer, as the rate of change in absorbance at 340 nm with the synthetic cofactor 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine hydrochloride (80 μM) and NADH (100 μM) as substrates at 37°C (Firgaira et al., 1979). A molar absorption coefficient of 6220 M$^{-1}\cdot$cm^{-1} for NADH at 340 nm was used in calculating enzymic activity (Horecker & Kornberg, 1948). One unit of enzyme activity represents 1 μmol of NADH oxidized/min, and specific activity is in units/mg of protein. Protein was determined by the method of Lowry et al. (1951), with bovine albumin stock solution 905–10 (Sigma) as standard.

Kinetic experiments were conducted essentially as described previously (Firgaira et al., 1979). Michaelis constants and maximal velocities were obtained from linear-regression analysis of Lineweaver–Burk double-reciprocal plots (Lineweaver & Burk, 1934).

To study the effect of various compounds, enzyme and reagent of interest were incubated for 5 min at 37°C in the assay mixture (without NADH or pterin). After incubation, enzyme activity was determined as usual by addition of NADH and pterin. The ability of NADH to protect the enzyme against inactivation was assessed by preincubating the enzyme in the assay mixture (without pterin);

then after 5 min the reagent of interest was added to the mixture, which was incubated for a further 5 min before determination of activity by addition of pterin substrate to the mixture.

Isolation of human liver dihydropteridine reductase

All procedures were performed at 4°C unless otherwise specified.

Extraction. Liver tissue (approx. 300 g) was cut into small pieces with a scalpel and homogenized in 0.1 M-Tris/HCl buffer, pH 8.6, containing 0.1 mM-EDTA (2 ml/g wet wt. of tissue) in an electric blender. The homogenate was centrifuged (at 30 000 g for 45 min), and the supernatant was filtered through cotton gauze to remove lipid material. The sedimented liver tissue was re-extracted in half the volume of the same buffer, and the two supernatants were pooled.

Affinity chromatography. The liver extract was adjusted to 0.8 M-NaCl and 0.1 mM-NADH, fine particulate material remaining in solution was removed by passage through a pre-column of Sepharose 4B-CL (6 cm × 3 cm, equilibrated in extraction buffer), and the eluate was applied directly to a column (4 cm × 4 cm) of 1,2-naphthoquinone-AH-Sepharose (Cotton & Jennings, 1978).

After passage of the extract, the column was washed successively with (1) 400 ml of buffer 1 (0.8 M-NaCl/50 mM-Tris/HCl buffer, pH 7.6) containing 0.1 mM-NADH, (2) 300 ml of buffer 2 (0.8 M-NaCl/0.1 M-Na$_2$CO$_3$/NaHCO$_3$ buffer, pH 10.9) containing 0.1 mM-NADH and (3) 300 ml of buffer 2 without NADH. The bound reductase was then eluted with 50 ml of buffer 1 followed by 300 ml of the same buffer containing 2 mM-dithiothreitol (see Fig. 1). Fractions eluted at pH 10.9 were adjusted immediately to pH 7.6 with HCl. The flow rate used was 3–4 ml/min throughout. Fractions containing dihydropteridine reductase activity were pooled and dialysed exhaustively against 50 mM-Tris/HCl buffer, pH 7.8.

The adsorbent was regenerated with dithiothreitol as a routine after each column run (Cotton & Jennings, 1978).

DEAE-Sephadex chromatography. The dialysed enzyme preparation was applied to a column (2 cm × 35 cm) of DEAE-Sephadex A-50 previously equilibrated with 50 mM-Tris/HCl buffer, pH 7.8. After application of the enzyme the column was washed with equilibration buffer until the absorbance at 280 nm was essentially zero. Elution of the enzyme was performed with a 500 ml linear gradient of KCl (0–250 mM) in the equilibration buffer. The flow rate was 15 ml/h, and 10 ml fractions were collected. Fractions were assayed for dihydropteridine reductase activity, and the absorbance at 280 nm was monitored. The reductase was eluted between

320 and 390 ml (150 mM-KCl), and the active fractions were pooled.

CM-Sephadex chromatography. The pooled active fractions were dialysed exhaustively against 50 mM-Tris/HCl buffer, pH 7.4, before application to a CM-Sephadex C-50 column (2 cm × 20 cm) equilibrated with the same buffer. The column was washed with 200 ml of the equilibration buffer, and the reductase was then eluted with a 300 ml linear gradient of KCl (0–250 mM) in the equilibration buffer. The flow rate was 10 ml/h, 10 ml fractions being collected. Dihydropteridine reductase was eluted as a symmetrical peak of enzyme activity and absorbance at 280 nm in the 150 mM-KCl region of the gradient. The active fractions were combined, dialysed against 50 mM-Tris/HCl buffer, pH 7.8, and concentrated to 4–5 ml by ultrafiltration (Sartorius collodion membrane with 50 mM-Tris/HCl buffer, pH 7.8).

Electrophoretic methods

Analytical discontinuous polyacrylamide-gel electrophoresis was performed at pH 9.4 as described by Ornstein (1964) and Davis (1964) and at pH 4.0 as described by Parish & Marchalonis (1970). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed in accordance with the procedures described by Weber & Osborn (1969). Cross-linking studies of the reductase with dimethyl suberimidate (Aldrich) were performed as described by Davies & Stark (1970).

Testing for anomalous behaviour in these gel systems was performed by measuring the electrophoretic mobility of dihydropteridine reductase and standard proteins, at a series of different gel concentrations [see Hedrick & Smith (1968) and Chrambach & Rodbard (1971) for strategy of the method]. The results were analysed in a manner first proposed by Ferguson (1964), by plotting the logarithm of relative mobility (R_F) against acrylamide gel concentration (Ferguson plot). Anomalous behaviour is indicated for proteins with an extrapolation point different from that of the standards on the Ferguson plot [as observed for the reductase in native disc electrophoresis (Fig. 4)].

Gradient gel (Gradipore) electrophoresis was performed as previously described (Cotton & Jennings, 1978), and in a second buffer with a pH 9.6 electrophoresis buffer (11.2 g of Tris, 0.96 g of EDTA and 0.36 g of boric acid/litre) and extended electrophoresis (6000 V-h; 150 V for 40 h at 4°C).

High-molecular-weight and low-molecular-weight calibration-kit protein standards (Pharmacia) were used in the above electrophoresis experiments.

Protein was stained with Coomassie Brilliant Blue G250 (0.04%) in 3.5% (v/v) HClO$_4$ and destained in 7% (v/v) acetic acid. Staining for enzyme activity

was as previously described (Cotton & Jennings, 1978).

Analytical ultracentrifugation

Sedimentation-equilibrium analysis was performed in a Beckman model E analytical ultracentrifuge equipped with the interference optical system. The molecular weight of the reductase (0.25 mg/ml in 50 mM-Tris/HCl buffer, pH 7.5) was measured by the meniscus-depletion method of Yphantis (1964). The column height was 3 mm and the speed was 24 000 rev./min.

Gel filtration

This was performed on a Sephadex G-100 column (2 cm × 74 cm) with 50 mM-Tris/HCl buffer, pH 7.8, as eluting buffer. The void volume (V_0) was determined with Blue Dextran and the total volume (V_t) with tritiated water. The column was calibrated with the following proteins (Stokes radii in brackets); bovine serum albumin [3.62 nm (36.2Å)], ovalbumin [2.73 nm (27.3Å)], chymotrypsinogen [2.09 nm (20.9Å)], ribonuclease [1.92 nm (19.2Å)] and cytochrome c [1.64 nm (16.4Å)]. The molecular weight of dihydropteridine reductase was calculated by the method of Andrews (1964). The Stokes radius of the enzyme was determined as described by Ackers (1964), and this was used to calculate the diffusion coefficient ($D_{20,w}$) as outlined by Svedberg & Pedersen (1940).

Amino acid analysis

Samples (50–100 μg) of native and S-carboxymethylated (Crestfield et al., 1963) preparations of the pure reductase were hydrolysed in 6 M-HCl/1% 2-mercaptoethanol in evacuated tubes at 110°C for 24, 48 and 72 h. Amino acid analysis of the hydrolysates was performed on a Beckman 120C analyser. Tryptophan was determined by spectrophotometric (Edelhoch, 1967) and fluorimetric (Pajot, 1976) procedures.

Two - dimensional polyacrylamide - gel electrophoresis

This was performed essentially as described by O'Farrell (1975). First-dimension isoelectric-focusing disc gels (0.2 cm × 12 cm) of 5% polyacrylamide containing 2% (w/v) Ampholines, pH 3.5–10 (LKB Produkter, Bromma, Sweden), and 9.5 M-urea (AristaR grade) were run at room temperature for 16–18 h at a constant voltage of 400 V. The second dimension used a 0.08 cm × 9 cm resolving gel of 9% polyacrylamide and a 4.8 cm stacking gel of 4.5% polyacrylamide. First-dimension and second-dimension gels were fixed in 3.5% HClO₄ overnight before the staining for protein as described above.

Measurement of isoelectric points was based on the method of Righetti & Drysdale (1974). Electro-

focusing was performed in 6% polyacrylamide gels containing 10% (v/v) glycerol and 2% (w/v) ampholyte [pH 3.5–10 (LKB) or pH 6.5–9 (Pharmacia)]. After electrophoresis (at 400 V for 16 h at room temperature), the gels were sliced into 0.2 cm sections and extracted in 0.5 ml of distilled water for 3 h. The pH and enzyme activity of each extract were measured.

Results

Isolation of dihydropteridine reductase from human liver

The procedure employed in the isolation of dihydropteridine reductase from human liver involved (1) affinity chromatography on 1,2-naphthoquinone–AH-Sepharose, and ion-exchange chromatography on (2) DEAE-Sephadex and (3) CM-Sephadex.

The purification achieved in the affinity-chromatographic step was improved from 300-fold (Cotton & Jennings, 1978) to 700-fold, through a modification of the buffers used in washing and eluting the reductase from the adsorbent (Fig. 1). A recovery of over 80% was obtained, and the enzyme showed a specific activity of 285 units/mg of protein.

The enzyme was further purified by chromatography on DEAE-Sephadex and CM-Sephadex columns, where it was eluted as a single symmetrical peak of protein and activity at the 0.15 M-KCl region of the salt gradient employed (results not shown). The specific activity of the final product varied from one preparation to another, but was usually between 380 and 450 units/mg (six experiments). The purification procedure (summarized in Table 1) typically yields 4–5 mg of 1000-fold-purified dihydropteridine reductase from 300–400 g of liver tissue. Purified enzyme has been stored frozen at −20°C for up to 2 years without loss of biological activity.

Purity of enzyme

Polyacrylamide-gel disc electrophoresis of the purified liver dihydropteridine reductase revealed the presence of only one protein band (Fig. 2a). Staining of a duplicate gel for enzyme activity gave a single band in a position corresponding to that of the protein band (Fig. 2b). On two-dimensional gel electrophoresis, which separates proteins according to their isoelectric point (pI) and subunit molecular weight, the purified enzyme preparation revealed a single spot (Fig. 2c). In one electrophoretic system (non-equilibrium pH-gradient electrophoresis; O'Farrell et al., 1977) the enzyme was resolved into two bands, the nature of which is discussed in the following paper (Firgaira et al., 1981).
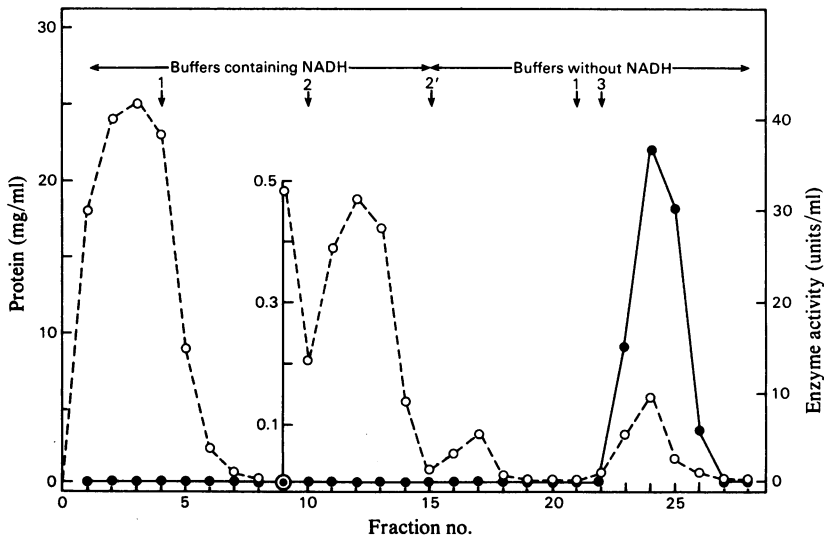
Unless otherwise specified, the purified enzyme

Fig. 1. *Affinity chromatography of human liver dihydropteridine reductase on 1,2-naphthoquinone–AH-Sepharose*
After the application of liver (particle-free) extract, the column was washed successively with buffer 1 (pH 7.6) containing NADH (arrow 1), buffer 2 (pH 10.9) containing NADH (arrow 2) and buffer 2 without NADH (arrow 2′). Buffer 1 was then applied to the column until the eluate was neutral before elution of the reductase by buffer 3 (arrow 3), which contains 2 mM-dithiothreitol. Fractions (1–5, 150 ml; 6–28, 50 ml) were assayed for protein (O) and dihydropteridine reductase activity (●).
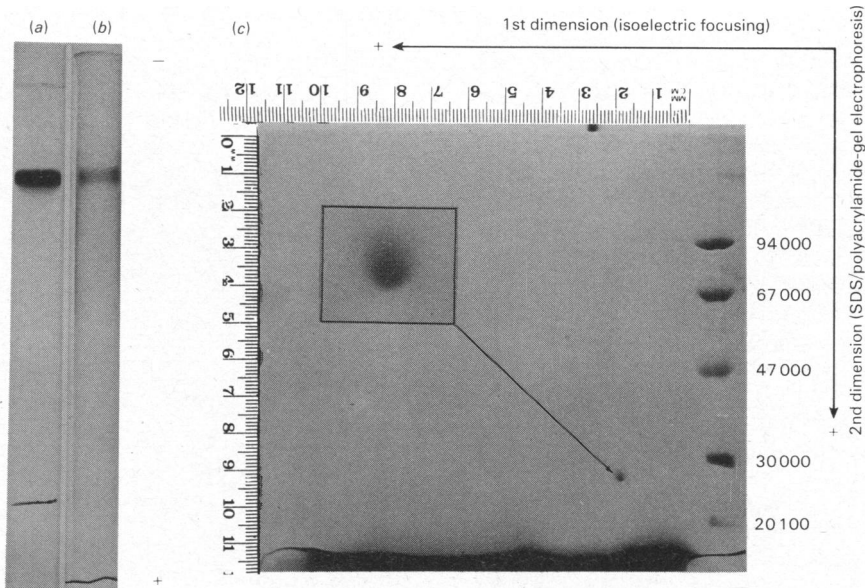

Fig. 2. *Electrophoretic analysis of purified human liver dihydropteridine reductase*
Disc electrophoresis at pH 9.4 of reductase (a) (25 μg) stained for protein and (b) (0.2 μg) stained for enzyme activity. (c) Two-dimensional polyacrylamide-gel electrophoresis of reductase (10 μg): isoelectric-focusing gel in first dimension and sodium dodecyl sulphate (SDS)/polyacrylamide (9%) slab gel in the second dimension. Insert shows a close-up of the dihydropteridine reductase spot.

Table 1. *Isolation of dihydropteridine reductase from human liver*
For experimental details see the text.

Purification stage	Volume (ml)	Total protein (mg)	Total enzyme activity (units)	Yield (%)	Specific activity (units/mg)	Purification (fold)
Crude extract	600	16094	6562	100	0.408	1
(1) Affinity chromatography	200	18.9	5380	82	285	699
(2) DEAE-Sephadex	72	10.2	3805	58	372	920
(3) CM-Sephadex	38	4.1	1732	26	422	1035

preparation was used for the following characterization studies.

Determinations of the molecular weight of the native enzyme

The molecular weight of native dihydropteridine reductase was determined by a number of procedures.

(1) *Sedimentation-equilibrium analysis.* This gave a linear plot of logarithm of the equilibrium protein concentration versus the square of radial distance from the centre of the rotor (results not shown), and indicates the absence of detectable contaminating protein material. From the slope of this plot and taking a partial specific volume for the enzyme of 0.736 ml/g [estimated from the amino acid composition (Table 2) by the procedure of Schachman (1957)], a molecular weight of 50000 was obtained for the enzyme.

(2) *Gel-filtration chromatography.* The molecular weight and the Stokes radius of the enzyme were determined by gel filtration on Sephadex G-100. A plot of log(molecular weight) of the standard proteins versus the partition coefficient (K_D) for each of the standards showed a linear correlation (results not shown). The molecular weight of the purified reductase was calculated from its observed K_D and a value of 47500 ± 3000 (six determinations) was obtained. The reductase activity from crude liver extract was found to be eluted from the column with the same elution volume (V_e) as the purified enzyme. A Stokes radius of 3.00 nm (30.0 Å) was obtained by analysis of these data (Ackers, 1964). The diffusion coefficient $(D_{20,w})$ was calculated to be $7.2 \times 10^{-7} \, cm^2/s$ from the Stokes radius (Svedberg & Pedersen, 1940).

(3) *Molecular-weight analysis on gradient polyacrylamide gels.* Our first experiments showed that the purified dihydropteridine reductase migrated to an apparent molecular weight of 150000 (i.e. slightly larger than lactate dehydrogenase) on analysis by gradient (2.5–27%) polyacrylamide-gel electrophoresis (Fig. 3a). This finding is very different from the molecular-weight estimates obtained by the sedimentation-equilibrium and gel-filtration studies reported above, and suggests that the reductase may

Table 2. *Amino acid composition of dihydropteridine reductase from various species*

The numbers of residues were calculated on the basis of a subunit molecular weight of 25000 for human dihydropteridine reductase. The values given by (1) Craine *et al.* (1972), based on 21000, (2) Cheema *et al.* (1973) and (3) Korri *et al.* (1977), based on 26000, and (4) Hasegawa (1977), based on 25000, have been adjusted where necessary to a subunit molecular weight of 25000 for comparative purposes.

	Residues per subunit (mol.wt. 25000)				
		Sheep liver		Bovine liver	
Residue	Human liver (present work)	(1)	(2)	(3)	(4)
Asp	16	18	19	17	15
Thr	17	15	18	15	18
Ser	18	21	20	18	22
Glu	22	26	26	23	21
Pro	9	10	9	10	9
Gly	26	26	24	20	22
Ala	31	30	32	26	32
S-Cm-Cys	3	1	—	—	—
Val	17	17	17	16	17
Met	4	4	5	5	5
Ile	8	6	7	7	6
Leu	21	20	25	21	22
Tyr	3	2	3	3	2
Phe	7	6	7	6	6
His	5	5	5	5	4
Lys	14	15	14	14	14
Arg	9	8	10	8	9
Trp	5	2	9	3	8
½Cys	—	—	3	9	1
Cys	—	1	—	—	—

be migrating in an anomalous manner in the gradient gel system.

Anomalous behaviour of dihydropteridine reductase on polyacrylamide gels

A good test for anomalous behaviour of proteins in polyacrylamide gels is the determination of the electrophoretic mobility of the protein of interest at a series of different polyacrylamide-gel concentrations (Chrambach & Rodbard, 1971). As shown in
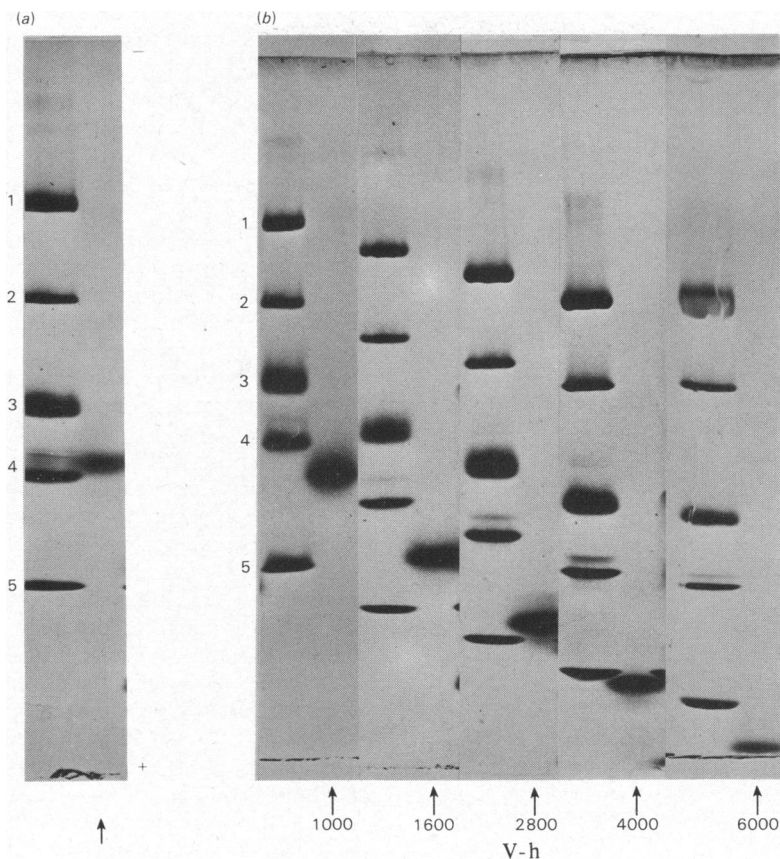
Fig. 3. *Gradient polyacrylamide-gel electrophoresis of dihydropteridine reductase*
(*a*) Electrophoresis at pH 8.7 at 4°C for 1200 V-h; (*b*) electrophoresis at pH 9.6 for increasing V-h periods. The dihydropteridine reductase track is marked by arrow and compared with protein standards (molecular weights in parentheses): 1, thyroglobulin (669 000); 2, ferritin (440 000); 3, catalase (232 000); 4, lactate dehydrogenase (140 000); 5, albumin (67 000).

Fig. 4, dihydropteridine reductase shows a markedly different extrapolation point from that of the standard proteins in the resultant Ferguson plot. This indicates that caution should be used when comparing this protein to others in polyacrylamide-gel systems. At low polyacrylamide concentrations (approaching zero) there is very little retardation of protein migration by the sieving effect of the gel matrix, and protein migration is largely dependent on the net charge of the protein. As shown in Fig. 4, the reductase showed a lower mobility than many proteins of larger size at low polyacrylamide concentration. At higher polyacrylamide concentrations, where a size-sieving effect becomes more important, the reductase begins to approach the migration expected by its size. The results indicate a low net charge/size ratio for human dihydropteridine reductase, and are consistent with the relatively high isoelectric point (pI

7.0 ± 0.1) found for this enzyme. These characteristics suggest that in the electrophoretic conditions used prevously (pH 8.7 for 1200 V-h) in gradient gel electrophoresis the enzyme bore a low relative charge and may not have reached its migration limit, leading to an erroneous estimate of its native molecular weight (Fig. 3*a*; Cotton & Jennings, 1978). Molecular-weight determination by polyacrylamide gradient gels are only accurate if the migration rates of unknown and standard proteins approach zero and depend on the free mobility of the proteins at the selected electrophoresis pH and the period of electrophoresis.

At higher electrophoretic buffer pH values the relative net charge/size ratio of the reductase begins to resemble more closely that of the standard proteins used in calibration of the gel. As presented in Fig. 3(*b*), electrophoresis at pH 9.6 for increasing periods showed that human dihydropteridine reduc-
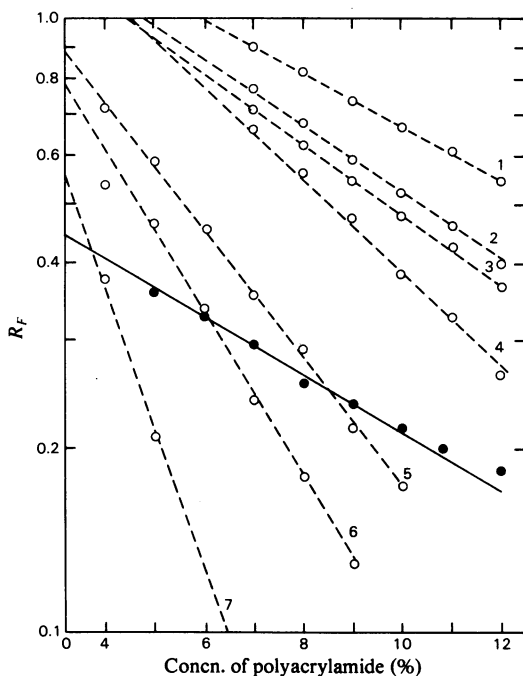
Fig. 4. *Ferguson plot of dihydropteridine reductase migration on disc gel electrophoresis*
Electrophoresis was performed at pH 9.4 at 22 °C in 0.5 cm × 10 cm gels; protein migration is expressed relative to Bromophenol Blue migration (R_F). Dihydropteridine reductase (●) migration is compared with that of protein standards (molecular weights in parentheses): 1, α-lactalbumin (14 400); 2, carbonic anhydrase (30 000); 3, ovalbumin (47 000); 4, albumin (67 000); 5, lactate dehydrogenase (140 000); 6, catalase (232 000); 7, thyroglobulin (669 000).

tase reaches its migration limit much more slowly than do the standard proteins, and begins to approach zero mobility only after 6000 V-h. At this stage calculation from the straight-line calibration curve obtained from a plot of log (molecular weight) against R_F for the standard proteins (Figure not shown) indicated an apparent molecular weight of 54 000 for the reductase.

A molecular weight of 50 000 (average from molecular-weight studies) was used with the Stokes radius and partial specific volume to compute a frictional ratio (f/f_0) of 1.23 for the enzyme by the method of Siegel & Monty (1966).

Subunit molecular weight

Analysis of the purified enzyme preparation by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed one protein band with an apparent molecular weight of 26 000 (Fig. 5). This value was

confirmed by a Ferguson plot, which showed a linear relationship of log R_F of dihydropteridine reductase versus polyacrylamide concentration with the same intercept $(R_F\ 1.5)$ as those for plots for standard proteins (Fig. 5d).

Dimethyl suberimidate, a protein-cross-linking reagent, was used in studying the subunit structure of the enzyme (Davies & Stark, 1970). When the reductase was treated with increasing concentrations of the suberimidate and analysed by sodium dodecyl sulphate / polyacrylamide - gel electrophoresis, a second protein band emerged at 58 000 mol.wt., with a concurrent decrease in the intensity of the monomer band at 26 000 mol.wt. (Figs. 5a–5c). The Ferguson plot of the cross-linked dimer (■ in Fig. 5d) shows the same intercept but a higher retardation coefficient than the monomer (● in Fig. 5d), indicating a difference in molecular weight and not in charge between the two.

Assay for reductase activity before and after treatment with suberimidate (4 mg/ml, which resulted in near complete formation of the cross-linked dimer) indicated no significant alteration of enzyme activity. Gel filtration of the cross-linked material on Sephadex G-100 gave one symmetrical peak of enzyme activity, eluted at the same elution volume (V_e) as untreated enzyme, indicating a molecular weight of 47 500 for the cross-linked dimer.

To test whether or not the two subunits were identical, polyacrylamide-gel electrophoresis was performed on the enzyme at pH 9.4 and pH 4.0 in 7.5% gels (as described in the Materials and methods section). Reductase samples (10 μg) were treated separately with (i) 9 M-urea, (ii) 9 M-urea and 0.2% 2-mercaptoethanol and (iii) 9 M-urea, 0.2% 2-mercaptoethanol and 1 mM-iodoacetamide. Electrophoresis of the treated samples in both the pH 9.4 and pH 4.0 systems showed only one protein band on the gels. This indicates that the two subunits have similar electrophoretic mobilities, and, since they also have similar molecular weights (see above), the two subunits are presumably identical. This contention is also supported by the single spot observed in two-dimensional electrophoresis (Fig. 2c).

Amino acid composition

Amino acid analysis of two different preparations of purified human liver dihydropteridine reductase gave essentially the same results. These are presented in Table 2 and are compared with the known amino acid compositions of sheep and bovine liver dihydropteridine reductases.

Subunit–NADH interaction

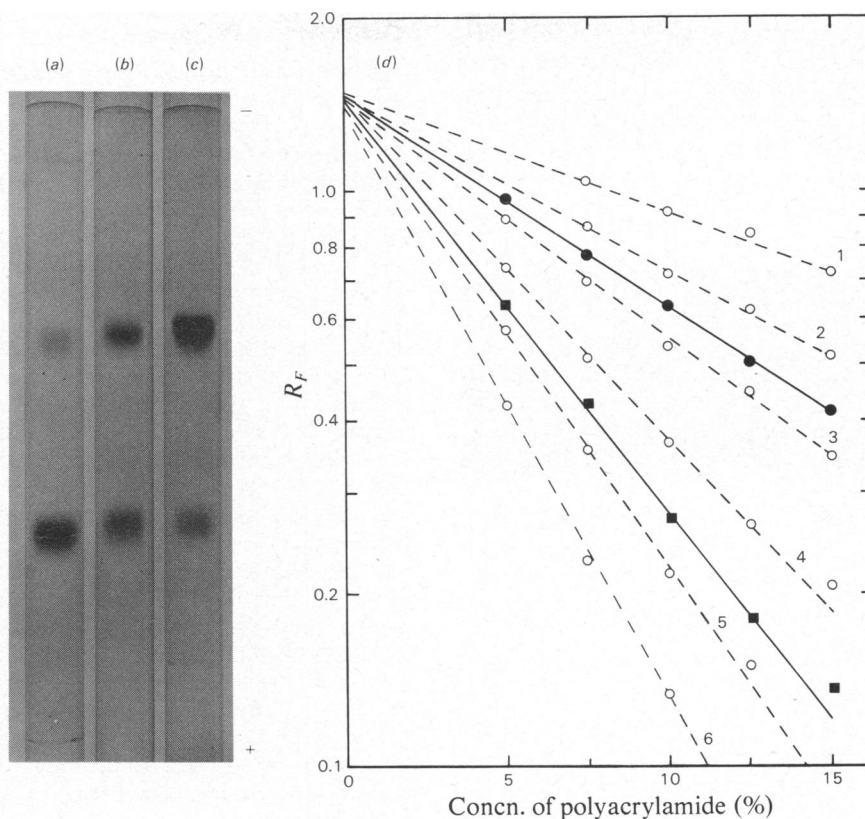Webber & Whiteley (1978) showed that the addition of NADH to rat liver dihydropteridine

Fig. 5. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of dihydropteridine reductase*
Electrophoresis was performed at room temperature in 0.5 cm × 10 cm gels; protein migration is expressed relative to Bromophenol Blue migration (R_F). Gels (a)–(c), purified enzyme (15 μg) treated with dimethyl suberimidate at 0.02 mg/ml, 0.2 mg/ml and 2 mg/ml respectively. (d) Ferguson plots of reductase (●) and dimethyl suberimidate-treated enzyme (■) migration, compared with protein standards (subunit molecular weights in parentheses): 1, α-lactalbumin (14 400); 2, trypsin inhibitor (20 100); 3, carbonic anhydrase (30 000); 4, ovalbumin (47 000); 5, albumin (67 000); 6, phosphorylase *b* (94 000).

reductase followed by analysis on polyacrylamide gels under non-denaturing conditions revealed two bands, the enzyme and a faster-migrating band of enzyme–NADH complex.

Addition of NADH to the human liver enzyme produced a faster-migrating band of reductase, as indicated by protein (Figs. 6a–6c) or enzyme-activity staining. To determine the nature of the observed heterogeneity in the presence of NADH, the mobilities of the two different dihydropteridine reductase bands were recorded as Ferguson plots. The faster-migrating band (enzyme–NADH complex) gave a line parallel to that observed for reductase alone (Fig. 4), indicating that the separation between these bands is caused by a difference in charge, not of molecular weight. As shown in Fig. 6, the intensity of the reductase–NADH band increased with the amount of NADH added. The conversion of enzyme into enzyme–NADH com-plex appeared to be nearly complete in the presence of 2 equiv. or more of NADH (Fig. 6c). A trace amount of the uncomplexed enzyme was still found with greater amounts (2–50 equiv.) of NADH.

These results suggest that each molecule of the human enzyme binds two molecules of NADH, which agrees with the findings made by Hasegawa (1977) for the bovine enzyme. No complexes with altered mobility could be detected when the reductase was incubated with up to a 100-fold molar excess of NADPH, NAD$^+$ or 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

Isoelectric point

The purified human liver dihydropteridine reductase was subjected to isoelectric focusing in a non-denaturing system (Righetti & Drysdale, 1974). Enzyme activity could be extracted from the gels at a position corresponding to the single band of

stained protein in duplicate gels. Use of broad-range ampholytes (pH 3.5–10) indicated one peak of enzyme activity corresponding to pI 7.0 ± 0.1 (six determinations). A similar result was obtained with narrow-range ampholytes (pH 6.5–9.0).
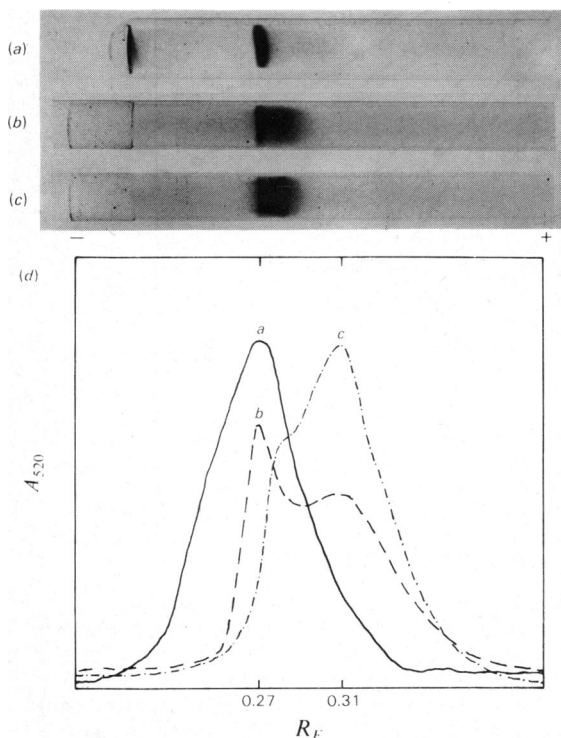

Fig. 6. *Binding of NADH to dihydropteridine reductase* Samples of purified dihydropteridine reductase (10 μg, 0.2 nmol) were incubated with various amounts of NADH (0.05–10 nmol) for 15 min at room temperature. These were analysed by disc polyacrylamide-gel electrophoresis at pH 9.4 with 7.5% polyacrylamide gels. Gel (*a*), reductase (0.2 nmol); gel (*b*), reductase (0.2 nmol) with NADH (0.2 nmol); gel (*c*), reductase (0.2 nmol) with NADH (0.4 nmol). (*d*) shows densitometric tracings obtained when gels (*a*)–(*c*) were scanned at 520 nm (relative scale).

Catalytic and kinetic properties

Optimum pH and substrate specificity. Measurement of dihydropteridine reductase activity at various pH values (Tris/HCl buffers, pH 5.8–9.8) in the presence of NADH or NADPH as the electron donor showed optimal activity at pH 7.2 for both nucleotides. The buffers used were adjusted with HCl to a desired pH at 20°C. In determining the actual assay pH at 37°C, a correction factor of 0.025/°C was applied (temperature coefficient of Tris buffer used). A buffer adjusted to pH 7.65 at 20°C is used in the standard assay. NADH was 20-fold more effective as an electron donor than NADPH for the reaction with the synthetic cofactor 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine. A similar pH–activity profile was observed with tetrahydrobiopterin, but NADH was only 2.5-fold more effective than NADPH in this reaction.

Temperature. The assay reaction mixture (see the Materials and methods section) containing equal amounts of the purified reductase (50 ng) was incubated at different temperatures (15–45°C) by means of a water-jacketed cell holder and thermostatically controlled circulating water bath. After 10 min incubation the reaction rate was determined by addition of the pterin to the mixture; this showed an increase in activity with temperatue, reaching a maximum at 37°C (results not shown).

These experiments are in agreement with our previous observations of dihydropteridine reductase activity in human cultured cells and liver extracts (Firgaira *et al.*, 1979).

Kinetic constants. The effect of varying the concentration of substrates on the measured enzymic activity was determined. This showed typical hyperbolic curves for plots of velocity versus substrate concentration. The K_m and $V_{max.}$ values for tetrahydrobiopterin, the synthetic cofactor, NADH and NADPH were determined from Lineweaver–Burk plots and are presented in Table 3. The lower K_m and $V_{max.}$ values observed for NADH show that this is a more effective substrate than NADPH in the reaction. This is in agreement with observations with other mammalian reductases (Nielsen *et al.*, 1969; Craine *et al.*, 1972; Cheema

Table 3. *Kinetic data for human liver dihydropteridine reductase*
Values were measured at 37°C at pH 7.2 (for further experimental details see the text).

Substrate	K_m (μM)	$V_{max.}$ (μmol/min per mg)	Concn. of second substrate
NADH	29	468	Synthetic cofactor* (75 μM)
NADPH	770	316	Synthetic cofactor* (75 μM)
Tetrahydrobiopterin	17	239	NADH (100 μM)
Synthetic cofactor*	36	612	NADH (100 μM)

* 2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

et al., 1973). The enzyme showed a higher affinity (lower K_m) for the natural cofactor tetrahydrobiopterin than for the synthetic cofactor; however, higher enzyme activity was observed with the latter, as indicated by the higher $V_{max.}$ observed with this substrate (Table 3). This has also been observed for the sheep liver enzyme (Craine *et al.*, 1972). On the basis of the observed $V_{max.}$ (Table 3) and a molecular weight of 50000, human liver dihydropteridine reductase shows a turnover number (molecules of product synthesized/min by each molecule of enzyme) of 1.20×10^4 and 3.06×10^4 for tetrahydrobiopterin and synthetic cofactor respectively.

Substrate inhibition by pterin was previously observed for the crude extract enzyme at concentrations of the synthetic cofactor above $100 \mu M$ (Firgaira *et al.*, 1979). This was not apparent with the pure enzyme. However, use of cofactor concentrations above approx. $100 \mu M$, though not affecting the initial velocity (over 1 min), caused a marked decrease in the reaction after this period. Higher concentrations ($>100 \mu M$) of substrates (synthetic cofactor and/or NADH) in our assay system produced disturbance of the spectrophotometric signal and recording. Substrate inhibition was observed with tetrahydrobiopterin concentrations above $50 \mu M$.

The effects of various compounds on dihydropteridine reductase activity were examined. The results are presented in parentheses after the specified compound and indicate the concentration of the compound required to produce the stated percentage inhibition of reductase activity. Among the thiol-blocking inhibitors tested HgCl$_2$ ($0.1 \mu M$; 60%), *p*-chloromercuribenzoate (0.01 mM; 70%) and 5,5'-dithiobis-(2-nitrobenzoic acid) (0.1 mM; 60%) markedly inhibited the enzyme, whereas *N*-ethylmaleimide (1 mM; 76%) and iodoacetamide (10 mM; 70%) showed less effect. Use of these compounds at concentrations one order of magnitude higher than those shown above produced total inhibition of activity. Preincubation of the enzyme with 0.1 mM-NADH provided complete protection from inhibition. We have found three *S*-carboxymethylcysteine residues per subunit (Table 2); the inhibition observed with the thiol-blocking reagents suggests that human dihydropteridine reductase activity is dependent on accessible thiol groups. These findings are in agreement with the observations made by Cheema *et al.* (1973) with the sheep liver enzyme and by Webber *et al.* (1978) with both sheep and rat liver reductases; however, Craine *et al.* (1972) could not detect inhibition of their sheep liver enzyme preparation by thiol-blocking reagents, and Aksnes *et al.* (1979) have indicated that cystine is not part of the active site of the bovine liver enzyme. The arginine-specific reagent butane-2,3-dione (Woodroofe & Butterworth, 1979) at 10 mM produced 90%

inhibition of activity, and complete protection from inactivation was observed by preincubation with NADH. Arginine has been implicated in the nucleotide-binding sites of many dehydrogenases (Lange *et al.*, 1974; Bleile *et al.*, 1975; Nagradova & Asryants, 1975), and this may also be true for the reductase.

The effects of metal ions were also examined: MgCl$_2$ (10 mM) and MnCl$_2$ (10 mM) had no effect on enzyme activity, but CoCl$_2$ was observed to inhibit by 30% at 0.01 mM concentration. Williams *et al.* (1976) found a reverse pattern of inhibition of bacterial reductase, which was unaffected by CoCl$_2$ but inhibited by MgCl$_2$, MnCl$_2$ and CdCl$_2$. Metal-ion-chelating agents EDTA (10 mM), *o*-phenanthroline (1 mM) and 2,2'-bipyridyl (1 mM) had no inhibitory effect on enzyme activity, indicating that dihydropteridine reductase does not have a metal-ion requirement, as seen for some dehydrogenase enzymes.

Discussion

The three-step chromatographic procedure described achieves efficient purification of homogeneous dihydropteridine reductase, which constitutes approximately 0.1% of the total soluble protein from human liver.

The molecular properties of human liver dihydropteridine reductase are summarized in Table 4. It shows major similarities in molecular weight (Table 4), amino acid composition (Table 2), dimeric structure (Fig. 5) and substrate specificity (Table 3) to the reductase from sheep (Craine *et al.*, 1972; Cheema *et al.*, 1973), bovine (Hasegawa, 1977; Aksnes *et al.*, 1979) and rat livers (Webber *et al.*, 1978).

However, distinct differences exist between the various dihydropteridine reductases. We found pI 7.0 for human liver dihydropteridine reductase, whereas the enzyme from sheep and rat liver showed pI 5.4 and 6.35 respectively (Webber *et al.*, 1978), and pI 5.7 has been shown for the bovine brain enzyme (Snady & Musacchio, 1978) and liver enzyme (Aksnes *et al.*, 1979). Differences have also been demonstrated in kinetic constants (K_m, K_i) for the reductase from different species (Craine *et al.*, 1972; Cheema *et al.*, 1973; Webber *et al.*, 1978; Firgaira *et al.*, 1979).

Human dihydropteridine reductase forms an enzyme–NADH complex that is stable to gel electrophoresis (Fig. 6). Hasegawa (1977) has suggested that bovine liver dihydropteridine reductase binds two molecules of NADH per enzyme molecule, and Aksnes *et al.* (1979) have indicated that the binding site on each of the two subunits are identical with each other and do not interact. Webber & Whiteley (1978) demonstrated that the

Table 4. *Molecular properties of human dihydropteridine reductase*

Molecular weight	
From sedimentation equilibrium	50000
From gel filtration	47500
From gradient polyacrylamide-gel electrophoresis	54000
Subunit molecular weight	
Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis	26000
Cross-linked dimer (dimethyl suberimidate)	58000
Stokes radius	3.0nm (30 Å)
Diffusion coefficient	$7.2 \times 10^{-7} cm^2/s$
Frictional ratio	1.23
Partial specific volume	0.736 ml/g
Isoelectric point	7.0 ± 1
K_m (NADH, 37°C, pH 7.2)	29 μM
K_m (NADPH, 37°C, pH 7.2)	770 μM
K_m (synthetic cofactor,* 37°C, pH 7.2)	36 μM
K_m (tetrahydrobiopterin, 37°C, pH 7.2)	17 μM

* 2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

rat liver reductase is capable of binding only one molecule of NADH per molecule of enzyme. Titration of the human enzyme with increasing concentrations of NADH suggests that it interacts with 2 equiv. of NADH (Fig. 6), but further studies are required to quantify this accurately.

Our sedimentation-equilibrium and gel-filtration studies indicated a native molecular weight of approx. 50000 for the purified human liver dihydropteridine reductase. Careful studies showed that the molecular weight of 100000, reported by Cotton & Jennings (1978) from studies with gradient polyacrylamide (Gradipore) gel, resulted from anomalous behaviour of the enzyme in this system. Use of the procedures outlined by Chrambach & Rodbard (1971) showed (Fig. 4) that the reductase exhibits a low net charge/size ratio. This indicates that in gradient polyacrylamide-gel electrophoresis human dihydropteridine reductase migrates more slowly than do the standards used to calibrate the gel. Margolis & Kenrick (1969), who developed this procedure, pointed out a similar anomalous behaviour for haemoglobin (mol.wt. 65000, pI 7.0), which migrated at an apparent higher molecular weight than transferrin (mol.wt. 90000). At higher electrophoretic buffer pH values the relative net charge/size ratio of the reductase would more closely resemble that of the standard proteins. By an increase of the buffer pH and allowing a longer period (V-h) for electrophoresis, dihydropteridine reductase was shown to migrate to its expected molecular weight in polyacrylamide gradient gels (Fig. 3). In contrast with the anomalous migration observed for the enzyme in non-denaturing polyacrylamide-gel electrophoresis, its migration was found to be normal when examined by sodium dodecyl sulphate / polyacrylamide - gel electro-

phoresis (Fig. 5); this is consistent with a masking of protein charge brought about by this detergent.

In summary, characterization of human liver dihydropteridine reductase has shown basic similarities of size, subunit structure and amino acid composition to the enzyme from a variety of other species; however, differences in isoelectric points, kinetic constants, NADH-binding ratios and mobility in polyacrylamide-gel systems suggest that distinct variation in physical characteristics exists among the various mammalian dihydropteridine reductase enzymes.

The isolation and characterization of normal human dihydropteridine reductase will now permit analysis of mutant dihydropteridine reductase from patients with deficiency of this enzyme.

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