Molecular and immunological comparison of human dihydropteridine reductase in liver, cultured fibroblasts and continuous lymphoid cells

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An antiserum was raised in a rabbit against highly purified human liver dihydropteridine reductase (EC 1.6.99.7). Dihydropteridine reductase from human liver, in human cultured fibroblasts and in continuous lymphoid cells all showed identical antigenic properties. The structural characteristics of the reductase from these three sources were further compared by the use of high-precision two-dimensional polyacrylamide-gel electrophoresis. The enzyme from radiolabelled fibroblasts and continuous lymphoid cells was isolated by immunoprecipitation or by affinity chromatography and compared with the purified liver enzyme. Two major polypeptide species were resolved, and polypeptides from all three sources co-migrated identically. Indirect evidence is presented indicating that one of the polypeptide species may have been derived from the other via a post-translational modification. These results support the concept that the same structural gene(s) encodes for dihydropteridine reductase in human liver, fibroblasts and lymphocytes.

Dihydropteridine reductase (EC 1.6.99.7) has been assigned a role in aromatic amino acid hydroxylations, where it recycles the pterin cofactor tetrahydrobiopterin. However, there is growing evidence that it may be involved in other metabolic pathways, which remain to be clarified. For example, Rembold & Buff (1972) have proposed the involvement of dihydropteridine reductase in the mitochondrial respiratory chain, and Pollock & Kaufman (1978) have suggested that the reductase can function as a means of salvaging quinonoid dihydrofolate obtained from the oxidation of tetrahydrofolate.

Studies of the tissue distribution of this enzyme in the rat have shown significant activities in all tissues examined, including many that are not known to hydroxylate the aromatic amino acids (Craine *et al.*, 1972; Urretia *et al.*, 1978). Within the rat brain, dihydropteridine reductase has been found in cell types other than adrenergic neurons, including regions lacking tyrosine hydroxylase and tryptophan hydroxylase (Turner *et al.*, 1974; Snady & Musacchio, 1978). In humans distribution of the reductase appears to be similarly widespread (Urretia *et al.*, 1978). Cell types that lack the aromatic amino acid hydroxylases, but possess dihydropteridine reductase, include cultured fibroblasts (Kaufman *et al.*, 1975), continuous lymphoid

cells (Firgaira *et al.*, 1979a) and peripheral-blood lymphocytes, granulocytes (Firgaira *et al.*, 1979b) and platelets (Abelson *et al.*, 1979; Firgaira *et al.*, 1979b). These findings have led to considerable speculation about the role of the enzyme in these cells and whether different forms of dihydropteridine reductase exist in different tissues and cell types.

In the present study we have compared the structural relationship between dihydropteridine reductase isolated from human liver (where it is involved in the hydroxylation of phenylalanine) and that from cultured human fibroblasts and lymphocytes (where the role of the enzyme is as yet unknown).

The study has involved the use of an antibody prepared against human liver dihydropteridine reductase and the development of procedures for the purification and characterization of small quantities of the enzyme from human cultured cells.

Materials and methods

Unless otherwise specified, the materials and methods employed in this study are as previously reported (Choo *et al.*, 1979; Firgaira *et al.*, 1981).

Preparation of antiserum

Dihydropteridine reductase purified from human

liver obtained at autopsy (Firgaira *et al.*, 1981) was used to raise antibodies in rabbits. Enzyme cross-linked with dimethyl suberimide (Firgaira *et al.*, 1981) was found to be more antigenic than was untreated enzyme or enzyme extracted from polyacrylamide gels, and was therefore used. The enzyme (400 µg) was emulsified in complete Freund's adjuvant and injected subcutaneously at multiple sites at the back. Two further booster injections with enzyme (200 µg) emulsified in incomplete adjuvant were given at monthly intervals. Animals were bled 10 days after the final injection, and samples of antiserum were stored at -20°C. Control serum was obtained from the same animals before immunization.

The specificity of the antiserum was assessed by Ouchterlony (1968) immunodiffusion analysis, with modification as described in Choo *et al.* (1979). Immunodiffusion gels to be stained for dihydropteridine reductase activity were prepared as described above but with omission of the dye, Methyl Orange, from the gel mixture. After immunoprecipitation reaction, the gel was washed by incubation in 0.9% NaCl solution for 2–4 h at room temperature followed by 50 mM-Tris/HCl buffer, pH 7.2, for 1 h. The gel was stained for enzyme activity essentially as described by Cotton & Jennings (1978), with the following modifications. The gel was allowed to take up the staining solution, which is composed of 50 mM-tris/HCl buffer, pH 7.2, containing 1 mM-NADH and 1 mM-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for 2 h. After this the quinonoid (substrate) form of 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride was added to the solution to approx. 1 mM. Purple–blue staining of a precipitin line indicated the presence of dihydropteridine reductase activity.

Immunochemical titrations

The antigenic relationship of dihydropteridine reductase from extracts of cultured fibroblasts, continuous lymphoid cells, the homogeneous liver enzyme and crude liver was examined. A fixed amount (0.04 unit) of enzyme from each of the above sources was treated with a range of volumes of antiserum. Each incubation mixture was adjusted to a final volume of 0.2 ml and equivalent protein concentration by addition of phosphate-buffered saline (20 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl) and bovine serum albumin. The solutions were incubated overnight at 4°C, after which they were centrifuged at 3000 g for 15 min at 4°C, and the supernatants were assayed for dihydropteridine reductase activity. The activity, determined relative to that obtained against control antiserum, was used to calculate the percentage inhibition observed.

One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 µmol of NADH/min in the assay system previously described (Firgaira *et al.*, 1981).

Labelling of cultured cells

Cells for enzyme purification were labelled with L-[³⁵S]methionine (1020 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.).

(a) Continuous lymphoid cells were grown in disposable 75 cm² Falcon flasks. Approx. 7×10^7 – 8×10^7 exponentially growing cells were harvested by centrifugation at 800 g for 5 min. The cells were washed twice, with resuspension and centrifugation in phosphate-buffered saline. After this, cells were incubated in 15 ml of methionine-free Eagle's basal medium (Commonwealth Serum Laboratories, Melbourne, Vic., Australia) at 37°C for 10 min before they were centrifuged down and transferred into 15 ml of fresh methionine-free medium containing 2.5 mCi of [³⁵S]methionine. Labelling was conducted at 37°C for 4–6 h. Cells were collected by centrifugation, washed twice with phosphate-buffered saline and stored at -70°C until use.

(b) Fibroblast cells were grown in 1585 cm² Bellico roller vessels. Approx. 1×10^7 – 3×10^7 exponentially growing cells were used in each vessel (approximately two-thirds confluence). The cell surface was rinsed twice with phosphate-buffered saline followed by incubation of the cells in 15 ml of methionine-free medium at 37°C for 10 min. Then 15 ml of fresh methionine-free medium containing 0.5 mCi of [³⁵S]-methionine was added, and the culture was incubated at 37°C for 24 h. At the end of this period, the cells were detached from the culture vessel by using a mixture of 0.02% EDTA and 0.025% trypsin in phosphate-buffered saline. Cells from three roller vessels were pooled, collected by centrifugation, washed twice in phosphate-buffered saline and stored at -70°C.

Purification of [³⁵S]methionine-labelled dihydropteridine reductase

Extracts of ³⁵S-labelled cells were prepared by freezing and thawing the cells three times followed by homogenization in 0.15 M-KCl (approx. 1 ml) in a hand-operated Kontes ground-glass homogenizer. The homogenates were centrifuged at 12000 g for 45 min at 4°C, and the supernatants were collected and frozen (-20°C) or used immediately.

Partial purification of reductase from the labelled cell extract was achieved by (1) immunoprecipitation with anti-(liver dihydropteridine reductase) serum or (2) affinity chromatography.

(1) Immunoprecipitation was performed as follows. A 25 µl volume of 10% (v/v) Triton X-100/10% (w/v) sodium deoxycholate was added to 500 µl of extract (approx. 1×10^8 acid-precipitable

c.p.m.). This was followed by 60 μ l of antiserum (or pre-immunization control serum). The mixture was left overnight (16–18 h) at 4°C, after which 50 μ l of goat anti-(rabbit γ -globulin) serum was added. The mixture was left for an additional 6 h. The resulting immunoprecipitate was collected by centrifugation at 3000 *g* for 15 min at 4°C and washed twice with 0.5 ml of cold 0.5% Triton X-100/0.5% sodium deoxycholate in 0.1 M-Tris/HCl buffer, pH 7.4. The pelleted immunoprecipitate was then used for two-dimensional gel-electrophoretic analysis as described below.

(2) Affinity chromatography was performed as described in the preceding paper (Firgaira *et al.*, 1981), with the following modifications. The [³⁵S]-methionine-labelled extracts (approx. 1 ml) containing 1×10^8 – 4×10^8 acid-precipitable c.p.m. from approx. 8×10^7 continuous lymphoid cells or fibroblasts were loaded on to 0.2 ml columns of 1,2-naphthoquinone–Sephadex in 1 ml disposable syringe barrels. The columns were washed with 6 ml of washing buffers as described previously and the reductase was eluted in a buffer containing dithiothreitol (Firgaira *et al.*, 1981). A peak of radioactivity was detected in the elution fractions, and this corresponded to the enzyme activity peak determined in a duplicate column involving the use of unlabelled cell extract. The peak fractions (approx. 1 ml) were pooled, dialysed against 50 mM-Tris/HCl buffer, pH 7.4, and concentrated to approx. 200 μ l in a Sartorius collodion membrane. This preparation was stored at –70°C.

Two-dimensional gel electrophoresis

The materials and procedures for two-dimensional polyacrylamide-gel electrophoresis were as published before (Choo *et al.*, 1979), with the following modifications. The first-dimensional gels were run under conditions of non-equilibrium pH-gradient electrophoresis (O'Farrell *et al.*, 1977). Gels (120 mm in length) were cast in glass tubes 150 mm long and 2 mm internal diameter. The gels contained 2% (w/v) pH 3.5–10 Ampholines. Samples contained approx. 50 000 c.p.m. of affinity-purified or immunoprecipitated enzyme and 5 μ g of purified (unlabelled) liver dihydropteridine reductase. These were freeze-dried, and then redissolved in 30 μ l of sample buffer containing 9.5 M-urea, 2% (w/v) pH 3.5–10 Ampholines, 5% (v/v) mercaptoethanol and 2% (v/v) Nonidet P-40 before they were applied on to the first-dimension gels and overlaid with 10 μ l of overlay solution (9 M-urea and 1% pH 3.5–10 Ampholines). After an additional overlay with 10 μ l of 8 M-urea solution, the tubes were filled with 10 mM-phosphoric acid. The upper reservoir was filled with 10 mM-phosphoric acid and the lower reservoir with 20 mM-NaOH. The gels were electrophoresed without pre-focusing and with the cathode

on the bottom and the anode on the top. Electrophoresis was at 450 V for 4½ h. At the end of the run, the gels were gently extruded from the tubes and stored at –20°C in 5 ml of a buffer composed of 10% (v/v) glycerol, 5% (v/v) mercaptoethanol and 2.3% (w/v) sodium dodecyl sulphate in 62.5 mM-Tris/HCl buffer, pH 6.8 (O'Farrell, 1975). The gels were slowly thawed at room temperature just before being loaded on to the second-dimension gels.

The second-dimension electrophoresis was performed under denaturing (sodium dodecyl sulphate) conditions as described before (Choo *et al.*, 1979), except that a resolving gel of 12% polyacrylamide was used.

At the end of the run, the gels were fixed in 3.5% (v/v) HClO₄ overnight, stained with 0.04% Coomassie Brilliant Blue G250 in 3.5% HClO₄ for 1 h, then destained in 7% (v/v) acetic acid. Photographs of the gels were taken at this stage if necessary.

Fluorographic detection of ³⁵S-labelled materials

The method of Chamberlain (1979) was used. Two-dimensional gels in destaining solution were first soaked in approx. 20 vol. of water for 20 min. The gels were then transferred into 10 vol. of 1 M-sodium salicylate (pH 5–7; Ajax Chemicals, Sydney, N.S.W., Australia). This was for 30 min before the gels were dried on to Whatman 3MM paper. Fluorographic exposure was made by placing Kodak X-Omat R film (XR-5) over the dried gel slab and holding the two in firm contact with two heavy glass plates. Films were exposed at –70°C for 2–3 weeks. Exposed films were developed for 6 min at 20°C in Kodak DX-80 developer.

Results

Antiserum to human liver dihydropteridine reductase

The specificity of the antiserum obtained was tested by Ouchterlony immunodiffusion reaction. A single precipitin band was observed against a particle-free liver extract, and this formed a line of identity with that formed when purified dihydropteridine reductase was used (Fig. 1a). The precipitin band was also identified by staining for dihydropteridine reductase activity (Fig. 1b); control precipitin lines produced by cross-reacting liver extract with an anti-albumin serum did not stain for activity.

No precipitin bands were observed against control serum obtained from rabbits before immunization.

Immunochemical titrations

The antigenic determinants of dihydropteridine reductase from human liver were compared with those of the enzyme from human cultured fibro-

blasts and continuous lymphoid cells. Fig. 2 shows the results of immunochemical titrations of enzymes from human liver, cultured fibroblasts and continuous lymphoid cells. The activity in fibroblasts and continuous lymphoid cells was inhibited by the antiserum, with titration curves nearly identical with that of the purified liver enzyme. A similar titration curve was observed against the crude extract liver enzyme (results not shown).

From Fig. 2 an equivalence value of 0.005 unit/ μ l of antiserum was obtained. This value agrees with the titration results obtained by immunodiffusion,

Fig. 1. Immunodiffusion of human liver dihydropteridine reductase against antiserum

Wells 1–5, titration of liver extract (30000g supernatant): 1, 0.16 unit (400 μ g); 2, 0.08 unit; 3, 0.04 unit; 4, 0.02 unit; 5, 0.01 unit. Well 6, purified dihydropteridine reductase, 0.08 unit (0.17 μ g). Centre well, rabbit antiserum (As, 20 μ l). Gel (a) was stained for protein and gel (b) for enzyme activity, as described in the Materials and methods section.

which indicate an equivalence value of 0.004 unit/ μ l of antiserum (Fig. 1a, wells 2 and 6).

Absorption of the antiserum with liver, fibroblast or lymphocyte extract before reaction with purified dihydropteridine reductase resulted in a decrease in immunoinhibition titre and an abolition of the precipitin line in immunodiffusion (results not shown).

Immunoprecipitation and two-dimensional polyacrylamide-gel electrophoresis

The antiserum was used to immunoprecipitate enzyme from extracts of ^{35} S-labelled cultured cells. The precipitated material was then examined by two-dimensional polyacrylamide-gel electrophoresis, and the migration was compared with that of unlabelled purified liver dihydropteridine reductase. Fig. 3 shows typical results of one such experiment.

As seen in Fig. 3(a), enzyme immunoprecipitated from fibroblast (detected by fluorography) resolved into two major spots (designated 1 and 2), which co-migrated to positions identical with those of purified liver enzyme (detected by Coomassie Brilliant Blue staining) (Fig. 3b).

Fig. 2. Immunochemical titration analysis of dihydropteridine reductase from human liver, cultured fibroblasts and lymphocytes

Samples (0.04 unit) of purified liver dihydropteridine reductase ($\bullet$), fibroblast extract ($\blacktriangledown$) and continuous lymphoid-cell extract ($\blacksquare$) were incubated with various amounts of antiserum and kept at 4°C overnight. Protein concentrations were kept constant in all incubations by addition of bovine serum albumin. The enzyme activity remaining in solution after centrifugation at 3000g was determined relative to control incubations containing non-immune serum and presented as a percentage value.

Fig. 3. Two-dimensional polyacrylamide-gel analysis of dihydropteridine reductase immunoprecipitated from ^{35}S -labelled human fibroblast-cell extract

(a) Fluorogram showing the fibroblast-cell enzyme polypeptide (designated as spot nos. 1 and 2; enclosed in box). (b) Coomassie Brilliant Blue-stained gel of (a) showing the position of pure human liver enzyme that has been co-electrophoresed with the fibroblast enzyme. (c) and (d) are close-ups of the dihydropteridine reductase regions in (a) and (b) respectively. A similar set of pictures (not shown) was obtained when enzyme immunoprecipitated from lymphocyte cells were co-electrophoresed with pure human liver enzyme. Other polypeptide spots seen in (a) were brought down non-specifically by the immunoprecipitation procedure; these were also seen in gels of control serum, which did not show the dihydropteridine reductase spots. Abbreviations: SDS, sodium dodecyl sulphate; Ig, immunoglobulin chains.

A similar experiment comparing immuno-precipitated dihydropteridine reductase from continuous lymphoid cells with the purified liver enzyme gave similar results (not shown).

Use of pre-immunization control serum in these experiments failed to immunoprecipitate the enzyme from the cell extracts.

Affinity chromatography and two-dimensional polyacrylamide-gel electrophoresis

Partial purification of the reductase from cultured cells was achieved by chromatography on 1,2-naphthoquinone-Sepharose. ^{35}S -labelled cell extracts and unlabelled extracts were chromatographed in

duplicate columns. The radioactivity and reductase activity in individual fractions of the respective columns were monitored. Results for purification from continuous lymphoid-cell extract are presented in Fig. 4. The elution profile for enzyme activity is similar to that observed for the liver enzyme (Firgaira *et al.*, 1981). The procedure typically yielded a 500–900-fold purification of radioactivity in the enzyme peak (relative to the total acid-precipitable radioactivity applied on to the column).

The material isolated by use of the affinity procedure was compared with the homogeneous liver enzyme by analysis on two-dimensional polyacrylamide-gel electrophoresis. Figs. 5(a) and 5(b) show the patterns obtained, and indicate two major polypeptide species of the lymphoid-cell reductase, which coincided with those seen for the hepatic enzyme. These results are consistent with those obtained by immunoprecipitation (Figs. 3a and 3b).

Affinity preparation of human fibroblast-cell extracts revealed a pattern (not shown) identical with those seen above for the lymphoid cells.

Nature of the two polypeptide spots observed for dihydropteridine reductase

Under conventional isoelectric-focusing conditions, dihydropteridine reductase showed one band, corresponding to a single spot on the second-

dimension gel (Fig. 2c in Firgaira *et al.*, 1981). In the present study we have switched to the use of a non-equilibrium pH-gradient electrophoretic procedure (O'Farrell *et al.*, 1977) in the first dimension, as this gave a greater reproducibility and resolution of gel patterns for the enzyme. Use of this procedure has resolved the pure enzyme into at least two major spots (Figs. 3 and 5), and a third very minor band (Fig. 6), which migrates to a more acidic position than species no. 1 at 26000 mol.wt., is present in some of the highly purified liver enzyme preparations. Note: (i) that the more basic subunit (no. 2) appears to be slightly smaller in molecular weight than the more acidic species (no. 1); (ii) that the relative intensities of the two species are reversed when fibroblast or continuous-lymphoid-cell enzyme is compared with the liver enzyme.

Indirect evidence indicates that polypeptide no. 1 may represent the primary molecular form of dihydropteridine reductase and that factors such as the extent of autolysis *post mortem* and/or period of storage of the liver tissue may modify this polypeptide to give rise to a more stable second form (no. 2). Fig. 6 shows the non-equilibrium pH-gradient focusing gels of two different preparations of purified human liver dihydropteridine reductase. The enzyme isolated immediately from a liver obtained 4 h *post mortem* showed predominantly

Fig. 4. *Affinity chromatography of dihydropteridine reductase from human cultured continuous lymphoid cells* An extract (1 ml; 400×10^6 acid-precipitable c.p.m.) prepared from [^{35} S]methionine-labelled continuous lymphoid cells was applied to a 0.2 ml column of 1,2-naphthoquinone-AH-Sepharose. The column was then washed successively with 2 ml each of buffers 1, 2 and 2', and dihydropteridine reductase was eluted with buffer 3 (buffer compositions described by Firgaira *et al.*, 1981). Radioactivity was measured (O). A duplicate column of unlabelled extract (0.25 unit of enzyme) was co-chromatographed and dihydropteridine reductase activity was monitored (●). The flow rate was 1 ml/h, and 0.33 ml fractions were collected.

Fig. 5. Two-dimensional polyacrylamide-gel analysis of dihydropteridine reductase purified from ^{35}S -labelled human lymphocyte-cell extract by affinity chromatography

(a) Fluorogram showing the lymphocyte-cell enzyme (polypeptides designated as spot nos. 1 and 2). (b) Coomassie Brilliant Blue-stained gel of (a) showing the position of pure human liver enzyme that has been co-electrophoresed with the lymphocyte enzyme. (c) and (d) are close-ups of the dihydropteridine reductase regions in (a) and (b) respectively. A similar set of pictures (not shown) was obtained when affinity-chromatography-purified fibroblast-cell enzyme was co-electrophoresed with pure human liver enzyme. Other polypeptide spots seen in (a) were contaminants co-eluted with the enzyme from the column. Abbreviation: SDS, sodium dodecyl sulphate.

subunit no. 1 (Fig. 6a), whereas that isolated from a liver obtained 12h *post mortem* and after a 1-week storage period at -20°C revealed predominantly subunit no. 2 (Fig. 6b). The enzyme isolated from livers obtained up to 24h *post mortem* and stored for up to 6 months at -20°C gave essentially the same pattern as that observed in Fig. 6(b). Polypeptide no. 1 is the predominant species in cultured cells (Figs. 3 and 5). We have on occasions also observed a two-dimensional gel pattern of enzyme immunoprecipitated from cultured cells showing only species no. 1 with no detectable amounts of species no. 2 (results not shown). Presumably, prompt preparation of the cell extract and rapid isolation of the enzyme by immunoprecipitation are factors that may minimize the appearance of polypeptide no. 2.

The smaller subunit (no. 2) form of the enzyme appears to be quite stable and retains full biological activity. This is seen by the fact that enzyme preparations from livers obtained late after death and that show predominantly polypeptide no. 2 do not appear to exhibit further autolysis, and that the specific activities of the enzyme isolated from these livers are similar to those in which polypeptide no. 1 is the predominant species.

Discussion

In the present study dihydropteridine reductase from human liver, cultured fibroblasts and cultured continuous lymphoid cells were compared by using immunological reactions and two-dimensional gel electrophoresis. The antiserum used was raised in

Fig. 6. Non-equilibrium pH-gradient focusing gels of dihydropteridine reductase

Gels: (a) enzyme (6 μ g) purified immediately after prompt (4h) post-mortem; (b) enzyme (6 μ g) purified from delayed-(12h)-post-mortem liver tissue that had been stored at -20°C for 1 week; (c) mixture of enzyme described in (a) 6 μ g and (b) 6 μ g. The arrows indicate polypeptide nos. 1 and 2 described in the legends to Figs. 3 and 5. Gels were stained for protein (see the Materials and methods section).

rabbits against highly purified human liver dihydropteridine reductase. This antiserum was shown to be mono-specific for the enzyme by double immunodiffusion (Fig. 1) and immunological titration of enzyme activity (Fig. 2).

Characterization of the reductase from cultured fibroblasts and continuous lymphoid cells by immuno-inhibition titration produced curves identical with those obtained against the liver enzyme (Fig. 2), and shows that enzyme from these three sources has the same antigenic determinants. During the preparation of the present paper, Narisawa *et al.* (1980) reported a similar finding showing that the equivalence points were the same for the reductase from human liver, fibroblasts and leucocytes with an antiserum prepared against bovine liver dihydropteridine reductase.

A more critical analysis can be obtained by examining the polypeptides of the enzyme. The isolation of dihydropteridine reductase from radioactively labelled fibroblasts and lymphocytes by immunoprecipitation or one-step affinity chromatography provided sufficient purification to allow high-resolution analysis of the enzyme on two-dimensional gels. Direct comparison (by co-electrophoresis) of the fibroblast and lymphocyte enzyme with purified human liver enzyme showed that all three co-migrated identically (Figs. 3 and 5).

Aksnes *et al.* (1979) have purified and compared bovine dihydropteridine reductase from liver, which has a phenylalanine-hydroxylating system, and adrenal medulla, which has a tyrosine-hydroxylating system, and concluded that the two enzymes are identical. The results obtained in the present study indicate that dihydropteridine reductase from human liver is identical with the enzyme found in cultured human fibroblasts and lymphocytes (these cells do not hydroxylate amino acids).

This suggests that the same structural gene(s) codes for dihydropteridine reductase in each of these tissues. Several other lines of evidence that further support this concept include: (a) the similarity of kinetic properties of dihydropteridine reductase from liver, cultured fibroblasts and lymphocytes [including K_m for substrates, pH optima, temperature optima and inhibitor-susceptibility (Firgaira *et al.*, 1979a, 1981)]; (b) similarity in electrophoretic mobility (under non-denaturing conditions) of enzyme found in the liver and that from peripheral-blood lymphocytes, platelets and granulocytes (Firgaira *et al.*, 1979b); (c) the fact that deficiency of enzyme activity is observed in all tissues and cell types examined in patients diagnosed to be dihydropteridine reductase-deficient (Kaufman *et al.*, 1975; Danks *et al.*, 1979; Firgaira *et al.*, 1979a,b).

Use of the non-equilibrium pH-gradient electrophoretic procedure of O'Farrell *et al.* (1977) resolved dihydropteridine reductase into at least two major polypeptides (nos. 1 and 2, Figs. 3, 5 and 6). This raises the question whether the two are related structurally (i.e. have similar amino acid sequence but post-translationally modified) or are structurally unrelated polypeptides present in the dihydropteridine reductase molecule. There are five pieces of evidence that suggest that they are structurally related. (1) Immunoprecipitates in some cases only gave polypeptide no. 1. (2) There was no constant ratio of the two, as may be expected if there were two non-identical subunits present. (3) Enzyme purified from livers obtained promptly (4h) after death contained mainly no. 1 whereas that purified from livers obtained later (24h) after death contained more of the no. 2 polypeptide. (4) Specific activity remains similar regardless of the ratio of polypeptides nos. 1 and 2 in enzyme purified from liver. (5) Both polypeptides disappear together in dihydropteridine reductase deficiency (F. A. Firgaira, K. H. Choo, R. G. H. Cotton & D. M. Danks, unpublished work).

This would lead us to the possibility that dihydropteridine reductase is specified by only a single gene if only one polypeptide contributes to the reductase molecule.

We do not know how widespread this heterogeneity is in other species, as the non-equilibrium pH-gradient electrophoretic procedure has not been

used by other workers. It is possible that species no. 2 is the first product of degradation of the enzyme but is still active, or it may of course be a normal species *in vivo*, but this would be difficult to prove.

The development of procedures for the rapid isolation and analysis of dihydropteridine reductase from cultured cells should facilitate the study of the mutation arising in patients with inherited catalytic deficiency of this enzyme.

Note added in proof (received 21 April 1981)

We have recently obtained unambiguous identification of a chemical relationship between polypeptides 1 and 2 of dihydropteridine reductase. Radio-iodinated preparations of these polypeptides were submitted to limited proteolysis with *Staphylococcus aureus* proteinase, and their cleavage fragments were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Cleveland *et al.*, 1977). This showed identical peptide 'maps' for both polypeptide species 1 and 2, with the major peptide fragments at molecular weights 22000, 17000, 16000, 12000, 11000 and 6000.

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