Heterogeneity of the molecular defect in human dihydropteridine reductase deficiency

Frank A. FIRGAIRA,* Kong H. CHOO,* Richard G. H. COTTON* and David M. DANKS*†

*Birth Defects Research Institute, Royal Children's Hospital Research Foundation, Parkville, Vic. 3052, and

†Department of Paediatrics, University of Melbourne, Parkville, Vic. 3052, Australia

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Radioimmunoassay, immunoprecipitation, affinity chromatography and two-dimensional gel electrophoresis were used to test cultured cells from three families with dihydropteridine reductase deficiency for a catalytically incompetent product of the mutant gene. No mutant enzyme was detected in one dihydropteridine reductase-deficient homozygote or in her parents. A second homozygote and both her parents had easily detectable concentrations of inactive mutant enzyme. In a third family one parent fitted into each of these categories.

Dihydropteridine reductase (EC 1.6.99.7) is a required enzyme for the hydroxylation of phenylalanine, tyrosine and tryptophan. A deficiency of this enzyme leads to hyperphenylalaninaemia (due to defective hydroxylation of phenylalanine) similar to that observed in phenylketonuria. These patients do not respond clinically to the dietary regime with which classical phenylketonuria is treated, and develop a progressive neurological disorder arising from deficiency of the neurotransmitter products of tyrosine and tryptophan hydroxylation (Danks *et al.*, 1978). To date, the mutational cause of deficient dihydropteridine reductase activity is not known.

We have previously described purification of normal human liver dihydropteridine reductase (Firgaira *et al.*, 1981a) and the development of procedures for the purification and characterization of small quantities of the enzyme from human cultured cells (Firgaira *et al.*, 1981b). Comparison of the purified liver enzyme with that derived from cultured fibroblasts and lymphocytes indicated identical antigenic properties and the enzyme from all three sources showed the same electrophoretic mobility in two-dimensional gel electrophoresis (Firgaira *et al.*, 1981b).

The present paper outlines a study of the mutation arising in patients with inherited catalytic deficiency of dihydropteridine reductase.

Materials and methods

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

Cultured cells

Cultured fibroblasts were available from patient S.P. (Case 4, Danks *et al.*, 1979) and both her parents, patient K.A. (Firgaira *et al.*, 1980) and both her parents; and the parents of patient M.Z. (Case 3, Danks *et al.*, 1979).

Continuous lymphoid cells were available from S.P. and her father.

Healthy volunteers and patients with unrelated diseases provided control cell lines.

Anti-(dihydropteridine reductase) immunoglobulin G

An antiserum to highly purified human liver dihydropteridine reductase was previously prepared and characterized (Firgaira *et al.*, 1981b). The immunoglobulin G fraction of this antiserum was purified by absorption to a column of protein A–Sephrose CL-4B (Pharmacia, Uppsala, Sweden) and eluted with 0.1 M-citrate, pH 3.5 (Ey *et al.*, 1978).

Radiolabelling

Purified human liver dihydropteridine reductase was radioiodinated with Na¹²⁵I by the chloramine-T method (Greenwood *et al.*, 1963). The specific radioactivity of ¹²⁵I-labelled dihydropteridine reductase was 0.35 µCi/µg. At these low values of specific radioactivity the reductase retained its catalytic activity and the protein showed a normal structure as revealed by autoradiography of two-dimensional electrophoretic mapping of the enzyme.

Fig. 1. Competitive radioimmunoassay of dihydropteridine reductase from cultured lymphocytes and fibroblasts from normal and dihydropteridine reductase-deficient subjects

Increasing amounts of test cell extract were added to tubes (1 ml, plastic) containing a fixed amount of ^{125}I -labelled dihydropteridine reductase (25 000 c.p.m.). Each mixture was adjusted to a final volume of 0.3 ml and to equivalent protein concentration by addition of phosphate-buffered saline (Dulbecco & Vogt, 1954) and bovine serum albumin before addition of 5 μl of anti-(dihydropteridine reductase) immunoglobulin G. The volume of immunoglobulin G used was chosen by previous titration experiments (results not shown), and corresponds to the amount that precipitates 50% of radioiodinated antigen in the absence of unlabelled antigen. After incubation overnight at 4°C, 50 μl of a 10% (v/v) solution of protein A ('immunoglobulin G sorb'; The Enzyme Centre, Boston, MA, U.S.A.) was added to the mixture to separate immunoglobulin G from free antigen (Jonsson & Kronwall, 1974; Frohman *et al.*, 1979). After incubation for 15 min at 4°C with gentle mixing, the tubes were centrifuged at 1000g for 15 min. The pellet was washed once in phosphate-buffered saline containing 0.02% Tween-20 and its radioactivity was determined in an LKB gamma-counter. The amount of radioactivity precipitated was corrected for non-specific binding in duplicate experiments with control rabbit immunoglobulin G. Symbols for competing lymphocyte extracts are as follows: ■, control normal; ●, dihydropteridine reductase-deficient homozygote S.P.; ▲, father of S.P. Symbols for competing fibroblast extracts are as follows: □, control normals N1–N6; ○, dihydropteridine

Radioimmunoassay of dihydropteridine reductase cross-reacting material

This was based on the capacity of unlabelled dihydropteridine reductase (from cultured cell extracts) to inhibit the reaction between labelled dihydropteridine reductase and anti-(dihydropteridine reductase) immunoglobulin G. (Experimental details are given in the legend to Fig. 1.)

Other methods

The procedures of affinity chromatography and immunoprecipitation of dihydropteridine reductase from [^{35}S]methionine-labelled cultured fibroblast and lymphoid cells, and analysis by two-dimensional polyacrylamide-gel electrophoresis have been described previously (Firgaira *et al.*, 1981b).

Results

Dihydropteridine reductase activity in cultured cells

The activities of dihydropteridine reductase in cell lines from the three families studied in the present paper have been reported before. In summary, no activity was detected in cultured cells from patient S.P., whereas her parents and the parents of patient M.Z. showed intermediate enzyme activity (approx. 50% of normal values) (Danks *et al.*, 1979; Firgaira *et al.*, 1979). Dihydropteridine reductase in a lymphocyte/platelet preparation isolated from peripheral blood samples from patient K.A. and her parents showed no detectable and intermediate activities respectively (Firgaira *et al.*, 1980). The same results were obtained in assays of cultured fibroblasts from this family (F. A. Firgaira, K. H. Choo, R. G. H. Cotton & D. M. Danks, unpublished work).

Competitive radioimmunoassay

As illustrated in Fig. 1, competition between increasing concentrations of immunoreactive dihydropteridine reductase from the control-normal lymphocyte extract and a fixed amount of ^{125}I -labelled dihydropteridine reductase is indicated by a progressive decrease in the radioactivity found in the corresponding precipitates. In contrast with this, an extract of lymphocytes from patient S.P. did not inhibit the reaction, whereas the lymphocyte extract from the father of this patient showed an intermediate level of inhibition and indicated that the

reductase-deficient homozygotes S.P. and K.A.; Δ, dihydropteridine reductase-deficient heterozygotes M.S.P., F.S.P., M.K.A. and F.K.A., and presumed heterozygotes M.M.Z. and F.M.Z.

amount of cross-reacting material present in the heterozygous father was proportionate to his enzyme activity. A preliminary application of this procedure to test six normal fibroblast lines and fibroblasts from dihydropteridine reductase-deficient individuals (described elsewhere in the text) was carried out and the results are presented in Fig. 1. Only enough fibroblast extract was available to carry out the competitive assay at one concentration for each of these. The values obtained with the six normals (N1–N6) fell within the competition curve obtained against normal lymphocyte extract. The fibroblast extract from patient S.P. showed no cross-reacting material and intermediate concentrations of cross-reacting material were seen for both the mother (M.S.P.) and father (F.S.P.) of this patient (Fig. 1). Fibroblasts of patient K.A. showed approx. 30% cross-reacting material and her parents (M.K.A. and F.K.A.) showed near normal values (Fig. 1), suggesting that the mutation in this patient is quite different to that in patient S.P. Fibroblast extracts from the presumed heterozygous parents of patient M.Z. showed an intermediate amount of cross-reacting material in the mother

(M.M.Z.) and near normal cross-reacting material in the father (F.M.Z.) (Fig. 1).

Structural studies of mutant dihydropteridine reductase

Radioactively labelled ($[^{35}\text{S}]$ methionine) cell extracts from the various mutant homozygous and heterozygous individuals were immunoprecipitated by using a specific anti-(human dihydropteridine reductase) antiserum and analysed by two-dimensional gel electrophoresis as described previously (Firgaira *et al.*, 1981b). Pure human liver dihydropteridine reductase was used as a position marker by co-electrophoresis.

S.P. family. Fig. 2 shows results obtained with immunoprecipitates of lymphocyte cell extracts from a normal subject, patient S.P. and the patient's father. In the fluorogram of the normal cell extract (Fig. 2a), only one polypeptide spot was observed in the dihydropteridine reductase region. Co-electrophoresis with pure human liver enzyme indicated that the polypeptide corresponded to spot no. 1 of the liver enzyme.

Fig. 2. Two-dimensional polyacrylamide-gel analysis of dihydropteridine reductase from $[^{35}\text{S}]$ methionine-labelled lymphocyte cell extracts

Fluorograms showing the dihydropteridine reductase region of two-dimensional polyacrylamide gels of immunoprecipitated [using anti-(dihydropteridine reductase)antiserum] polypeptides from the following lymphocyte lines: (a) control-normal; (b) dihydropteridine reductase-deficient patient S.P.; (c) heterozygote father (F.S.P.). (d) shows a fluorogram of dihydropteridine reductase isolated by affinity chromatography from lymphocytes of a heterozygote (F.S.P.). Designation for the polypeptide spots as nos. 1 and 2 was established by co-electrophoresis with pure human liver enzyme as exemplified in (e), which shows the Coomassie Brilliant Blue-stained gel of (d). A major set of contaminant polypeptides (designated M) in immunoprecipitation experiments was included as a useful position reference marker.

Analysis of the immunoprecipitate obtained from the cell extract of patient S.P. revealed no dihydropteridine reductase polypeptide spot(s) that corresponded to the normal enzyme spots or that had an altered electrophoretic mobility in the dihydropteridine reductase region (Fig. 2*b*). Only one polypeptide spot was immunoprecipitated from the heterozygote father (F.S.P.) (Fig. 2*c*) and this corresponded to spot no. 1 of the co-electrophoresed normal liver enzyme. No other major polypeptide spots were observed in the reductase molecular-weight region and a similar result was obtained when labelled fibroblasts from the heterozygous mother (M.S.P.) were examined.

The possibility that the S.P. family may have a mutant enzyme that is not recognized by our antiserum but that is still able to bind to 1,2-naphthoquinone affinity adsorbent (Cotton & Jennings, 1978; Firgaira *et al.*, 1981*a,b*) was tested by isolation of dihydropteridine reductase from labelled lymphocyte and fibroblast cells from patient S.P., and fibroblasts from F.S.P. and M.S.P., by the one-step affinity-chromatographic procedure described previously (Firgaira *et al.*, 1981*b*). The column fractions corresponding to those that gave enzyme activity, as determined by using an unlabelled normal cell extract, were pooled, concentrated and analysed on two-dimensional polyacrylamide gels.

With cells from patient S.P., none of the reductase polypeptide spots, nor any altered mutant spots, were observed in the reductase region of the gel (results not shown). With the cells from the parents of patient S.P. the enzyme polypeptides had electrophoretic mobilities identical with those of the normal human liver dihydropteridine reductase (Fig. 2*d* shows result obtained with the father F.S.P.).

K.A. family. Fig. 3 shows results of analysis of the reductase in fibroblasts from patient K.A. and her parents. As can be seen, a single polypeptide spot with electrophoretic mobility corresponding to polypeptide spot no. 1 of normal enzyme is immunoprecipitated from cells of these three individuals including the affected homozygote K.A. (Figs. 3*a*–3*c*). The identity of the spot was established by co-migration with normal liver dihydropteridine reductase and by two other means, (i) competition with an excess of unlabelled, highly purified liver dihydropteridine reductase, and (ii) use of a control, pre-immunization serum. In both cases, the designated spot was the only polypeptide that was absent from the gel (Figs. 3*d* and 3*e* respectively).

M.Z. family. When extracts of labelled fibroblast from the parents of patient M.Z. were analysed after immunoprecipitation or affinity-chromatographic purification, dihydropteridine reductase polypeptides with normal two-dimensional electro-

Fig. 3. Two-dimensional polyacrylamide-gel analysis of dihydropteridine reductase immunoprecipitated from [^{35}S]methionine-labelled fibroblast extracts

Fluorograms showing the immunoprecipitated polypeptides from (a) dihydropteridine reductase-deficient patient K.A., (b) heterozygote mother (M.K.A.) and (c) the heterozygote father (F.K.A.). Designation for the polypeptide spot as no. 1 was established by co-migration with pure enzyme (result not shown). (d) Extract from F.K.A. that was incubated with the antiserum in the presence of 5 μg of pure liver dihydropteridine reductase. (e) Immunoprecipitation of extract from F.K.A. using control preimmunization serum. A major set of contaminant polypeptides (designated M) was included as a useful position reference marker in the close-ups shown (a–e) of the dihydropteridine reductase region of the gels.

phoretic mobility were observed, giving pictures (results not shown) similar to those shown in Figs. 3(a)–3(c) and Figs. 2(a), 2(c) and 2(d). In none of the cases studied was the mutant enzyme with altered electrophoretic mobility observed.

Discussion

The primary goal of this work was to examine the nature of the molecular defect in the human inborn error of metabolism arising from catalytic deficiency of the enzyme dihydropteridine reductase. We have studied two unrelated affected children, their respective parents, and the parents of a third patient with presumed dihydropteridine reductase deficiency.

One patient (S.P.) appears to have a complete deficiency of any immunoreactive dihydropteridine reductase. No cross-reacting material was found in lymphocyte or fibroblast extracts using a competitive radioimmunoassay (Fig. 1). No normal or mutant dihydropteridine reductase could be seen in two-dimensional electrophoretic peptide mapping of immunoprecipitates of extracts of radioactively labelled cultured cells (Fig. 2b). No enzyme protein could be purified by an affinity chromatographic procedure. The parents of patient S.P. (who are consanguineous) show approx. 50% of immunoreactive enzyme (as judged by competitive radioimmunoassay; Fig. 1) that is structurally normal (as judged by peptide mapping on two-dimensional polyacrylamide-gel electrophoresis; Figs. 2c and 2d).

Other workers (e.g. Gudas *et al.*, 1978) have shown that mutant polypeptides present in heterozygous cells may be co-purified in association with the normal polypeptides if a protein has a multimer structure. Human dihydropteridine reductase shows two subunits of equal molecular weight (Firgaira *et al.*, 1981a). Analysis of the reductase from lymphocytes or fibroblasts of the (S.P.) parents after purification by either immunoprecipitation (Fig. 2c) or affinity chromatography (Fig. 2d), failed to demonstrate co-purification of an electrophoretically altered enzyme polypeptide with the normal polypeptide.

The failure to detect a mutant molecule in the cells from this family is consistent with a mutation that leads to a total absence of enzyme protein (gene deletion or early frameshift mutation) or a regulatory mutation causing greatly decreased production of enzyme. However, it remains possible that the mutation may have led to the production of an altered molecule that is very unstable or structurally so altered as to be unrecognised by the antibody used or by the affinity adsorbent. Milstein & Kaufman (1975) also failed to detect any antibody cross-reacting material in another patient with dihydropteridine reductase deficiency, when an extract of a liver biopsy was tested by double-

immunodiffusion against an antiserum to sheep liver dihydropteridine reductase.

The second patient (K.A.) in this study has shown approx. 30% of immunoreactive dihydropteridine reductase (Fig. 1), which migrates with the same pI and subunit molecular weight as the normal enzyme (Fig. 3a). The parents (consanguineous) of this patient showed near-normal amounts of cross-reacting material in competitive radioimmunoassay (Fig. 1) and the reductase from these individuals showed normal migration on two-dimensional gel electrophoresis (Figs. 3b and 3c).

The presence of a mutant enzyme with no catalytic capability offers strong evidence for a mutation in the structural locus for dihydropteridine reductase in this family. The alteration of the protein subunit has not been recognized by the electrophoretic systems used and presumably does not involve a change in charge or size.

The reductase from the parents of patient M.Z. (who are not consanguineous) showed normal migration on two-dimensional polyacrylamide-gel electrophoresis, and analysis of affinity-purified enzyme from both parents failed to demonstrate any protein altered in charge or size. The mother of this patient showed an intermediate amount of cross-reacting material, whereas the father had near normal amounts (Fig. 1). Since both parents exhibited approx. 50% enzyme activity the results suggest the presence of mutant cross-reacting material in the father's cells. In the absence of cultured cells from the affected individual (M.Z.), one cannot draw a firm conclusion about the alteration of dihydropteridine reductase in this family, but the results raise the possibility of compound heterozygosity in M.Z.

In summary, heterogeneity of gene mutations is apparent within three patients with dihydropteridine reductase deficiency. We hope to characterize further the extent and nature of mutational heterogeneity at the dihydropteridine locus by protein analysis (of the mutant reductase from patient K.A.) and by studies of cultured cells from other cases of dihydropteridine reductase deficiency.

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