Immunochemical analysis of normal and mutant forms of human pyruvate dehydrogenase

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Pyruvate dehydrogenase (PDH) deficiency has been described in many patients with primary lactic acidosis. However, there are very few cases in which a structural defect in the complex has been clearly demonstrated. Measurement of the activity of the PDH complex in cultured human cells has proved unreliable, and a combination of structural and functional studies are required to make a definitive diagnosis. For this reason, an immunochemical strategy has been developed to complement direct enzyme assay in the detection and further characterization of PDH deficiency. We illustrate the usefulness of this approach by describing defects in the α -subunit of the pyruvate decarboxylase component of the PDH complex in two patients with primary lactic acidosis. In one patient, there is no immunologically cross-reacting material corresponding to this subunit. In the second patient, there appears to be an intrinsic structural defect in the subunit which restricts dephosphorylation (and hence activation) of the inactive phosphorylated complex.

INTRODUCTION

The mammalian PDH complex consists of three enzymes which, acting in sequence, catalyse the oxidative decarboxylation of pyruvate to acetyl-CoA (reaction 1):

The three enzyme components, pyruvate decarboxylase (E1) (EC 1.2.4.1), dihydrolipoamide transacetylase (E2) (EC 2.3.1.12) and dihydrolipoamide dehydrogenase (E3) (EC 1.8.1.4), are arranged in a highly ordered multienzyme structure with a total M_r of approx. 7×10^6 (reviewed by Reed, 1974; Reed *et al.*, 1978). Both the E2 and the E3 components consist of a single type of polypeptide chain, whereas the E1 component is a tetramer of two α and two β subunits.

The activity of the mammalian PDH complex is modulated by two specific regulatory enzymes, a phosphatase and a kinase (Linn *et al.*, 1969). Phosphorylation of serine residues in the α -subunit of pyruvate decarboxylase causes inactivation of the PDH complex (Yeaman *et al.*, 1978), and is catalysed by the MgATP^{2-} -dependent kinase. Dephosphorylation and subsequent re-activation is catalysed by PDH phosphatase.

The PDH complex occupies a key metabolic position between the glycolytic pathway and the tricarboxylic acid cycle. Consequently, mutations affecting the functional integrity of the complex usually have a profound metabolic effect, which manifests as severe lactic acidosis.

Deficiency of the PDH complex is the most commonly defined enzyme defect in patients with primary lactic acidosis (Robinson *et al.*, 1980). However, in spite of the relatively high frequency of this enzyme defect and the importance of PDH in intermediary metabolism, there have been remarkably few structural studies of this enzyme complex in man.

A structural analysis of mutant forms of PDH subunits may provide direct confirmation of PDH deficiency and help to define the basic genetic defects.

The specific structural alterations produced by mutation also offer a unique opportunity to analyse some of the relationships between the structure and function of the normal human PDH complex.

As a further practical consideration, direct demonstration of deficient PDH activity in cultured cells and tissues is often unreliable (Blass, 1980; Sheu *et al.*, 1981). Thus we consider it essential that functional defects of the PDH complex be correlated with structural abnormalities of individual components before a confident diagnosis of PDH deficiency can be made.

In this paper we describe the immunochemical characterization of purified human heart PDH by using antibodies raised in rabbits to the purified bovine heart complex. The immunochemical strategy developed for this study has also been used to demonstrate structural defects in the PDH complex from cultured fibroblasts of two patients with primary lactic acidosis.

CASE HISTORIES

Patient 1

This patient developed a profound lactic acidosis in the newborn period. Delivery was by elective caesarian section at 37 weeks gestation at the Queen Victoria Maternity Hospital, Adelaide, South Australia. At birth he weighed 1700 g and his length was below the third percentile. Some minor dysmorphic features (short fingers, coronal hypospadias and a broad nasal bridge) were noted.

Abbreviation used: PDH, pyruvate dehydrogenase.

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Over the first 4 h of life, the patient developed a metabolic acidosis, subsequently shown to be due to accumulation of lactate and pyruvate. The acidosis was initially corrected by infusion of bicarbonate, and he tolerated some oral feeds. However, on day 3, the plasma [lactate] was 11.2 mmol/l (normal range 0.2–2.0 mmol/l), and he was given intravenous fluids, biotin, thiamin and pyridoxine.

In spite of these measures the acidosis persisted, and glutamate and aspartate were added to the treatment. He remained severely acidotic on day 6 and over the next 24 h developed clinical and radiological evidence of necrotizing enterocolitis. He died on day 8, and post-mortem examination confirmed necrotizing enterocolitis with no other structural abnormalities found.

Patient 2

This patient has a more prolonged course with less severe acidosis. She is the ninth child of unrelated parents. One sibling was born at 24 weeks gestation and died a few hours after birth; another was stillborn at term.

Pregnancy, labour and delivery were normal at 42 weeks gestation, and the patient weighed 2180 g at birth. She fed poorly and by day 6 had lost 300 g weight. Throughout this period she had a mild metabolic acidosis (pH 7.36, $p\text{CO}_2$ 34 mmHg, bicarbonate 20 mmol/l and base excess –6), and this was found to be due to accumulation of lactate and pyruvate [blood lactate 6.1 mmol/l (normal 1.0–1.8 mmol/l), pyruvate 0.37 mmol/l (normal 0.04–0.07 mmol/l)].

She was irritable and hypotonic, with poor visual and auditory responses. An electroencephalogram showed multifocal sharp waves and spikes.

The lactate accumulation did not respond to dietary manipulation or administration of thiamin, but the acidosis could be readily controlled with oral bicarbonate.

The patient is now 13 years old. She has chronic lactic acidosis [blood lactate 2.5 mmol/l, pyruvate 0.29 mmol/l; cerebrospinal-fluid lactate 8.6 mmol/l (normal 1.1–2.3 mmol/l), pyruvate 0.42 mmol/l (normal 0.07–0.11 mmol/l)]. She is profoundly retarded, dwarfed, microcephalic and blind and has a marked spastic tetraparesis.

MATERIALS AND METHODS

Chemicals

Foetal-calf serum and Eagle's modified basal medium were from Flow Laboratories Australasia Pty. Ltd. [$1\text{-}^{14}\text{C}$]pyruvate, [$\gamma\text{-}^{32}\text{P}$]ATP and ^{125}I -labelled protein A were purchased from Amersham International (Amersham, Bucks., U.K.). Hydroxide of Hyamine 10-X was obtained from Packard, and Sepharose 2B was from Pharmacia (Uppsala, Sweden). Bovine serum albumin was purchased from Calbiochem (San Diego, CA, U.S.A.). All other chemicals were of analytical reagent grade.

Purification of bovine heart PDH complex

This was done as described by Stanley & Perham (1980). The only modifications were the omission of phenylmethanesulphonyl fluoride, benzamidinium hydrochloride and silicone antifoam, and the substitution of Sepharose 2B for Sepharose CL-2B in the gel-filtration step.

The activity of the PDH complex could not be assayed in crude tissue extracts. At later stages in the purification, samples were assayed by the method of Brown & Perham (1976), with the reaction mixture modified to 50 mM-potassium phosphate buffer (pH 8.1), 2 mM-NAD⁺, 0.225 mM-thiamin pyrophosphate, 1.25 mM-MgCl₂, 0.15 mM-CoA, 2.6 mM-cysteine and 2.0 mM-pyruvate. Assay was initiated by the addition of enzyme, and the increase in A_{340} was monitored with a Varian-Techtron 635 double-beam recording spectrophotometer, with full scale expanded to 0.5 absorbance unit.

Preparation of human heart PDH complex

Human heart tissue was obtained, with consent, at autopsy. The PDH complex was isolated by the same procedure as that described above for the bovine heart preparation.

Incorporation of [^{32}P]phosphate into the PDH complex

Both purified bovine and human heart PDH preparations contained endogenous PDH kinase activity, which co-purifies with the complex. Therefore incorporation of [^{32}P]phosphate into the E1 α subunit could be achieved by incubating these preparations with [$\gamma\text{-}^{32}\text{P}$]ATP. The reaction mixture contained 50 mM-Tris/HCl, pH 7.5, 80 μM -[$\gamma\text{-}^{32}\text{P}$]ATP (1.5 Ci/mmol), 0.5 mM-MgCl₂ and the PDH preparation. This was incubated for 1 h at 25 °C, and unchanged ATP was removed by dialysis for 16 h at 4 °C against 10 mM-potassium phosphate (pH 7.4)/1 mM-EDTA/0.1 mM-dithiothreitol. The phosphorylated preparations had no enzyme activity; however, this could be restored by incubating the samples with a partially purified preparation of PDH phosphatase.

SDS/polyacrylamide-gel electrophoresis

One-dimensional SDS/polyacrylamide-gel electrophoresis was performed with the Laemmli (1970) Tris/glycine buffer system. Two-dimensional isoelectric focusing/SDS/polyacrylamide-gel electrophoresis was performed by a modification (Choo *et al.*, 1981) of the method of O'Farrell (1975). The isoelectric focusing was carried out for 16 h at constant 450 V, then at 800 V for 1 h, both at room temperature. The SDS/polyacrylamide-gel electrophoresis was for 5–6 h at 25 mA constant current.

Immunization schedule

Initially 100 μg of purified bovine heart PDH complex was emulsified in complete Freund's adjuvant and injected intramuscularly into a rabbit. After 1 month, booster injections of 100 μg of protein in incomplete Freund's adjuvant were given intramuscularly at irregular intervals. The animal was bled from the ear vein approx. 10 days after each boost. Pre-immune serum was collected from the same animal before immunization.

The IgG fraction was collected from both pre- and hyper-immune sera by overnight dialysis against 18% (w/v) Na₂SO₄ at room temperature (Keckwick, 1940). Batches were stored frozen at –70 °C.

'Western blot' analysis

Immediately after fractionation by SDS/polyacrylamide-gel electrophoresis, protein samples were electrophoretically transferred to nitrocellulose (Millipore Corp., Bedford, MA, U.S.A.) with 30 V for 18 h at

4 °C, in 25 mM-Tris/192 mM-glycine/20% (v/v) methanol (pH 8.3). Nitrocellulose filters were subsequently probed with antibody by a modification of the methods of Towbin *et al.* (1979) and Burnette (1981). Excess protein-absorption sites were blocked by incubation for 2 h in 0.9% NaCl/10 mM-Tris/HCl, pH 7.4 (Tris/saline) containing 3% (w/v) Carnation skim-milk powder (Johnson *et al.*, 1984) and 0.5% gelatin. Filters were then incubated for 2 h in a fresh solution of Tris/saline/3% Carnation skim-milk powder containing the appropriate antibody solution, followed by three washes (10 min each) in excess volumes of Tris/saline, Tris/saline + 0.05% (w/v) Nonidet P40, and Tris/saline respectively. Filters were then immersed for 30 min in Tris/saline/5% (w/v) bovine serum albumin containing ^{125}I -labelled protein A (0.1 μCi /one-dimensional-electrophoretic track; 0.5 μCi /two-dimensional gel). After washing as above, filters were blotted dry and autoradiographed on Kodak XAR X-ray film with an intensifying screen (DuPont Lightning Plus) at -70°C .

Cell culture and preparation

Fibroblast lines were from the tissue-culture collection of the Birth Defects Research Institute, Royal Children's Hospital, Melbourne, Australia. Cells were grown from forearm skin biopsies in Eagle's modified basal medium supplemented with 10% foetal-calf serum, neomycin sulphate (12.5 $\mu\text{g}/\text{ml}$) and polymyxin B sulphate (12.5 $\mu\text{g}/\text{ml}$). All lines were shown to be free of mycoplasma by Hoechst 33258 staining (Chen, 1977).

Confluent cells were harvested with trypsin/EDTA, and the pellets were collected and washed once by centrifugation (800 g, 7 min) in sterile phosphate-buffered saline (0.137 M-NaCl/2.7 mM-KCl/9.6 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 , pH 7.40). For enzyme assay, pellets were homogenized with glycerol as described for platelets by Schofield *et al.* (1980). Mitochondrial extracts for Western blotting analysis were prepared from fibroblasts by the method of Zuurendonk & Tager (1974).

Assay of total PDH complex activity in fibroblasts

The method used was a modification of that described by Schofield *et al.* (1980), with $[1-^{14}\text{C}]$ pyruvate substituted for $[1-^{14}\text{C}]$ lactate. In a total volume of 0.5 ml, the reaction mixture contained 40 mM-potassium phosphate (pH 7.4), 1.3 mM-EDTA, 2.5 mM- MgCl_2 , 8 mg of albumin/ml, 10 mM-2-mercaptoethanol, 2.5 mM- CoA , 1.6 mM- NAD^+ , 0.9 mM-thiamin pyrophosphate, 1 mM- Na_2SO_3 , 5 μl of dialysed rat serum (Hyland & Leonard, 1983) and 50–120 μg of cell protein. Reaction was initiated by the addition of 0.26 mM-pyruvate labelled with 0.9 Ci of $[1-^{14}\text{C}]$ pyruvate/mol (0.25 $\mu\text{Ci}/\text{ml}$) and allowed to continue for 90 min at 37°C in a shaking water bath. The $^{14}\text{CO}_2$ liberated was trapped with hydroxide of Hyamine 10-X by the method of Fox (1971). Reaction was terminated with 1.0 ml of 50% acetic acid. After a further 15–30 min incubation, trapped radioactivity was quantified in a liquid-scintillation counter. To account for non-specific production of $^{14}\text{CO}_2$, controls with heat-denatured cell protein were assayed simultaneously.

PDH phosphatase assay

The $[^{32}\text{P}]$ phosphate-labelled purified PDH preparations were used to develop a sensitive assay for PDH phosphatase which could be used to measure this enzyme

in extracts of fibroblast mitochondria. Preparations of the $[^{32}\text{P}]$ phosphate-labelled PDH complex with approx. 1000 d.p.m. of $^{32}\text{P}/\mu\text{g}$ of protein were used. The reaction mixture contained approx. 15 μg of this preparation in 50 mM-Mops (pH 7.2)/10 mM- MgCl_2 /7.8 mM- CaCl_2 /8 mM-EGTA/0.4 mM-EDTA/0.5 mM-dithiothreitol. Mitochondrial extracts were added and, after various periods of incubation at 37°C , samples were removed for the determination of trichloroacetic acid-precipitable radioactivity.

Protein determination

All protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

Purified bovine heart PDH complex

The PDH complex was purified from bovine heart, to a final specific activity of 16.5 $\mu\text{mol}/\text{min}$ per mg of protein. When fractionated on a one-dimensional SDS/polyacrylamide gel, the purified complex showed five major bands (Fig. 1b). Four of these bands corresponded closely in M_r to previously published values for the electrophoresed components of the bovine heart PDH complex (Barrera *et al.*, 1972). Thus bands that migrate with estimated M_r values of 72000, 55000, 42000 and 34500 were said to correspond respectively to dihydrolipoamide transacetylase, dihydrolipoamide dehydrogenase and the α and β subunits of pyruvate decarboxylase. The remaining band, which migrates in the Laemmli electrophoretic buffer system with an apparent M_r of approx. 52000 has not been identified, but its consistent presence has led to its designation as 'component X' (De Marcucci *et al.*, 1985). It has been suggested that this band corresponds to the regulatory kinase specifically associated with the mammalian PDH complex (Stanley & Perham, 1980); however, the apparent M_r does not correspond to that of either of the subunits of the purified kinase (Stepp *et al.*, 1983).

Immunological profile

One-dimensional Western-blot analysis revealed that antibodies raised against purified bovine heart PDH react with all components of the complex. Fig. 1 compares an immunoassayed strip of nitrocellulose (track c) with a similar strip stained for protein after electrophoretic transfer (track b). The various components differed in their antigenicity, as measured by the amount of antibody bound/unit of protein.

Antibody binding was determined by cutting out the bands on the nitrocellulose filters after Western transfer and counting the radioactivity present. The protein content of each component was measured by densitometry after protein staining. The antibodies preferentially recognize the E2 and E1 α components, with relative amounts of antibody bound/unit of protein of 100, 46, 16 and 15 for the E2, E1 α , E1 β and E3 components respectively.

Purified human heart PDH complex

The human heart PDH complex preparation had a final specific activity of 6.7 $\mu\text{mol}/\text{min}$ per mg of protein. When analysed by SDS/polyacrylamide-gel electrophoresis, the overall pattern of subunits was very similar to

Fig. 1. Comparison of antibody-probed and protein-stained nitrocellulose after transfer of proteins from an SDS/polyacrylamide-gel

Purified bovine and human heart PDH complexes (1.6 μ g and 3.5 μ g; tracks *c* and *d* respectively) were fractionated on a one-dimensional SDS/9% polyacrylamide gel and transferred to nitrocellulose. They were probed with a 1:200 dilution of anti-PDH IgG, followed by 125 I-labelled protein A, and autoradiographic exposure for 7 h. When IgG prepared from pre-immune rabbit serum was substituted for the specific antibody, no bands were observed (not shown in this Figure). The sample of bovine heart PDH (10 μ g) shown in track (*b*) and the accompanying M_r standards (track *a*) were stained for protein with 0.1% Amido Black in 45% methanol/10% acetic acid. Complete transfer of proteins from the gel to the nitrocellulose was achieved, and the profile shown in track (*b*) is identical with that seen on a gel stained with Coomassie Blue. Numbers adjacent to track (*a*) are the M_r values of the marker proteins.

that of the bovine heart enzyme, but all components except the E1 α subunit migrated to different positions. A comparative densitometric tracing of Coomassie-Blue-stained gel tracks of the two preparations is shown in Fig. 2. The apparent M_r values of the human heart PDH components are: E1, 69500; E3, 56500; E1 α , 42000; E1 β , 35000.

All components of the human PDH complex were recognized by antibodies raised against the bovine heart preparation; a comparison of Western-blot analyses of the two preparations is shown in Fig. 1(*d*). In addition to the differences in apparent M_r described above, there are also differences in the relative reactions of the different components with the antibodies. All components of the human enzyme bound less antibody/unit of protein than did the corresponding component of the bovine enzyme.

The difference was greatest for the E2 component, where the amount of antibody bound by the human protein was only 24% of that bound by the bovine heart

Fig. 2. Densitometric tracing of human (—) and bovine (---) heart PDH complexes

Equal amounts (10 μ g) of purified human and bovine heart PDH complexes were fractionated by SDS/polyacrylamide-gel electrophoresis, and the gels were stained with Coomassie Blue. The gels were then scanned with an Helena Quickscan R and D densitometer.

preparation. The human E3, E1 β and E1 α , components bound 30%, 55% and 58% respectively of the amount of antibody bound by the corresponding bovine PDH subunits.

Two-dimensional electrophoresis of purified PDH

The bovine and human heart pyruvate dehydrogenase complexes were analysed by two-dimensional gel electrophoresis, before and after incorporation of [32 P]phosphate. Both preparations showed considerable charge heterogeneity in the E1 α component and a lesser degree in the E2. The profile was almost identical in the two species, and that of the human heart preparation is shown in Fig. 3.

The forms of the E1 α subunit with the lowest pI values are associated with different states of phosphorylation of this component. These more highly charged forms were shown to contain phosphate groups by labelling with [γ - 32 P]ATP (Fig. 3). In active purified preparations of both human and bovine heart PDH complexes, a form of relatively high pI, which is not phosphorylated, predominates. When the preparations are inactivated by incubation with MgATP $^{2-}$, this form disappears and the forms with lower pI values are augmented (Fig. 3). The proportions of the different forms of the E1 α subunit could also be altered by changing the state of phosphorylation of the complex by other methods (see below). No other subunits were labelled in the presence of [γ - 32 P]ATP.

As there are three phosphorylation sites on the E1 α subunit (Yeaman *et al.*, 1978) there are seven possible phosphorylated forms with different arrangements of occupancy of the sites. In fact, only three major spots of different pI are observed, as the presence or absence of a phosphate group has a much greater effect on the pI than does its position. The full pH range of the isoelectric-focusing gel is 7.0 to 4.3, and the difference in pI between the different phosphorylated forms is ~ 0.2 pH unit. This is comparable with previously

Fig. 3. Two-dimensional isoelectric focusing/SDS/polyacrylamide-gel electrophoresis of purified human heart PDH

Two 70 μ g portions of purified human heart PDH were analysed by two-dimensional isoelectric focusing/SDS/polyacrylamide gel electrophoresis. One sample (a) was left in the active, non-phosphorylated state. The other (b) was inactivated and labelled with [32 P]phosphate by incubation with [γ - 32 P]ATP as described in the Materials and methods section. The resulting gels were stained with Coomassie Blue and photographed. The gel with the labelled sample was then dried down and exposed to Kodak XAR X-ray film. The autoradiograph was photographed and superimposed on the photograph of the corresponding protein-stained gel (b) with slight displacement to show phosphorylated forms of the E1 α subunit. The arrow indicates the position of the non-phosphorylated component, which is the major form of the E1 α subunit in active preparations. Parallel isoelectric-focusing gels were run simultaneously, then sliced at 0.5 cm intervals, and the Ampholines were eluted with degassed distilled water. The pH of the solutions was measured, and at the bottom of the Figure is a scale of pH as a function of distance for these gels.

demonstrated shifts in the pI of proteins on phosphorylation, of between 0.05 and 0.27 pH unit (Garrison & Wagner, 1982).

Analysis of human cultured cell lines

Enzyme assay. Activity of the total PDH complex was virtually undetectable in the cell line derived from Patient 1 (Table 1). Cell lines from both his parents showed no evidence of deficient activity compared with 12 control cell lines. Amniotic cells obtained from the

Table 1. Total PDH complex activity in cultured fibroblasts

PDH activity was measured in sonicated preparations of cultured fibroblasts with [1 - 14 C]pyruvate as substrate (see the Materials and methods section). The results are the mean of duplicate determinations, except for the controls (*) where the results are the mean activity $\pm$ s.d. for 12 normal fibroblast lines, and the results for Patient 2 ($\dagger$), where the mean activity $\pm$ s.d. is given for eight separate determinations, each in duplicate.

Cell line	PDH activity (nmol/min per mg of protein)
Controls	0.78 ± 0.22 (12)*
Patient 1	0.08
Mother	0.84
Father	0.55
Amniotic cells from subsequent pregnancy	0.64
Control amniotic cells	0.51
Patient 2	$0.32 \pm 0.07\dagger$

mother during a subsequent pregnancy have also been assayed for PDH activity. No difference was found between these cells and control amniotic cells and fibroblasts. Cultured fibroblasts from Patient 2 showed significant residual activity (Table 1). Cell lines were not available from the parents of this patient.

Immunochemical studies. Mitochondrial extracts prepared from cultured human fibroblasts were probed with antibodies to the bovine heart PDH complex, by the technique of Western blotting. When extracts from control cell lines were probed after fractionation on one-dimensional gels, E1 α and E2 components of the PDH complex reacted strongly (Fig. 4). The unidentified (X) band was also recognized by the antiserum, and those corresponding to the E1 β and E3 components were detected after longer autoradiographic exposure. When extracts from Patient 1 were analysed in the same way, the band corresponding to the E1 α subunit could not be detected (Fig. 4). With prolonged exposure, both the E1 β and E3 components could be demonstrated. As exactly comparable amounts of mitochondrial protein were loaded on all tracks, it appears that there is also a secondary decrease in the other subunits of the complex in this patient relative to control lines. Again, as with enzyme-assay results, both parents showed normal patterns, as did the amniotic cells from the subsequent pregnancy (results not shown).

With one-dimensional Western-blot analyses, no abnormality could be detected in mitochondrial extracts from cells of Patient 2. However, when these extracts were subjected to two-dimensional electrophoresis before blotting, the profile differed from that of control cell lines.

The pattern of spots in control fibroblast lines is comparable with that of purified human heart PDH (Fig. 5a). Mitochondria prepared under identical conditions from five independent control lines consistently gave this same pattern.

When the analysis was repeated with mitochondrial extracts from cells grown in the presence of dichloro-

Fig. 4. Western-blot analysis of human fibroblast mitochondria

Extracts of fibroblast mitochondria (100 μ g) were fractionated on SDS/9%-polyacrylamide gels, transferred to nitrocellulose and probed with antibody as in Fig. 1. Tracks 1 and 3 are control fibroblast lines, track 2 is for patient 1, and track 4 is the purified bovine enzyme. The exposure time for autoradiography was 4 days. With further exposure, bands corresponding to the E3 and E1 β subunits could also be detected.

acetate, a compound which inhibits the regulatory PDH kinase (Whitehouse *et al.*, 1974), a different pattern of the spots corresponding to the E1 α subunit was found (Fig. 5*b*). Under these conditions, forms of higher pI predominated, particularly the form of relatively high pI which corresponds to the non-phosphorylated form of the purified human enzyme. None of the other PDH components showed any alteration in position after treatment of the cells with dichloroacetate.

Mitochondrial extracts of cells from Patient 2, prepared under standard conditions, showed a predominance of highly charged forms (Fig. 5*c*). After treatment of the cells with dichloroacetate, there was a partial shift to less charged forms, but not to the high-pI non-phosphorylated form found in control cells under these conditions (Fig. 5*d*). To investigate the possibility of a primary defect in PDH phosphatase, the ability of mitochondrial extracts to release [32 P]phosphate from purified PDH labelled with [γ - 32 P]ATP was measured. There was no difference between the patient's mitochondrial extracts and those of control cell lines in this assay of PDH phosphatase activity (Fig. 6). Release of 32 P from a non-specific phosphoprotein of comparable specific radioactivity (32 P-labelled phenylalanine hydroxylase) was 25% of that released from phosphorylated PDH by mitochondrial extracts.

DISCUSSION

Antibodies to purified bovine heart PDH have been used to analyse the structure of the normal human heart

Fig. 5. Two-dimensional isoelectric focusing/SDS/polyacrylamide-gel electrophoresis and Western transfer of fibroblast mitochondria

Mitochondrial extracts (300 μ g) from a control fibroblast line (*a*, *b*) and fibroblasts from Patient 2 (*c*, *d*) were analysed by two-dimensional isoelectric focusing/SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. The proteins were transferred to nitrocellulose and probed with the antibody as in Fig. 1. Profiles (*a*) and (*c*) are of mitochondrial extracts prepared from cells grown under normal conditions. Profiles (*b*) and (*d*) are from extracts of cells cultured for 24 h before harvesting in 1 mM-dichloroacetate. This compound was also included in all solutions used to harvest the cells and prepare the mitochondrial extracts. For each pair of extracts, (*a*) and (*c*) and (*b*) and (*d*), the isoelectric-focusing gels were run in parallel and the SDS/polyacrylamide gels were run simultaneously under identical conditions. Between each pair is a scale of pH determined as described in the legend to Fig. 3.

Fig. 6. PDH phosphatase activity in fibroblast mitochondria

Mitochondrial extracts from a control fibroblast line (●) and from fibroblasts from Patient 2 (■) were incubated with [^{32}P]phosphate-labelled purified bovine heart PDH prepared as described in the Materials and methods section. The amount of ^{32}P remaining in trichloroacetic acid-insoluble material after the times shown was measured by trapping the labelled precipitates on glass-fibre filters and counting the radioactivity by liquid-scintillation spectrometry.

complex and mutant forms of the enzyme in cultured fibroblasts from patients with primary lactic acidosis.

The fact that antibodies produced against purified bovine heart PDH cross-react with the corresponding subunits of the human enzyme is consistent with the belief that the PDH complex is similar in all mammalian species and tissues. The most striking findings in the present study were the apparent differences in M_r between all but the $\text{E1}\alpha$ subunit of the human and bovine complexes. These differences were not detected in immunoprecipitation experiments by Robinson (1983), but a similar finding has been reported for the E2 component in rat tissues (De Marcucci *et al.*, 1985; Matuda *et al.*, 1983; Sheu & Young, 1984). Whether this represents a true difference in M_r is not known, however, as this component is thought to migrate anomalously on SDS/polyacrylamide-gel electrophoresis. More reliable methods of M_r determination have led to an estimate of 52000 for the E2 component of bovine kidney and heart PDH, as opposed to approx. 74000 determined by SDS/polyacrylamide-gel electrophoresis (Barrera *et al.*, 1972; Pettit & Reed, 1982).

Not all components of the PDH complex are equally immunogenic. In particular, the E3 component, which is common to all 2-oxoacid dehydrogenases, has been found to be an extremely weak immunogen (De Marcucci *et al.*, 1985). We also found an apparently decreased immunogenicity of the E3 component relative to the E2 and $\text{E1}\alpha$, although immunological cross-

reactivity was clearly evident with adequate protein loading and autoradiographic exposure of Western blots. Our antibodies also appear to have relatively weak reactivity towards the $\text{E1}\beta$ component, as shown by the relative binding results. Antibodies raised against purified bovine heart PDH recognize all of the components of the human heart complex, but with lower affinity. Cross-reaction is least for the E2 component, which shows the greatest difference on SDS/polyacrylamide-gel electrophoresis, and greatest for the $\text{E1}\alpha$ subunit, which behaves identically with the bovine $\text{E1}\alpha$ on one- and two-dimensional electrophoresis.

The immunochemical reagents described in the present paper were primarily developed to study structural defects in individual subunits of the PDH complex in patients with PDH deficiency. As an example of their usefulness in this type of analysis, results are presented of two such patients.

In these patients, a functional defect of PDH was detected by measurement of the activity of the complex in extracts of cultured cells. This was then shown to be associated with structural defects involving the α -subunit of the E1 or pyruvate decarboxylase component.

In fibroblast extracts from Patient 1, no cross-reacting material corresponding to the $\text{E1}\alpha$ subunit could be detected by Western-blot analysis, using a polyclonal antiserum which recognizes this subunit in normal individuals. This suggests that the $\text{E1}\alpha$ subunits are completely lacking or that, if present, they are either decreased to an undetectable amount or are structurally altered so as to be unrecognized by the antiserum. The second patient appears to have a defect in dephosphorylation and subsequent activation of the $\text{E1}\alpha$ subunit. As a primary deficiency of PDH phosphatase seems unlikely, a detailed analysis of this subunit is needed to determine whether there is an intrinsic structural abnormality which prevents activation by the specific PDH phosphatase once the enzyme is in the inactive, phosphorylated, form.

Confidence in making a diagnosis of PDH deficiency from measurements of enzyme activity in extracts of cultured cells is limited by the intrinsic variability of the usual assay system, based on release of $^{14}\text{CO}_2$ from [$1\text{-}^{14}\text{C}$]pyruvate (Silverstein & Boyer, 1964; Blass *et al.*, 1972; Willems *et al.*, 1978). The interpretation of assay results is further complicated by uncertainty about the state of activation of the PDH complex in different cell extracts. We have found that pretreatment of the cells with dichloroacetate to convert all of the complex into the fully active form (Sheu *et al.*, 1981) is not sufficiently reliable to overcome this problem. In view of the difficulties associated with direct enzyme assay, the immunochemical techniques described in the present paper provide a valuable means of detection, confirmation and further characterization of PDH deficiency.

The combination of enzyme assay and immunochemical analysis also gives a greater degree of confidence for pre-natal diagnosis of PDH deficiency. In previous patients we have not considered results of enzyme assay alone to be sufficiently reliable for this type of investigation. For Patient 1, analysis of amniotic cells from a subsequent pregnancy demonstrated both normal enzyme activity and a normal immunochemical pattern of subunits, giving an additional degree of certainty that this foetus is not affected.

Further characterization of the structural defects in

these patients and the genetic basis for them is needed. In addition, we have obtained antisera to the individual components of the PDH complex for further studies of these and other patients.

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