Optimized Spectrophotometric Assay for the Completely Activated Pyruvate Dehydrogenase Complex in Fibroblasts

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Background: Analysis of the pyruvate dehydrogenase complex (PDHc) activity in human skin fibroblasts is hampered by low enzyme activity in the cells. The most commonly used radiochemical method detects the formation of 14CO2, an endproduct of the E1 component of PDHc, from [1-14C]pyruvate.

Methods: Here we report a spectrophotometric method for the analysis of PDHc activity in fibroblasts based on detection of NADH formation via a p-iodonitrotetrazolium violet (INT)-coupled system. We investigated in detail the specific requirements of this assay, such as cofactor requirements and the effects of suggested stimulatory compounds and different cell disruption procedures. The reliability of the optimized assay was studied by investigation of patients previously diagnosed with PDHc deficiency and by comparison with results from the radiochemical method.

Results: Mean (SD) total PDHc activities were 136 (31) and 58 (21) mU/U of citrate synthase in fibroblast homogenates from 10 healthy volunteers and 7 PDHc-deficient patients, respectively, by the spectrophotometric assay. Similar results were obtained in a mitochondrial fraction. Dithiothreitol (DTT) increased the nonspecific inhibitor-insensitive rate with less pronounced effect on the specific rate of PDHc activity. Administration of DTT increased PDHc activity to 193 (3)% of control activity (without DTT), but decreased the inhibitor-sensitive rate from 99 (0.3)% (without DTT) to 69 (2)% (with 0.3 mmol/L DTT).

Conclusion: The simple, optimized spectrophotometric assay for PDHc analysis allows reliable investigation of the enzyme complex in human skin fibroblasts.

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Many disorders caused by or associated with a disturbance in pyruvate metabolism have been reported (1, 2). The pyruvate dehydrogenase complex (PDHc)5 plays a pivotal role in energy metabolism: it catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA, which is an important entry point for activated two-carbon acetyl units into the tricarboxylic acid cycle. Thus, PDHc plays a key role in controlling the gateway for complete carbohydrate oxidation.

PDHc is a multienzyme complex localized in the inner mitochondrial membrane. It consists of six different components: pyruvate decarboxylase (E1; EC 1.2.4.1), dihydrolipoic transacylase (E2; EC 2.3.1.12), dihydrolipoyl dehydrogenase (E3; EC 1.8.1.4), two regulatory enzymes [PDH kinase (EC 2.7.1.99) and PDH phosphatase (EC 3.1.3.43)], and the E3-binding protein (3).

The first three subunits (E1, E2, E3) are the major components of PDHc.
catalytic components and act in sequence. PDHc is regulated mainly by a phosphorylation-dephosphorylation cycle catalyzed by PDH kinase or phosphatase, respectively (4). PDHc activity is also regulated by feedback inhibition by NADH and acetyl-CoA (5). PDHc deficiency is a frequent cause of inherited mitochondrial disorders, mostly affecting the E1 component (6).

The central role of PDHc in mitochondrial energy metabolism and the severe consequences of PDHc deficiency (7) underline the necessity of having a reliable method for PDHc analysis in the diagnostic work-up of mitochondrial disorders. Existing methods for the analysis of PDHc activity are based on measurement of the end-products of the enzymatic cascade, i.e., NADH, acetyl-CoA, and CO₂. The formation of NADH or acetyl-CoA end-products of the enzymatic cascade, i.e., NADH, acetyl-CoA, is a coupled enzyme assay using pigeon liver arylamine acetyltransferase for measurement of acetyl-CoA (9). A particular disadvantage of this method is that it does not reliably determine PDHc activity in fibroblast extracts (13). Therefore, the major aim of the present study was to develop a reliable spectrophotometric assay with high sensitivity and specificity for the analysis of completely activated PDHc activity in tissues low in mitochondria, such as human skin fibroblasts.

**Materials and Methods**

**Materials**

Pyruvic acid sodium salt, 3-fluoropyruvic acid sodium salt, α-L-carnitine, CoA lithium salt, NAD⁺, thiamine pyrophosphate (TPP), phenazine methosulfate (PMS), p-iodonitrotetrazolium violet (INT), crystallized bovine serum albumin (BSA), Triton X-100, sucrose, oxalacetic acid, 5,5-dithio-bis(2-nitrobenzoic acid), acetyl-CoA, uridine, dichloroacetic acid (DCA), Tris, HEPES, EGTA, dithiothreitol (DTT), and monobasic potassium phosphate were all purchased from Sigma Aldrich. EDTA was obtained from Roth. Magnesium chloride hexahydrate, calcium chloride, and potassium chloride were from Merck. All chemicals obtained were of the highest purity available.

PDHc activities were recorded with a computer-controllable spectrophotometer (Spectramax PLUS 384 Microplate Reader; Molecular Devices) operating in the dual wavelength mode. DMEM, MEM, and phosphate-buffered saline (PBS) were obtained from Cell concepts. Trypsin-EDTA, amphotericin B, penicillin/streptomycin, RPMI 1640, and fetal calf serum were purchased from PAA Laboratories GmbH. Gentamicin was obtained from Invitrogen, and the mycoplasma test was from Gen-Probe.

**CONTROLS AND PATIENTS**

Human skin fibroblast cell cultures from seven patients with PDHc deficiency were included into this study. All patients had decreased PDHc activity as measured by radiochemical analysis in different tissues, i.e., muscle and fibroblasts. In two patients, PDHc deficiency was confirmed to result from disease-causing mutations in the E1α gene of the PDH complex. In five patients, mutation analysis of this gene did not reveal any disease-causing mutation, suggesting an involvement of other components of the PDHc. Mutation analysis of the E1α gene was performed according to Brown et al. (14). In parallel to the spectrophotometric analysis, PDHc deficiency was investigated in fibroblasts and/or muscle tissue of these patients by a radiochemical method with [1-14C]pyruvate as substrate, as described by Robinson et al. (15) with the modifications of Sperl et al. (16). Control human skin fibroblasts from forearm skin biopsies were obtained from 11 healthy control individuals. Skin biopsies and subsequent experiments in human skin fibroblast cultures were performed after receipt of informed consent.

**HUMAN SKIN FIBROBLAST CULTURES**

Human skin fibroblasts from forearm skin biopsies were cultivated to confluency under standard conditions at 37 °C in DMEM supplemented with 100 mL/L fetal calf serum, 100 mg/L penicillin, 100 mg/L streptomycin, 2.5 mg/L fungizone, and 200 μmol/L uridine (17, 18). The medium was changed twice a week. Each cell culture was tested for contamination with Mycoplasma spp. before enzyme measurement.

**Preparation of submitochondrial particles from bovine heart**

To establish and optimize the spectrophotometric PDHc analysis, we used submitochondrial particles (SMPs) from bovine heart as a model for PDHc in our study. SMPs were isolated as described previously (19) and were stored in liquid nitrogen until they were used for the experiments.

**Preparation of crude tissue homogenate**

Confluent fibroblast cell cultures (~1–2 mg of total cell protein; one or two 75-cm² flasks) were harvested with 10
mL of 0.5 g/L trypsin–0.2 g/L EDTA, washed twice with PBS, and subsequently centrifuged (3000gmax). Cells were then suspended to a final protein concentration of 1 g/L in PBS. DCA (5 mmol/L) was added to specifically inhibit the inactivating kinase (but not the activating phosphatase) of PDHc, as described previously (20). The suspensions were incubated at 37 °C for 15 min. After activation with DCA, cultured fibroblasts were collected by centrifugation (3000gmax). The cells were washed once with PBS, and the cell pellet was resuspended in 3 mL of buffer containing 250 mmol/L sucrose, 1 mmol/L EGTA, and 10 mmol/L HEPES (adjusted to pH 7.5) and homogenized by 20 strokes in a tight-fitting ground-glass Potter-Elvehjem. The homogenization procedure was carried out at 0–4 °C. After centrifugation at 13 000gmax (4 °C), the supernatant was discarded. The pellet was stored in liquid nitrogen until measurement. The enzyme measurements were performed within 3 months after freezing. Immediately before PDHc measurement, the crude tissue pellet was frozen and thawed three times (−196 and 25 °C) and suspended at a protein concentration of ~4 g/L in 1 mL/L Triton X-100, 1 mmol/L CaCl2, 5 mmol/L MgCl2, 50 mmol/L KCl, 250 mmol/L sucrose, and 20 mmol/L Tris-HCl (pH 7.5). For some experiments (Table 1), the suspended samples were sonicated for 20 s (power output control 20% of maximum value) rather than being subjected to the freezing-thawing procedure.

To stimulate the phosphatase, which activates the E1 component of the PDHc, by calcium and magnesium, we preincubated the cell suspension at 37 °C for 10 min before measurement (10). To measure basal PDHc activity, we preincubated the cell pellet with DCA, calcium, and magnesium. The PBS used for our experiments was free of calcium and magnesium. The citrate synthase activity and protein concentration of each sample were determined to normalize PDHc activity (21, 22).

PREPARATION OF A MITOCHONDRIA-ENRICHED FRACTION

Confluent fibroblasts (~4–5 mg of total protein; five to six 75-cm² flasks) were harvested with trypsin and washed twice with PBS. To activate PDHc, the pellet was incubated at 37 °C with DCA as described above. The cells were then washed once more with PBS. Isolation of mitochondria from the cell pellet was performed at 4 °C according to Krähenbühl et al. (23) with some modifications. Briefly, the cell pellet was frozen at −80 °C for at least 15 min. After thawing, the pellet was suspended in 2 mL of isolation buffer containing 250 mmol/L sucrose, 1 mmol/L EGTA, 10 mmol/L HEPES, and 5 g/L BSA (pH 7.5) and then was centrifuged at 5000gmax for 2 min. The supernatant was discarded, and the remaining pellet was suspended in 3 mL of isolation buffer. The cell suspension was homogenized in a tight-fitting ground-glass Potter-Elvehjem. After centrifugation at 1500gmax for 10 min, the supernatant was kept on ice. The pellet was homogenized and centrifuged as described above. The two supernatants were pooled and centrifuged at 10 000gmax for 10 min. The resulting mitochondrial pellet was washed twice with BSA-free isolation buffer and stored in liquid nitrogen until measurement. For the analysis of PDHc activity the mitochondrial pellet was thawed and suspended at a protein concentration of ~4 g/L in 1 mL/L Triton X-100, 1 mmol/L CaCl2, 5 mmol/L MgCl2, 50 mmol/L KCl, 250 mmol/L sucrose, and 20 mmol/L Tris-HCl (pH 7.5). Similar to the preparation of tissue homogenates, the mitochondrial suspension was preincubated for 10 min at 37 °C before PDHc measurement, and an aliquot of the mitochondrial suspension was used for measurement of citrate synthase activity and protein content (21, 22).

SPECTROPHOTOMETRIC ASSAY FOR MEASUREMENT OF PDHc ACTIVITY

The reaction mixture contained 5 mmol/L l-carnitine (10), 2.5 mmol/L NAD, 0.2 mmol/L TPP, 0.1 mmol/L CoA, 5 mmol/L pyruvate, 1 mL/L Triton X-100, 1 mmol/L MgCl2, 1 g/L BSA, 0.6 mmol/L INT, and 6.5 μmol/L PMS in 0.05 mol/L potassium phosphate buffer (pH 7.5) according to Hinman and Blass (24) with some modifications. For some experiments, 25 mmol/L oxamate (Fig. 1 and Table 1) (8) was added to the test buffer. Experiments in the presence of DTT (Fig. 2) (24) were performed by adding 0.3 mmol/L DTT just before enzyme measurement. Assays were performed at 25 °C in a dual-wavelength spectrophotometer; we monitored the increase in absorbance at 500–750 nm, which was attributable to reduction of INT, the final electron acceptor (12.4 mmol·L⁻¹·cm⁻¹). Stock solutions of INT were prepared at a saturating concentration of 3 g/L in 0.05 mmol/L potassium phosphate, stirred at 4 °C for 1 h to aid solubilization, and then filtered (Whatman no. 1 filter paper; 0.22 μm pore size). PMS stocks were prepared at a concentration of 0.2 g/L. Both solutions were prepared fresh daily. A stock solution of 3-fluoropyruvate at a concentration of 600 mmol/L in 0.05 mol/L potassium phosphate (pH 7.5 at 25 °C) was prepared. Aliquots of 3-fluoropyruvate were stored in liquid nitrogen until measurement.

The enzyme activity measurements were performed in thermostated 96-well microtiter plates in at least three independent experiments. The PDHc activity was measured in two patient samples, with each experiment consisting of at least six to eight single measurements: three to four measurements without inhibition, and three to four measurements with inhibition by fluoropyruvate. Briefly, 50 μL of cell suspension [mitochondria-enriched fraction (MEF) of fibroblasts (200 μg of protein), fibroblast homogenate (200 μg of protein), or SMPs (60 μg of protein)] was added to the wells of the microtiter plate. To start the enzyme reaction, we added 250 μL of the reaction mixture to each well. To examine the specificity of the determined enzyme activity, we inhibited PDHc activity with 3-fluoropyruvate (5 mmol/L), a potent inhibitor of
the E1 component (25); for some experiments with arsenite (1 mmol/L), we added a specific inhibitor of the E2 component (26). These two components were added separately to the well (2.5 μL of a 600 mmol/L stock solution). Fluoropyruvate revealed a good inhibitory response (95–100% of control activity). The total volume of each well was 300 μL.

**DATA ANALYSIS**

Data are expressed as the mean (SD). Experiments were performed at least in triplicate. pH dependence of PDHc activity and Michaelis–Menten parameters were analyzed by use of PsIPPlot software package 5.02a. Statistical analysis was performed by ANOVA followed by the post hoc Bonferroni multiple comparison test or the Student t-test (for two groups). Statistics were calculated using SPSS for Windows 10.0 software. P <0.05 (Student t-test) and P <0.001 (ANOVA) were considered significant.

**Results**

**SPEKTROMETRICKÝ ANALÝZIS ZA SLOŽENÝCH PDHc**

The spectrophotometric assay showed appropriate cofactor requirements based on the known properties of PDHc (27). The addition of pyruvate, addition of CoA and NAD* to SMPs was obligatory for the detection of PDHc activity (Fig. 1; n = 3–8). Omission of TPP led to significantly decreased enzyme activity. In addition, carnitine and magnesium/calcium also increased PDHc activity (Fig. 1). The specificity of PDHc activity was confirmed by complete inhibition with the specific E1 inhibitor 3-fluoropyruvate (Fig. 1) (25). NADH is oxidized to NAD by mitochondrial complex I and lactate dehydrogenase (LD). We investigated whether inhibition of these enzymes influenced PDHc activity in our assay. However, 2-n-decyquinazolin-4-yl-amine (DQA), a potent inhibitor of complex I, and oxamate, an inhibitor of LD, did not significantly influence PDHc activity (Fig. 1). Furthermore, DCA increased PDHc activity in fibroblasts, as described previously (Table 1) (20).

We observed similar effects in MEFs and homogenates from human skin fibroblasts (Table 1), except for oxamate (25 mmol/L), which had no effect in MEFs but mildly decreased PDHc activity in homogenates (Table 1) and in SMPs (Fig. 1). In addition to tissues with low mitochondrial content, we also measured PDHc activity in tissues with a high mitochondrial content, such as liver, with the optimized spectrophotometric assay (data not shown).

**INFLUENCE OF pH ON PDHc ACTIVITY**

We determined the pH optimum (pH_{opt}) for the spectrophotometric assay in SMPs, varying the pH from 6.5 to 8.2 (n = 9). The pH_{opt} for PDHc activity was 7.5. We calculated the pH_{opt} according to the method of Brandt and Okun (28) and obtained a pK_a of 6.85 and a pK_a of 8.14 (see Figs. 1 and 2 in the Data Supplement that accompanies the online version of this article at http://wwwclinchem.org/content/vol51/issue1/). In a previous study using spectrophotometric analysis of PDHc, pH 7.8 was used (24). Notably, we demonstrated that even a small shift from pH 7.5 (i.e., pH_{opt}) to pH 7.8 decreased the mean (SD) V_{max} to 72 (10)% of pH_{opt}. Because this effect was more pronounced in control cells than in PDHc-deficient cells, measurement at pH 7.8 also decreased the diagnostically relevant difference between both groups (see Fig. 4 in the online Data Supplement).

**MICHAELIS–MENTEN PARAMETERS**

The Michaelis–Menten constant (K_m) was calculated at 27 (3) μmol/L pyruvate [V_{max} = 347 (2) μU/U of citrate synthase; n = 8]. A Lineweaver–Burk plot for pyruvate indicated nonlinearity (see Fig. 3 in the online Data Supplement).

**CORRELATION BETWEEN PDHc ACTIVITY AND PROTEIN CONCENTRATION**

In fibroblasts and SMPs, absorbance correlated with the protein concentration (up to 320 μg of protein; r^2 = 0.99). Cell homogenates showed similar results (see Figs. 7, 8, and 9 in the online Data Supplement).

**INFLUENCE OF DIFFERENT PREPARATION PROCEDURES ON PDHc ACTIVITY**

To allow reliable PDHc measurements, sufficient disruption of cells before PDHc activity measurement is necessary, particularly when crude tissue homogenates are...
used. For this purpose, we compared standard methods, i.e., the freezing-thawing procedure, sonication of samples, and the use of a detergent (Triton X-100). The freezing-thawing procedure increased PDHc activity in MEFs and fibroblast homogenates (Table 1; n = 3). In contrast, sonication of samples decreased PDHc activity in all investigated tissues (Table 1; n = 3), most likely because of partial disintegration of PDHc. Omission of the polyoxyethylene detergent Triton X-100 significantly decreased PDHc activity in all investigated tissues (n = 3; Fig. 1 and Table 1; see also Fig. 14 in the online Data Supplement). On the basis of the results of these experiments, we chose the following standard conditions for spectrophotometric PDHc analysis: freezing-thawing, addition of CaCl2, MgCl2, DCA, Triton X-100, and carnitine; and adjustment to pH 7.5 (Table 1).

**DTT Increases Nonspecific, Inhibitor-insensitive PDHc Activity.**

It has been suggested in a previous study (24) that the addition of sulfhydryl group-protecting agents, such as DTT, is necessary for INT-coupled spectrophotometric measurement of PDHc activity. We demonstrated that the addition of DTT to the test buffer increased PDHc activity in SMPs and decreased the inhibitory response to 3-fluoropyruvate (Fig. 2A; n = 4), i.e., DTT mostly increased nonspecific PDHc activity, whereas the increase in the specific activity was less pronounced. This notion was further supported by the finding that reduction of INT occurred even in the absence of SMPs after addition of DTT to the test mixture (Fig. 2A); we therefore suspected that this reduction is artificial. In contrast, the rate for nonspecific binding in a blank containing test buffer was <1% of control activity when DTT was omitted (Fig. 2A).

Finally, we demonstrated that the DTT-induced increase in absorbance at 500–750 nm was concentration-dependent: absorbance correlated to the amount of DTT in the test buffer up to a DTT concentration of 0.3 mmol/L (r² = 0.99; Fig. 2B). It can be suggested from these results that the nonspecific, inhibitor-insensitive enzyme activity in the presence of DTT is induced by direct reduction of INT by DTT. This effect was amplified by administration of the electron mediator PMS (see Figs. 11 and 12 in the online Data Supplement).

**PDHc Activity in Human Skin Fibroblasts.**

We next investigated PDHc activity in fibroblasts from seven patients with PDHc deficiency. The results from the spectrophotometric analysis were subsequently compared with those obtained by the radiochemical method in the same patients (Tables 2 and 3; also see Fig. 10 in the online Data Supplement). The comparative investigations were not performed with the same fibroblast homogenates, but all experiments were performed at least in triplicate, and our assay showed a low interday variability (<5%). In all patients, mutation analyses and/or enzymatic PDHc measurements in muscle and/or fibroblasts were performed in parallel. The results of the radiochemical assay in fibroblasts (Table 2) indicated a significant decrease in PDHc activity in patients 1–3 (Table 3). Patient 4 had a mutation in the thiamine-binding site of the E1α gene (640T>C), which led to an amino acid substitution (W214R) (29); spectrophotometric, but not radiochemical analysis, demonstrated a significant decrease in total PDHc activity. Whereas basal PDHc activity in fibroblasts was decreased in both methods (Tables 2 and 3), radio-

### Table 1. Influence of different preparation procedures and substrates on PDHc activity.

<table>
<thead>
<tr>
<th>Material</th>
<th>DCA</th>
<th>Oxamate</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;/Ca&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Carnitine</th>
<th>Freeze/Thaw&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sonication&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Triton X-100</th>
<th>Activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<td>−</td>
<td>+</td>
<td>86&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>+</td>
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<td>+</td>
<td>62&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>+</td>
<td>60&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

<sup>a</sup> PDHc activity was measured under standard conditions as described in the Materials and Methods and was normalized to 100%. Two tissue preparation forms of fibroblasts, MEFs and cell homogenates, were investigated. +, increases activity; −, decreases activity.

<sup>b</sup> Three freeze-thaw cycles.

<sup>c</sup> Sonication for 20 s with a sonicator equipped with a microtip (power output control, 20% of maximum value).

<sup>d</sup> Compared with controls (Student’s test; n = 3); <sup>e</sup>P <0.001; <sup>f</sup>P <0.05; <sup>g</sup>P <0.01.
chemical analysis of fresh muscle tissue showed normal PDHc activity in this patient (data not shown). Patient 1 had a polymorphism on the cDNA level (291G\textarrowright H11022\textarrowleft\textarrowleft A) in the E1\textarrowright H9251 gene that was present in the control population and did not lead to an amino acid substitution (G97G). It is therefore unlikely that this polymorphism is pathogenic. Notably, the patient had decreased total PDHc activity in fibroblasts and in fresh muscle tissue (data not shown) by radiochemical analysis. Spectrophotometric analysis gave results similar to the results of the radiochemical investigations in fibroblasts (Table 2). We found no mutation in the E1\textarrowright H9251 gene in patient 2, but radiochemical and spectrophotometric analysis in fibroblasts from the same patient showed clearly decreased PDHc activity (Tables 2 and 3).

No muscle tissue was available for PDHc measurement in patient 2. Patient 3 had a disease-causing mutation in the E1\textarrowright H9251 gene of the PDHc complex in fibroblasts (1133 G\textarrowright H11022\textarrowleft\textarrowleft A), which led to an amino acid substitution (R378H) (30). This finding was confirmed by spectrophotometric and radiochemical analysis in fibroblasts (Tables 2 and 3) as well as in muscle (radiochemical analysis; data not shown).

Patients 5–7 had significantly decreased fibroblast PDHc activity, as measured by the spectrophotometric method (Table 3). Radiochemical analysis confirmed the decreased PDHc activity in muscle samples from these patients, whereas the PDHc activity in fibroblasts was normal (Table 2). However, mutation analysis in the E1

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Table 2. Radiochemical analysis of PDHc activity using [1-\textsuperscript{14}C]pyruvate as substrate.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>PDHc activity</th>
<th>Mutation analysis of the E1\textarrowright H9251 gene\textsuperscript{b}</th>
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<tr>
<td>Patients</td>
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<td>Basal</td>
<td>Total</td>
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<td>1</td>
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<td>NM\textsuperscript{c}</td>
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</tr>
<tr>
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<td>2.7</td>
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<tr>
<td>7</td>
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<td>49</td>
<td>65</td>
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</table>

\textsuperscript{a} Analysis was performed in homogenates (n = 3) from human skin fibroblast cultures. Mutation analysis of the E1\textarrowright H9251 gene was performed in human skin fibroblasts from the patients.

\textsuperscript{b} Amino acid substitution in parentheses.

\textsuperscript{c} NM, not measured.

\textsuperscript{d} mU/U citrate synthase.

\textsuperscript{e} nmol \cdot h\textsuperscript{−1} \cdot (mg protein\textsuperscript{−1}).

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Fig. 2. Influence of DTT on PDHc activity (A), and concentration-dependent increase in absorbance at 500 nm after addition of DTT to PDHc-free medium, mimicking PDHc activity (B).

(A), PDHc activity measured under standard conditions in SMPs (60 \mu g) was normalized to 100%. Values are the mean (SD; error bars; n = 8). DTT was added to the test mixture just before enzyme measurement. DTT increased PDHc activity in SMPs (193 (3)% but dramatically decreased the inhibitory response to 3-fluoropyruvate [99 (0.3)% of the original activity with DTT added vs 69 (1)% without DTT added]. Samples containing no SMPs showed no measurable enzyme activity in the absence of DTT (1 (0.2)% but surprisingly high PDHc activity (179 (1)% in the presence of DTT, which was not inhibited by 3-fluoropyruvate (181 (2)%, P < 0.001 vs control (Student t-test; n = 8). (B), absorbance increased in a concentration-dependent way after addition of DTT (0–0.3 mmol/L) to SMP-free medium (r\textsuperscript{2} = 0.99). Each value represents the mean (SD; n = 3–4)
component could not confirm PDHc deficiency in these patients.

Shown in Fig. 3 is an overview of total PDHc activities in MEFs (Fig. 3A) and homogenates (Fig. 3B) from human skin fibroblasts of healthy controls (n = 110–11) and patients with PDHc deficiency (n = 7) as measured by the spectrophotometric method. The measured PDHc activities in fibroblast cell lines from controls and PDHc-deficient patients are summarized in Table 3. In all fibroblast cultures from PDHc-deficient patients, citrate synthase activity was within range of values obtained for the controls and was used as the mitochondrial reference enzyme (data not shown).

**Discussion**

Measurement of PDHc activity in crude fibroblast extracts, which are less invasive to obtain and are available more often than muscle tissue, is hampered by low PDHc activity in this tissue. In the present study, we report a sensitive and reliable spectrophotometric assay for the measurement of completely activated PDHc activity in fibroblasts. Spectrophotometric methods for PDHc measurement described in the literature during the last decades have not been adapted to fibroblasts but only for tissues with a high content of mitochondria, such as skeletal muscle (8) or kidney (24). Alternatively, PDHc activity was measured in purified enzyme (31). Two commonly used assays allow the measurement of PDHc activity in crude fibroblast extract: a radiochemical method using [1-14C]pyruvate as substrate (10), and a kinetic spectrophotometric assay based on the monitoring of acetyl-CoA production by acetylation of a variety of aromatic amines by acetyl-CoA in the presence of acetyl-CoA:arylamine N-acetyltransferase (EC 2.3.1.5) (9, 32). Both methods have major disadvantages. In the radiochemical method, a high blank rate is achieved because of spontaneous decarboxylation of [1-14C]pyruvate, which limits the measurement of specific enzyme activity by this assay. In the arylamine acetyltransferase-based method, the activity of arylamine acetyltransferase prepared by acetone extraction of pigeon liver is not well standardized and shows large variations, increasing the range of PDHc activity (10, 32). In addition to these methodologic shortcomings, none of these assays allows analysis of the complete reaction of PDHc, i.e., production of NADH via three enzymatic steps.

In the present study, we describe a sensitive and specific spectrophotometric assay for measurement of completely activated PDHc that can be used to measure PDHc activity in tissues with low activity, such as fibroblasts. Measurement of PDHc activity in tissues rich in mitochondria, such as liver, can also be performed with this optimized assay.

To investigate the sensitivity and specificity of our

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**Table 3. Spectrophotometric analysis of PDHc activity.**

<table>
<thead>
<tr>
<th>PDHc activity, U/U CS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Controls (total activity)</th>
<th>Homogenates</th>
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</table>

<sup>a</sup> Analysis was performed in homogenates (n = 4) and MEFs (n = 4) from human skin fibroblast cultures.

<sup>b</sup> CS, citrate synthase.

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**Fig. 3. PDHc activity in control and PDHc-deficient human skin fibroblasts.**

CS, citrate synthase. (A), overview of measured PDHc activity in a MEF of control human skin fibroblasts (n = 11; ▲) and PDHc-deficient fibroblasts (n = 7; ■). (B), PDHc activity in human skin fibroblast homogenate of control cell (n = 10; △) and PDHc-deficient fibroblasts (n = 7; ●). The dotted line indicates the lower limit of the reference interval. PDHc activities in deficient cell lines are given in Table 3 (mean of at least three measurements). The horizontal bar indicates the mean of the control group.
assay, we omitted the known cofactors of the PDHc (TPP, NAD, and CoA) and inhibited the enzyme with 3-fluoropyruvate, a specific inhibitor of the E1 component (25), and with arsenite, a specific inhibitor of the E2 component (26). We demonstrated that this spectrophotometric PDHc assay revealed an appropriate cofactor requirement and a good inhibitory response to 3-fluoropyruvate (inhibition >95%), a well-described inhibitor of the E1 component, confirming the specificity of the assay (Fig. 1). The assay had a negligible blank rate.

To determine maximum PDHc activity, many different variables, such as pH, incubation period, disruption procedure, and phosphorylation/dephosphorylation of the PDHc, dramatically influence enzyme activity and must be considered carefully. Hinman and Blass (24) used pH 7.8 for the INT-coupled assay adapted for mouse brain homogenate and rat kidney mitochondria without exact calculation of the pHopt of their assay. We demonstrated that even a slight pH shift from pH 7.5 (i.e., pHopt) to 7.8 significantly decreased the PDHc activity to 72% of Vmax (Fig. 4 in the online Data Supplement) and thus would impair the diagnostic work-up of PDHc deficiency in fibroblasts. Furthermore, a prolonged incubation period up to 60 min, as is often required for the radiochemical method, decreased PDHc activity (Figs. 5 and 6 in the online Data Supplement). In addition to pH- and time-dependent effects, the method used to disrupt the mitochondrial membrane is of utmost importance. As described previously for the measurement of E1 activity (33), a freezing–thawing procedure significantly increased PDHc activity, whereas the addition of Triton X-100 was less effective (Table 1 and Fig. 1). In the present study, we demonstrated that a combination of freezing–thawing and Triton X-100 further increased the PDHc activity compared with use of either of these alone, whereas the addition of a sonication step decreased the PDHc activity.

PDHc activity is regulated mainly by phosphorylation and dephosphorylation and is strongly inhibited by NADH and acetyl-CoA via a feedback loop. Considering these regulation mechanisms, we tested the influence of different compounds (magnesium, calcium, DCA, carnitine, and oxamate) suggested to activate PDHc activity. In accordance to other reports, magnesium (5 mmol/L) and calcium (1 mmol/L) increased PDHc activity (Table 1 and Fig. 1) (10), most likely through stimulation of the endogenous PDH phosphatase and subsequent dephosphorylation of the E1 component (34). In line with the report by Sheu et al. (20), activation of PDHc could also be induced by the addition of DCA, an inhibitor of PDH kinase (Table 1). We added L-carnitine to the test mixture to prevent feedback inhibition by acetyl-CoA. In fact, L-carnitine increased the enzyme activity in our assay, whereas this effect was lower (64% vs 29% in our assay) than that reported previously in another study (10). In contrast to the results obtained by Chretien et al. (8), who found increased PDHc activity in the presence of oxamate, an LD inhibitor that prevents LD-induced NADH reoxidation, we could not confirm a significant effect of this compound in our assay (Table 1 and Fig. 1). To exclude oxidation of NADH by mitochondrial complex I, we inhibited this multiprotein complex with the specific inhibitor DQA. However, this compound also had no significant influence on PDHc activity (Fig. 1).

In a previous study (24) in which PDHc activity was measured by an INT-coupled spectrophotometric assay, the sulfhydryl group-protecting agent DTT was suggested as a necessary ingredient in the test buffer for PDHc measurements; there was a complete loss of PDHc activity in the absence of DTT. Unfortunately, the specificity of this finding has not been confirmed by analysis of the inhibitor-sensitive rate of this assay. In our study, DTT mostly increased the inhibitor-insensitive, i.e., nonspecific, rate of PDHc activity from 1% (without DTT) to 31% (with DTT), whereas it had a less pronounced effect on the inhibitor-sensitive rate. We demonstrated that DTT increased the absorbance at 500–750 nm in a concentration-dependent way even in the absence of PDHc protein in the assay system, thereby mimicking PDHc activity (Fig. 2B). To further unravel the underlying mechanism of this effect, we investigated changes in absorbance at 500–750 nm by combining DTT with different components in the absence of SMPs according to the previously published protocol of Hinman and Blass (24). Notably, we could demonstrate that DTT directly reduces INT in the test system, explaining the inhibitor-insensitive enzyme activity (Fig. 11 in the online Data Supplement). Because these results highlight a direct reaction between DTT and INT, an indicator of specific PDHc activity in the presence of DTT, we concluded that addition of DTT is not suitable for this assay although it increases the absolute PDHc activity.

Using fibroblasts from patients with PDHc deficiency, we demonstrated that all of these patients had significantly decreased PDHc activity, as measured by our spectrophotometric assay (Table 3). Radiochemical analysis revealed decreased PDHc activity in muscle tissue from five of the seven patients (patients 1, 3, and 5–7). In four patients, basal and/or total PDHc activity was decreased in fibroblasts, as measured by the same method (patients 1–4). Notably, in one patient, who had a mutation in the thiamine-binding site of the E1α subunit of the PDHc, spectrophotometric but not radiochemical analysis revealed a significant decrease in total PDHc activity in fibroblasts. In contrast, in both assays basal PDHc activity was decreased in fibroblasts from this patient (Tables 2 and 3). The two patients with pathogenic mutations in the E1α gene (patients 3 and 4) and the patient with the polymorphism in the E1α gene (patient 1) had decreased PDHc activities in fibroblasts, as measured by both methods (Tables 2 and 3). Radiochemical analysis of muscle specimens from patients 1 and 3 confirmed the PDHc deficiency (data not shown). Although patients 5–7 had decreased PDHc activity in muscle tissue, as measured by
the radiochemical assay, and in fibroblasts, as measured by the spectrophotometric assay, mutation analysis revealed no mutation in the Elα gene. This phenomenon is possibly attributable to pathogenic mutations in the Elα gene (e.g., mutations in the promoter site of the Elα gene) or in the localization of the disease-causing mutation in the remaining subunits (E2, E3, or E3-binding protein) or regulatory enzymes (PDH phosphatase and PDH kinase) of the PDHc. It can be concluded from these results that the optimized spectrophotometric assay has at least the same sensitivity and specificity as the radiochemical method. However, because the present investigations have been performed in a relatively small group of PDHc-deficient patients in two different laboratories, our comparisons regarding the reliability and validity of these methods has some limitations. This should be investigated in future studies in greater detail.

PDH Elα subunit (pyruvate decarboxylase; EC 4.1.1.1) deficiency (McKusick 312170) is an X-linked error of metabolism, and diagnosis is usually based on measurement of enzyme activity. This may present difficulties in females because of random X inactivation patterns (35). The X inactivation occurs rather frequently in fibroblast cultures (~25% of cases), making exclusion of the diagnosis in females rather impossible (35). Patient 2, the sole female patient in our study, had dramatically decreased PDHc activity in fibroblasts, as measured in both the radiochemical and spectrophotometric assays. Mutation analysis revealed no mutation in the Elα gene. The X inactivation pattern and the variable tissue expression of PDHc activity can limit usefulness of enzyme measurements in human skin fibroblasts to exclude the diagnosis of PDH Elα subunit deficiency. Because fibroblasts are easier to obtain than muscle tissue and can be expanded in cell culture, diagnostic work-up is often initially performed in this tissue. If clinical and biochemical features strongly suggest PDHc deficiency and the fibroblast enzyme activity is within the reference interval, screening for mutations in the PDH Elα gene or other investigations, such as immunohistochemical analysis or enzyme measurement in muscle tissue, are necessary because normal PDHc activity in human skin fibroblasts does not exclude a PDHc deficiency.

In conclusion, our results confirmed that the spectrophotometric assay adapted for the analysis of PDHc activity in human skin fibroblasts reliably measured PDHc activity in tissues with low PDHc activity. Because spectrophotometric measurement in fibroblast homogenates is less time-consuming and gives enzyme activity results identical to those for a MEF (Fig. 13 in the online Data Supplement), we propose to use fibroblast homogenates for spectrophotometric analysis of PDHc complex activity. PDHc activities under different assay conditions reflect both tissue-specific differences (e.g., higher activity in muscle than in fibroblasts) and loss of function or incomplete activation of maximum PDHc activity as a result of methodologic differences (e.g., PDHc leaks from the mitochondria during the isolation of MEFs). Thus, the measured PDHc activity is the result of these two different dimensions. Van Laack et al. (33) observed that the activity of the E1 component of PDHc, as measured by radiochemical analysis of fresh human skeletal muscle, was considerably higher in homogenates than in the mitochondria-enriched supernatants. They also proposed the use of homogenates for E1 measurement in human skeletal muscle. Compared with previously described methods using radioactive-labeled pyruvate or the spectrophotometric arylamine acetyltransferase reaction, our assay has several advantages, i.e., no need for radioactive compounds, a low blank rate, a high specific activity, use of commercially available reagents of standardized quality, and low costs. It is rapid and sensitive and could be easily implemented as a valid and reliable powerful tool in the diagnostic work-up of patients suspected of having a mitochondrial disorder.

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References


