A HUMAN ENERGY CRISIS:

DEFECTIVE REGULATION OF

CELLULAR ENERGY LEVELS

by

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A THESIS

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Like the world we live in, the human body needs energy to operate. An energy shortage in the body may be catastrophic, or may simply be limiting, depending on the nature and severity of the shortage. Energy generation from carbohydrates is controlled in large part by the enzyme complex pyruvate dehydrogenase (PDH), and defects in this protein can lead to serious illness. Defects are usually assessed by studying the enzymatic activity of the complex and by analyzing the presence of subunits in the mitochondria. Immunoprecipitation is a technique that provides additional information about assembly of the complex that may be overlooked by these two methods. I have tested the use of

immunoprecipitation to isolate whole PDH complex from mitochondria and cells and have applied the method to cell lines from PDH deficient patients. My results indicate that immunoprecipitation can extract an intact PDH complex and can detect patients who have assembly mutations in PDH.

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

II. BACKGROUND 1-10

A. METABOLISM 1

B. PYRUVATE DEHYDROGENASE (PDH)
1. Metabolic Role 2-3
2. Structure and Mechanisms 3-4

C. PDH DEFICIENCIES 5-10
1. A Patient 5
2. Clinical Considerations 6
3. Mutations 7
4. Diagnosis 8-9

D. IMMUNOPRECIPITATION OF PDH 10

III. MATERIALS AND METHODS 11-19

A. MATERIALS 11-15
1. Source of PDH for Studies 11-12
2. Antibodies 12-13
3. Protein-A Sepharose Beads 13-14
4. Buffers 14
5. Protease Inhibitors 15
6. Detergents 15

B. METHODS 16-19
1. Immunoprecipitation 16
2. SDS-PAGE 17-18
3. Silver Staining 18
4. Western Blotting 18-19

IV. PROCEDURES..... 20-25

A. IMMUNOPRECIPITATION..... 20-22
1. Preparation of Cells and Mitochondria..... 20
2. Preparation of Beads..... 21
3. Immunoprecipitation..... 21-22

B. SDS-PAGE..... 23

C. SILVER STAINING..... 24

D. WESTERN BLOTS..... 25

V. RESULTS..... 26-31

A. BOVINE HEART MITOCHONDRIA..... 26-27

B. MRC5 MITOCHONDRIA AND CELLS..... 28-29

C. PATIENT MITOCHONDRIA..... 30-31

VI. DISCUSSION..... 32-35

A. EXTRACTION..... 32

B. CONSIDERATION OF SOURCE..... 33

C. OUTCOME OF BLIND STUDY..... 34

D. IMMUNOPRECIPITATION FOR CLINICAL APPLICATION.... 35

VIII. REFERENCES..... 36-37

LIST OF FIGURES

Figure		Page
1.	Mitochondria Within a Cell.....	2
2.	Metabolic Pathway.....	2
3.	PDH Structure.....	3
4.	PDH Mechanisms.....	4
5.	Antibody.....	12
6.	Protein-A Sepharose Beads.....	14
7.	Immunoprecipitation.....	16
8.	SDS-PAGE Gel.....	17
9.	Metric Scale, Units.....	20
10.	Bovine Heart Mitochondria.....	26
11.	MRC5 Mitochondria and Cells.....	28
12.	PDH Patient Mitochondria.....	30

BACKGROUND

METABOLISM

It does not take a scientist to understand that people get energy from food. Science is helpful, though, in understanding how that energy is derived. The major source of food energy for most people is carbohydrates. Carbohydrates are converted into a molecule called glucose, which is used in every cell of the body to make energy. The cells have two energy generating pathways available to them. One, a high energy pathway[1], generates 100% of the energy possible from each glucose. The other, a low-energy pathway[2], generates only 5% of the total energy possible. One enzyme[3] complex in the cell controls this pivotal point, directing the flow of glucose into the high energy pathway. That enzyme complex is the pyruvate dehydrogenase (PDH) complex.

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[1] The high-energy pathway is called cellular respiration.

[2] The low-energy pathway is called fermentation.

[3] An enzyme is a protein that accelerates the rate of a reaction. An enzyme complex is an assembly of proteins into an enzymatic super-strucutre.

PYRUVATE DEHYDROGENASE (PDH)

Metabolic Role

The PDH complex is located in the mitochondrion (*pl. mitochondria*), a cellular organelle specialized for energy production (Fig. 1). Glucose does not enter the mitochondria, however, so PDH does not work directly on glucose. When it first enters a cell, glucose is split into two identical molecules called pyruvate. The PDH complex uses the pyruvate to make a substance called acetyl-Coenzyme A (acetyl-CoA) (Figure 2). Acetyl-CoA is the metabolite that begins the high energy pathway. If PDH is

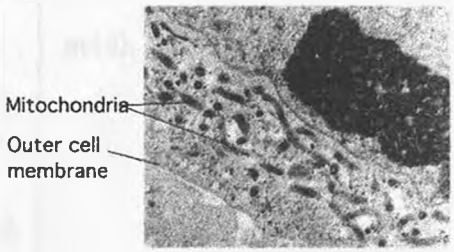


Figure 1: Mitochondria are organelles within a cell that are specialized for making energy (1).

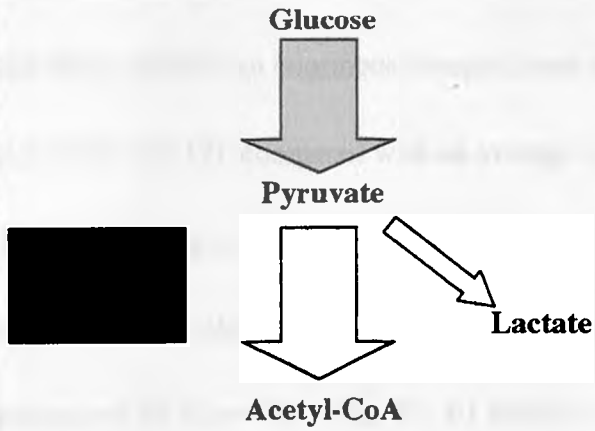


Figure 2. Pyruvate Dehydrogenase (PDH) Complex converts pyruvate to acetyl-CoA, a crucial step in metabolism. A defective PDH enzyme causes pyruvate and lactate to build up in cells.

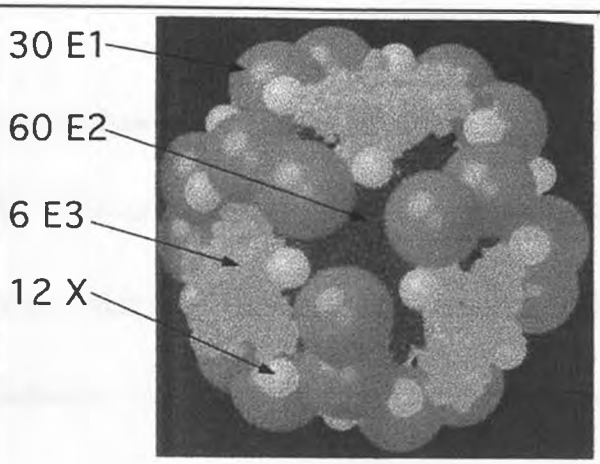


Figure 3. The PDH complex consists of 30 E1, 60 E2, 6 E3, and 12 X. Each E1 consists of 2 E1-alpha and 2 E1-beta. Each E3 consists of 2 E3 subunits.

not working, pyruvate instead goes through the low energy pathway, producing an end product called lactate (lactic acid).

Structure and Mechanisms

Correlating with its large role in metabolism, PDH is an enormous complex, one of the largest known. Its molecular weight is 7000 kDa (2), compared with an average protein weight of 50 kDa. However, PDH has a surprisingly simple structure, given the importance of its role in the cell. It has only four subunits: E1, E2, E3, and X, and two regulatory components: E1-phosphotase and E1-kinase[4] (3) (Fig. 3). E1 and E2 contain the catalytic sites for the reaction, while X holds the E3 subunit in the complex (Fig. 4). E2 is the structural core of the complex, and E3 acts directly on E2 to restore its activity after a reaction has been completed. The E1 subunit is itself composed of four small

[4] The phosphotase activates the complex; the kinase de-activates it.

the catalytic sites for the reaction, while X holds the E3 subunit in the complex (Fig. 4). E2 is the structural core of the complex, and E3 acts directly on E2 to restore its activity after a reaction has been completed. The E1 subunit is itself composed of four small subunits: two copies of an E1α subunit and two copies of an E1β subunit. Thus, the overall ratio of subunits in the complex is E2 (60) : E3 (12) : X (12) : E1α (60) : E1β (60). The subunits have molecular weights of 72, 55, 50, 41, and 36 kDa[5], respectively.

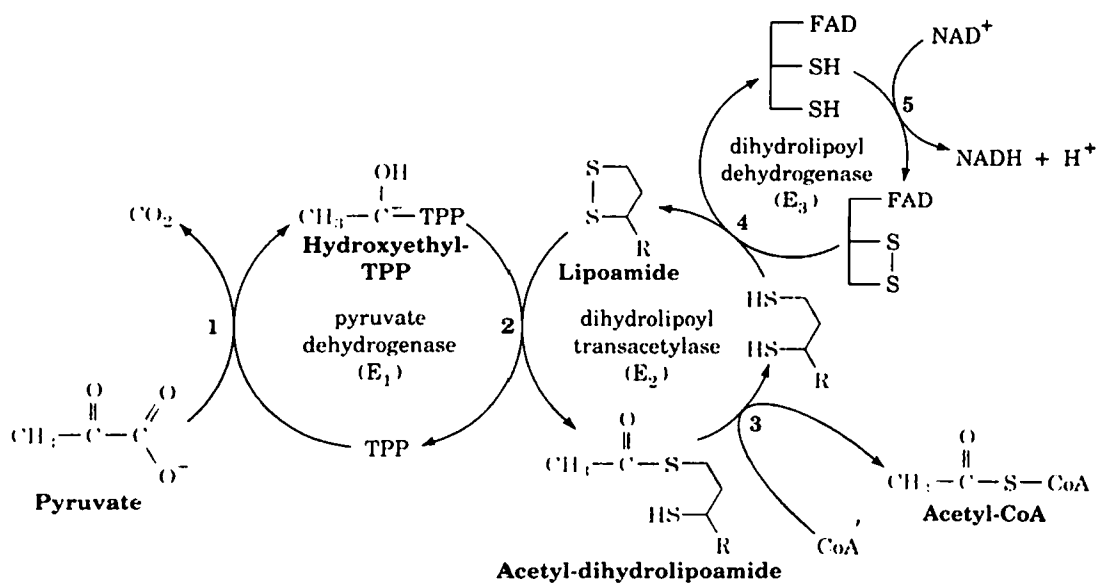


Figure 4. PDH Mechanisms. E1 removes a CO_2 from pyruvate, the reaction's irreversible step. E2 forms acetyl-CoA, and E3 returns E2 to its initial state.

[5] **kDa**, kiloDalton. Daltons are a unit of measure for molecular weight.

PDH DEFICIENCIES

A Patient

Maintenance of this pathway is crucial for the body. A case of PDH deficiency might look similar to this (4):

An infant girl was born to a mother who had previously had one still-birth and another child delivered 12 weeks early, who died after 2 hours. Pregnancy and delivery for the girl had been uncomplicated, but she was underweight and showed poor feeding, losing 16% of her body weight over the first four days of life. She showed high levels of pyruvate and lactate in blood and had highly acidic body fluids (acidosis). Her auditory and visual responses were poor and she had a notable lack of muscle tone (hypotonia). Her acidosis responded well to treatment with bicarbonate, but the lactate and pyruvate levels remained high and were unresponsive to treatment. At thirteen years old she was still living, but was dwarfed, blind, severely retarded, and had malformed body features and episodes of seizures.

Clinical Considerations

The condition of this girl and the outcome of her siblings illustrate the range and severity of effect that PDH deficiency can have. The condition is usually noticed in the first few months of life, and is frequently fatal early in life (5). PDH deficiency is first suspected when an infant shows lactic acidosis in the blood or cerebrospinal fluid accompanied by high levels of pyruvate and lactate. Those who survive with a PDH deficiency are often severely disabled, particularly with neurological defects. The severity can range widely however, and one adult male with PDH deficiency is known to be living with a mild and intermittent movement disorder (ataxia) as his only symptom (6). The most successful treatments for PDH deficiency rely on a high fat, no-carbohydrate diet, and require very early detection of the disease (7). Even these treatments have not been hugely successful, however, because much of the damage caused by PDH deficiency occurs before birth. Prognosis and treatment are difficult because of the multiple genetic, environmental, and nutritional factors that can affect the course of the disease (5).

Mutations

A significant amount of the variation lies in genetics. Defects in the PDH complex arise when a gene[6] is mutated, and the severity of a defect can depend on the nature and location of the genetic mutation. The overwhelming majority of PDH defects lie in the gene for E1α, the active site of the reaction's committed step. The gene for E1α is located on the X-chromosome and shows a remarkably high frequency of mutations (8). X-linked mutations are typically expressed preferentially in males, who have only one copy of the X-chromosome. PDH deficiency does not follow this pattern, however, and both males and females show PDH deficiency with equal frequency and severity (9). This expression pattern in females has been attributed to X-inactivation, a phenomenon whereby one copy of the X-chromosome is inactivated in all cells (10). X-inactivation is a normal cellular phenomenon, however, and many X-linked mutations are not expressed in females. Because females do express E1α mutations on the X-chromosome, the gene has been called "X-linked dominant" or "X-linked lethal" (11).

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[6] Proteins are created from the information encoded in genes.

Diagnosis

Though mutations in PDH occur at the genes, the search to identify a PDH deficiency begins with the protein complex. The genes for the subunits are scattered about the chromosome (12), and human genes have a significant amount of natural, harmless variation. Identifying a pathogenic mutation is not always possible from a purely genetic analysis. Studies begin with assessment of PDH activity, generally by measuring release of radioactively labeled carbon dioxide from pyruvate (13). The activity study can indicate whether a defect in PDH accompanies the clinical symptoms presented, but it does not give insight into the molecular nature of the defect. For this purpose, immunological[7] studies are used, which identify presence of the subunits based on antibody recognition (14).

Immunological studies are generally done with western blots against mitochondria from cells of PDH patients. Presence of all five subunits is taken as an indication that the mutation affects only the regulatory components (phosphotase and kinase) or occurs at

the active sites of subunits without affecting assembly of the complex. Lack of a subunit or subunits on the blot is taken to mean that the mutation affects assembly of the complex or production of the subunits. However, it is possible that immunoblotting techniques could incorrectly support a conclusion of complete assembly of the complex. If the subunits are present in the mitochondria, but not assembled in the complex, a western blot could show a false positive result for assembly. At least four cases are known of a genetic mutation that has affects assembly of the subunits, yet the subunits levels all appear normal on a western blot (7).

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[7] Immunological studies use antibodies, components of the immune system, as a testing basis. Antibodies and the immunological method of western blotting are discussed more thoroughly in the Materials and Techniques section.

IMMUNOPRECIPITATION OF PDH

One way to avoid this ambiguity is to use immunoprecipitation[8] to isolate the whole, intact complex from cells, thus identifying only those components that are present in the assembled complex. I have tested the use of immunoprecipitation with monoclonal antibodies for extracting intact PDH complex from mitochondria and from cells. I have also tested the applicability of this method for identifying PDH patients. The results show that immunoprecipitation is a viable method for isolating intact PDH complex and can identify patients with assembly defects in PDH.

[8] Also explained in Methods section.

MATERIALS AND METHODS

MATERIALS

Source of PDH for Studies

Pyruvate dehydrogenase was extracted from four different experimental systems: 1) purified mitochondria from bovine heart tissue; 2) purified mitochondria from human MRC5 fibroblast cells[9]; 3) whole MRC5 cells; and 4) mitochondria from patients with PDH deficiency. The cells were grown in culture and mitchondria extracted by Kathy Chicas-Cruz and Margarita Lib. Patients cells were given by Dr. Nancy Kennaway, Oregon Health Sciences University, for a blind study[10] from four cell lines. PDH was released from the cells and mitochondria with a detergent lysis[11], leaving the PDH in solution.. The PDH complex is soluble in water, so I use the liquid portion of the lysate

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[9] MRC5 is a common line of human cells used in research. Fibroblasts are cells from connective tissue, such as skin.

[10] Identity of the patients among the cells was not disclosed. My task was to identify the patients by immunoprecipitation.

for the immunoprecipitation. The control[12] for isolation of PDH was purified porcine PDH (Sigma Chemical Co., P-5194).

Antibodies

Antibodies[13] are agents of the immune system that bind to foreign particles, targeting them for elimination by other parts of the immune system (Fig. 5). The recognition of an antibody for a particle (antigen) is specific. Recognition occurs between a three amino acid sequence on the antibody and on the protein.

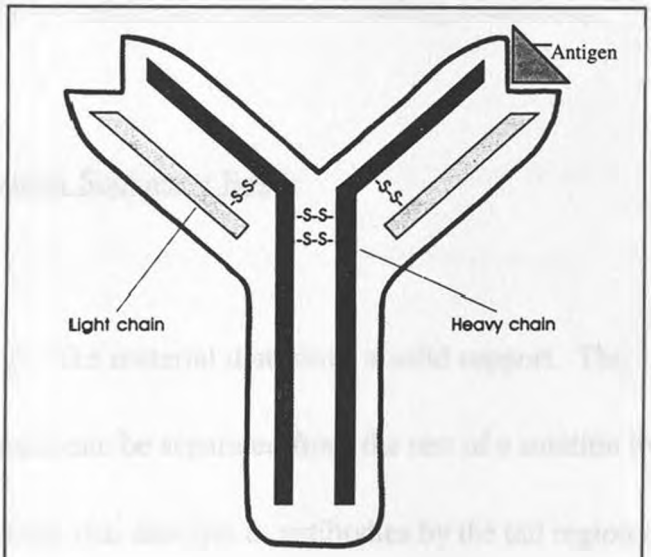


Figure 5. An antibody is a Y-shaped protein that binds specifically to an antigen. The antibodies used here are made of two heavy chains (55 kDa each) and 2 light chains (22 kDa each) (15).

Antibodies are produced by B-cells, and monoclonal antibodies are raised from clones of

[12] The control is a sample known to be PDH. I compare the appearance of my extracted product to the known PDH.

[13] Like enzymes, antibodies are proteins. Proteins are composed of chains of amino acids.

a single B-cell, giving only one recognition site. Polyclonal antibodies are raised from clones of several cells, so more than one recognition site exists in a polyclonal mix. Antibodies for these experiments were raised in mice against purified PDH at the Monoclonal Antibody Facility, University of Oregon. Monoclonal antibodies were obtained for the E2 and E1β subunits of PDH. A non-specific normal mouse antibody[14] was used as a control in the immunoprecipitations for random binding to antibody.

Protein-A Sepharose Beads

These are beads made of agarose, a gel-like material that forms a solid support. The beads are large on a molecular scale and can be separated from the rest of a solution by centrifugation[15]. Protein-A is a molecule that attaches to antibodies by the tail region (F_c region), which is common to all antibodies within a given class. It binds antibodies without changing the affinity of the antibodies for their antigens. Adding a monoclonal antibody to a protein-A sepharose beads essentially creates a heavy magnet for the

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[14] Normal mouse antibody is an antibody representative of the antibodies coming from mouse. Use of this antibody allowed me to see if proteins in solution may bind non-specifically to the mouse antibody for E2.

molecule to be purified. I used a chemical cross-linking procedure to firmly attach the antibodies to the beads and increase the yield of the immunoprecipitation.

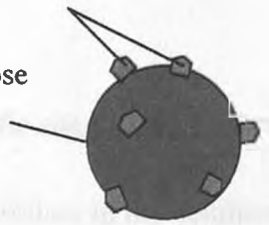


Figure 6. Protein-A Sepharose beads have molecules of protein-A attached to the surface of a large sepharose bead (not to scale).

Buffers

The acidity of a solution is measured as pH, and buffers are used to maintain a solution at a desired pH. Proteins and other biological materials are extremely sensitive to pH, and I have used buffers to maintain a pH close to that of the cell (7.0 - 7.5). My primary buffer was phosphate buffered saline (PBS), a common physiological buffer of pH 7.4. Another buffer used solutions was Tris-acetate, at pH 6.8 or 8.8. This buffer was used when conditions were designed to denature[1] the protein.

[1] Proteins are long chains of amino acids wrapped and twisted into secondary shapes. Denaturing refers to unraveling the secondary structure of the protein so that it becomes linear.

Protease Inhibitors

Proteases are enzymes that cut apart proteins at specific sites. They are present in both mitochondria and cells, so I used several protease inhibitors in my solutions to avoid degradation of the PDH complex. The inhibitors I used were EDTA, PMSF, leupeptin, and pepstatin-A. Each substance inhibits a different type of protease.

Detergents

Detergents are used to break apart biological membranes and release the contents of a cell or organelle. Detergents may also be used at low concentrations to keep protein soluble. I used lauryl maltoside (LM) as a gentle detergent for lysing the cells and mitochondria, and used sodium dodecyl sulfate (SDS), a harsher detergent when I wanted to denature the proteins. Tween, an extremely gentle detergent, was used in the western blots when I didn't want to interrupt protein binding or shape.

METHODS

Immunoprecipitation

Immunoprecipitation uses antibodies attached to a heavy bead as magnets to isolate a given protein from a mix of materials in a solution (Fig 7). A high speed centrifugation is used to separate the beads with the protein bound, then the protein is released from the beads with detergent. Washes are done before the protein is released to clear out any material not bound to the beads.

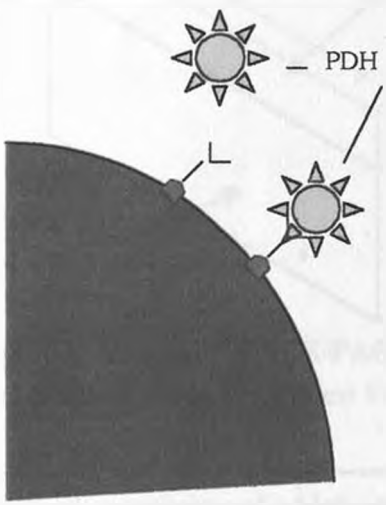


Figure 7. Immunoprecipitation uses the antibodies attached to the sepharose bead to isolate PDH from solution.

SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

SDS-PAGE is used to separate proteins based on molecular weight. The polyacrylamide gel is a matrix whose pore size can be increased by decreasing the percentage of acrylamide in the gel. Controlling the pore size controls how fast proteins can move through the gel. Generally, large proteins are hindered by the matrix and move slowly, while small proteins move quickly. A bi-layer gel is often used, consisting of a high percentage acrylamide to separate the proteins (separating gel) topped with a low percentage acrylamide gel (stacking gel). The stacking gel allows all proteins to run quickly and stack up against the separating gel, resulting in a more even distribution in the separating gel. SDS detergent is used to denature the proteins and give them an even ratio of mass to electric charge. An electrical current is run through the gel, polarizing it and causing the proteins to run in one direction, towards the positive end of the gel. The molecular

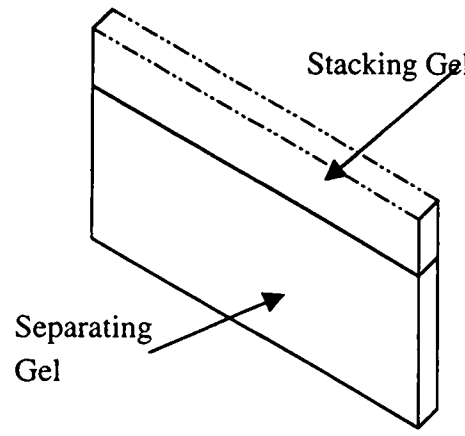


Figure 8. Diagram of SDS-PAGE gel. The dimensions of the gel are 1x4x6 mm.

weight of the proteins is estimated by running a sample of proteins having known molecular weights.

Silver Staining

Proteins in an SDS-PAGE gel can be visualized by silver staining. Silver staining uses chemical processes to fix the proteins in the gel and attach silver atoms to them. The silver attached to the proteins makes a dark band on the gel wherever proteins are present, and all proteins in a gel can be seen with silver staining. Within a certain range, the darkness of the band corresponds to the amount of protein present, so the technique can be roughly quantitative.

Western Blotting

Western blotting relies on antibody recognition to visualize bands of protein on a gel. The protein is first transferred from the gel to a membrane, so that the proteins are on an accessible surface. The membrane is adhesive to all proteins, so empty space on the membrane is blocked from antibodies (which are also proteins) using a solution of milk

protein. Antibodies are then added, and if the protein that the antibody recognizes is present on the membrane, the antibody will bind to it. A secondary antibody having a fluorescent signal is attached to the first antibody, and the presence of this signal is used to indicate whether a protein is present. Western blotting has the advantage over silver staining that it is specific for selected proteins.

PROCEDURES

IMMUNOPRECIPITATION

Preparation of cells and mitochondria

Both cells and mitochondria were lysed in a PBS solution of 0.1% lauryl maltoside containing protease inhibitors 1 mM EDTA, 1 mM PMSF, 0.5 µg/ml leupeptin, and 0.5 µg/ml pepstatin-A. Lysis was done at 4°C while gently agitating the samples. Mitochondria were lysed for 30 minutes, cells for 45 minutes. The lysate was centrifuged for 10 minutes at 4°C at 12,000 rpm and the super-natant[17] removed and used for immunoprecipitation.

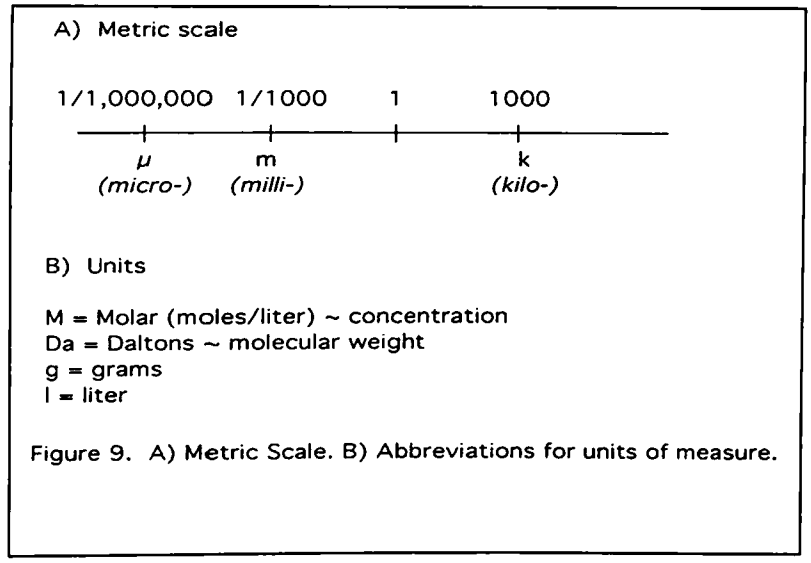


Figure 9. A) Metric Scale. B) Abbreviations for units of measure.

[17] After a centifugation, the heavy material at the bottom of the tube is called the pellet, or precipitate. The liquid material above the pellet is called the supernatant, or supe.

Preparation of beads

A monoclonal antibody against E2 (MM#3 15D3C9G11) was coupled to protein-A sepharose beads (Amersham Pharmacia 17-0780-01) at 10 μg antibody / 100 μg beads. Normal mouse antibody (# 015 000 003) was also attached to beads at 10 μg antibody / 100 μg beads. All antibodies were obtained from the Monoclonal Antibody Facility, University of Oregon. Antibodies were incubated with the beads for two hours at room temperature. Coupling was done as previously described (15)[18].

Immunoprecipitation

Lysate from the mitochondria was added to beads at a ratio of 1 mg mitochondrial protein / 100 μg beads. For the cells, a volume 50 μl cells / 100 μg beads was used. Lysate was incubated with the beads for 2 hours at 4°C then separated by a 30 second spin at 10,000 rpm at 4°C. The supernatant was removed and the pellet washed three times with a 10x volume PBS. Fifty μl dissociation buffer (2x Laemmli buffer) was added to each pellet and briefly vortexed. The pellet was heated for 3 minutes at 100°C and briefly vortexed

again. Beads were separated by a 3 minute spin at 10,000 and the supernatant removed and applied to SDS-PAGE.

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[18] Modification: Sodium borate tetrahydrate was used at 0.16 M, which is the solubility limit at room temperature (16).

SDS-PAGE

A 10% separating gel and 4% stacking gel were used. The separating gel contained 10% polyacrylamide, 0.375 M Tris buffer at pH 8.8, 0.1% SDS detergent, 0.05% ammonium persulfate (APS), and 0.05% (v/v) TEMED. The stacking gel was 4% polyacrylamide, 0.125 M Tris buffer at pH 6.8, 0.05% ammonium persulfate (APS), and 0.1% (v/v) TEMED. Gels were run for 1 hour at 200 V in a running buffer of 1.4 % glycine, 0.3% Tris, and 0.1% SDS.

SILVER STAINING

(The volumes given here are for 1 minigel, but can be scaled up for larger gels or for more gels.)

Proteins were fixed in the gel with a 30 minute incubation in solution of 400 ml ethanol and 100 ml acetic acid, dH_2O[19] added to a total volume of 1 L. The gel was then incubated for 30 minutes in priming solution: 3.4 g anhydrous sodium acetate, 100 mg sodium thiosulfate•$5H_2O$, 15 ml ethanol, and 260 µl glutaraldehyde, topped to 50 ml with dH_2O. The gel was washed 3 times in dH_2O, then incubated for 40 minutes in silver solution: 50 mg silver nitrate, 10 µl formaldehyde, and dH_2O to 50 ml. The gel was then transferred to developing solution: 1.25 g anhydrous sodium carbonate and 5 µl formaldehyde, topped to 50 ml with dH_2O, and incubated approximately 10 minutes, until bands appeared. Development was quenched in a stop solution: 0.73 g disodium EDTA in 50 ml dH_2O, for 10 minutes.

[19] dH_2O is deionized water.

WESTERN BLOTS

Proteins were transferred to a PVDF membrane (Millipore IPVH00010) for 1 hour at 200 mA. A transfer buffer of 1.4 % glycine, 0.3% Tris 0.1% SDS, and 25% (v/v) methanol was used. The membrane was blocked overnight at 4°C with 5% milk (5 g powdered milk, 100 ml PBS). Anti-E2 (MM#3 15D3C9G11) and anti-E1β (MM#3 17A5E2H8) monoclonal antibodies were used (Monoclonal Antibody Facility, University of Oregon). Antibodies were added to 10 ml 5% milk (0.5 µl anti-E2 [1.29 mg/ml], 20 µl anti-E1 β [2.2 mg/ml]). The membrane was incubated with these antibodies for 1 hour at room temperature, then washed three time with T-PBS[20]. The secondary antibody was GAM-HRP (Monoclonal Antibody Facility, University of Oregon), which was added to the membrane at 4 µl GAM-HRP in 10 ml 5% milk. The membrane was incubated with GAM-HRP for 40 minutes at room temperature, then washed 3 times with T-PBS and once with PBS. The HRP signal was developed with ECL-Plus (Amersham Pharmacia RPN 2132) for 3 minutes and read on STORM 860[21] with blue fluorescence scanning.

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[20] T-PBS: PBS containing 0.05% Tween detergent.

[21] STORM 860 is a computer that can detect the fluorescence from HRP and translates it into an image.

RESULTS

BOVINE HEART MITOCHONDRIA

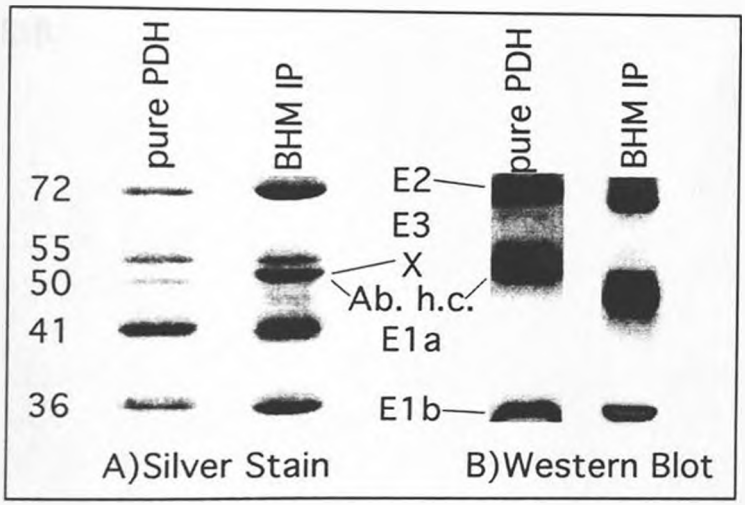


Figure 10. Immunoprecipitations from bovine heart mitochondria (BHM). A) Silver staining shows proteins in the immunoprecipitation of weights similar to the subunits of pure PDH (Sigma Chem. Co.). B) Western blotting with monoclonal antibodies against E2 (72 kDa) and E1β (36 kDa) show bands of similar weight in immunoprecipitated material and pure PDH. An extra band at 50-55 kDa in both lanes may be due to contamination by antibody heavy chain.

As the silver stain in Figure 10.A shows, the bands of protein immunoprecipitated from bovine heart mitochondria (BHM) are of comparable weight to those appearing in pure PDH. The weights of bands in both lanes correspond to the expected weights for the

subunits of PDH: 72 kDa (E2), 55 kDa (E3), 50 kDa (X), 41 kDa (E1α), and 36 kDa (E1β). The western blots (Fig. 10.B.) show that monoclonal antibodies against the E2 and E1β subunits of PDH react with both pure PDH and immunoprecipitated material. The weights of bands in the western blot correspond to the expected weights for E2 and E1β.

MRC5 HUMAN MITOCHONDRIA AND CELLS

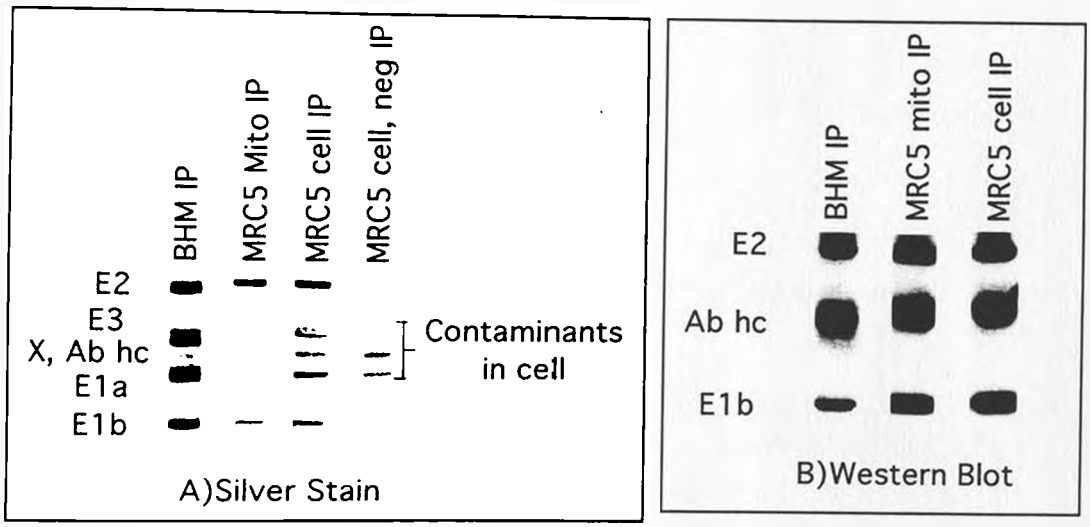


Figure 11. Immunoprecipitions from MRC5 mitochondria and cells. A) Silver staining shows bands at 72, 54, 41, and 36 kDa in the MRC5 mitochondrial and cell immunoprecipitations. The cell immunoprecipition also shows two additional bands and 56 and 45 kDa which correspond to bands found in the negative control. B) Western blotting with monoclonal antibodies against E2 and E1β shows bands of 72 kDa and 36 kDa in immunoprecipitations from both MRC5 mitochondria and cells.

Immunoprecipitation from MRC5 mitochondria shows bands on silver staining at 72 kDa, 54 kDa, 41 kDa, and 36 kDa (Fig. 11.A.). The immunoprecipitate from MRC5 cells also shows these bands, but additionally has bands at 56 kDa and 45 kDa. The bands at 56 and 45 kDa also appear in the negative control with normal mouse antibody. A 41 kDa band in the negative control also likely contributes to the density of the 41 kDa band

in the cell immunoprecipitate. The western blot (Fig. 11.B.) with monoclonal antibodies against E2 and E1β shows bands of 72 and 36 kDa in the immunoprecipitate from both MRC5 mitochondria and cells.

PDH PATIENT MITOCHONDRIA

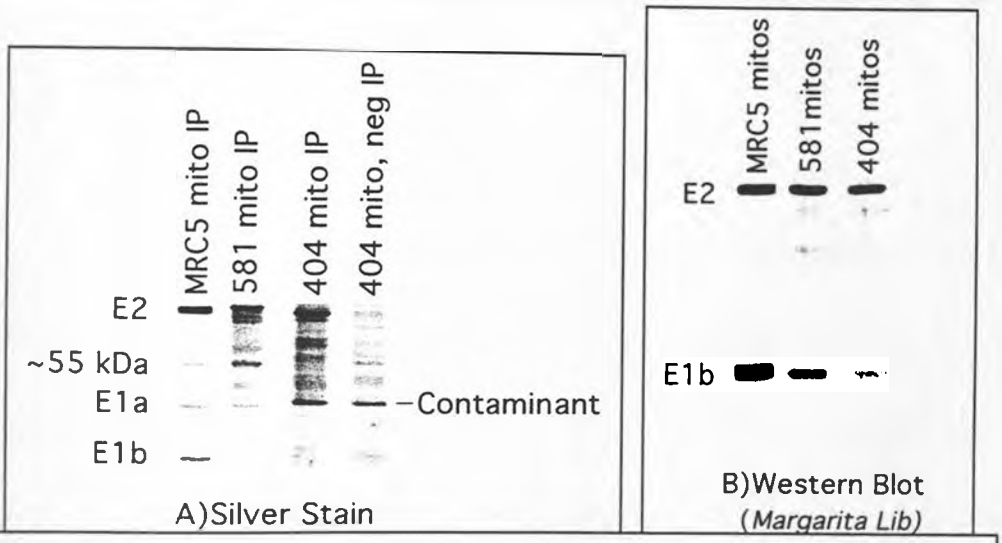


Figure 12. Mitochondria from patients with PDH deficiency. A) Silver stain of immunoprecipitation from two cell lines, 404 and 581, shows E1β notably lacking compared to control. A non-specific binding of a 41 kDa protein obscures presence/absence of E1α. B) Western blot against whole mitochondria from these cells using E2 and E1β monoclonal antibodies shows lowered levels of E1β.

Immunoprecipitation on the four cell lines showed two of the lines, 404 and 581, to be lacking the 36 kDa band when compared to control MRC5 inmmunoprecipitation (Fig. 12.A). The presence of a protein binding non-specifically to normal mouse antibody at 41 kDa obscures interpretation of whether an E1α band at 41 kDa is present in these mitochondria. Western blotting was done against the whole mitochondria, and illustrated that both cell lines have lowered levels of 36 kDa E1β band by immunoreactive measures

(Fig. 12.B.). Not enough patient mitochondria were available at the time of this writing to test a western blot against immunoprecipitation from these mitochondria.

DISCUSSION

EXTRACTION

Immunoprecipitation of PDH from bovine heart mitochondria shows a robust signal on both silver staining and western blotting, indicating that this technique can immunoprecipitate the complex in large quantity. The human materials likewise show a strong signal from western blots against E2 and E1β, but the silver stain is less robust. The amount of E2 present in the silver stain is far greater than what would be expected from the ratio of subunits in the complex *(E2 (60) : E3 (12) : X (12) : E1α (60) : E1β (60))* It appears that immunoprecipitating with an E2 antibody is extracting some E2 not complexed to the other subunits. This result could come from the presence of free E2 subunit in solution, or it could be that the human PDH complex is less stable than the bovine under the conditions used, so the complex partially disassembles during the immunoprecipitation.

CONSIDERATION OF SOURCE

The success from bovine and human materials is not directly comparable, however, because the bovine extraction was done from heart cells, whereas the immunoprecipitation with human material was against fibroblast (skin) cells. Expression of PDH is much stronger in heart cells (13), and it has been shown that expression of PDH in fibroblasts may not reflect the expression of PDH in other tissues (9,17). Immunoprecipitating against human heart cells may show a yield as high as the bovine immunoprecipitations. For clinical applications, obtaining human heart cells for study is usually not possible, unless a patient has died. However, examining heart tissue from a deceased patient may provide information that can be valuable to the family members.

OUTCOME OF BLIND STUDY

The two patients in the blind study were correctly identified with immunoprecipitation, showing promise for immunoprecipitation as a tool for identifying PDH deficiencies. The two patients had different mutations, both affecting E1α, so the absence of E1β suggests an assembly defect preventing the α and β subunits from forming the whole E1 subunit. Obtaining an E1α monoclonal antibody would be most useful for further examination of these patients, because determination of E1α levels on the silver stain is complicated be the presence of a non-specifically binding 41 kDa protein.

IMMUNOPRECIPITATION FOR CLINICAL APPLICATION

The clinical outcome of the two patients was significantly different, emphasizing the variety of course that PDH deficiency may have. Patient 404, who appears to have the lowest level of E1 assembly from the western blot (Fig. 12.B.), lived to twelve years old (18), while patient 581, who had higher E1β levels on the western blot, died at 5 months old (personal communication, Dr. N. Kennaway). Treatments for PDH deficiency are still not sufficient to correct the disease, but the most effective treatments currently rely on early diagnosis. Immunoprecipitation from whole human cells, as illustrated here, can eliminate the time needed for extracting mitochondria and can speed diagnosis. This method provides information about assembly of the complex that may not be evident from activity studies and western blots of whole mitochondria. Immunoprecipitation is a tool that can be useful is diagnosing patients with PDH deficiency and understanding the nature of the disorder.

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