FEBS 26504 FEBS Letters 529 (2002) 173–178

Quantitative proteomics: the copy number of pyruvate dehydrogenase is more than 10^2-fold lower than that of complex III in human mitochondria

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Received 19 July 2002; accepted 8 August 2002

First published online 6 September 2002

Edited by Vladimir Skulachev

Abstract Pyruvate dehydrogenase (PDH) and complex III are two key protein complexes in mitochondrial metabolic activity. Using a novel quantitative Western blotting method, we find that PDH and complex III exist at a steady-state ratio of 1:100, 1:128 and 1:202 in HeLa cell extracts, fibroblast mitochondria and heart tissue mitochondria, respectively. This difference in stoichiometry is reflected in the immunogold labeling intensities of the two complexes and by the much more sparse distribution of PDH in fluorescence microscopy. In Rho0 fibroblasts there is a 64% reduction of complex III but the concentration of PDH remains the same as wild-type.

Key words: Mitochondria; PDH; Complex III; Quantification; Proteomics; Immunogold labeling

1. Introduction

The full catalog of human genes is now known from automated genome analysis [1]. Newly developed proteomics approaches are identifying multiple gene products for many of the genes related to alternative splicing and post-translational modification [2,3]. Further, the cellular interactions of many proteins are being revealed by two-hybrid and immunocapture approaches in conjunction with proteomic analysis [4]. What is currently missing from studies of cellular protein content is quantification of the amounts of proteins in cells that can vary by as much as 10^7-fold [5]. It will also be important to measure the changes in amounts of certain proteins that underlie many normal cellular processes, as well as pathological conditions.

Our studies are on the structure and functioning of mitochondria in human cells. Proteomics studies have identified many of the proteins permanently or transiently a part of the organelle [6,7]. These include the components of oxidative phosphorylation (OXPHOS), other enzymes involved in energy metabolism such as components of the Krebs cycle, proteins involved in mitochondrial DNA replication, as well as transcription and translation of this DNA, and proteins involved in apoptosis. To establish suitable methods for determining the amounts of specific proteins, we have focused initially on two complexes involved in energy metabolism in mitochondria, the pyruvate dehydrogenase complex (PDH) and a respiratory chain complex – the ubiquinol cytochrome *c* oxidoreductase (complex III). PDH is a large complex (M_r 8 million) involved in funneling pyruvate formed in glycolysis into the Krebs cycle by conversion to acetyl CoA [8]. When OXPHOS is inhibited or reduced by hypoxia and many other conditions, PDH is inhibited and the glycolytically produced pyruvate is converted to lactic acid by lactate dehydrogenase [9]. Complex III is one of four complexes of the respiratory chain. As the name implies, it transfers electrons from reduced ubiquinone to cytochrome *c* [10] and is, therefore, at the center of the respiratory chain, and its amount is a good measure of respiratory chain content of cells. We have measured the levels of these two protein complexes in three human cell types – heart cells, fibroblasts and HeLa cells. Adult heart cells are fully differentiated and rely almost exclusively on OXPHOS for energy [11]. By contrast, cells in tissue culture, such as primary fibroblasts, and HeLas, which are cancer cells, are rapidly dividing and use glycolysis almost exclusively when grown in high glucose [12]. Therefore, we anticipate that the levels of enzyme involved in energy metabolism will be very different in these cell types.

We have used a novel quantitative Western blotting electrophoresis approach. This method uses monoclonal antibodies (mAbs) highly specific to each protein to detect and quantify them. The specific antigenic protein, once over-expressed and purified, is used as a calibration reference. To complement these measurements of amounts, we have examined the distribution of both protein complexes by fluorescence and electron microscopy (EM) techniques. These show the much more sparse distribution of PDH compared with complex III, consistent with the stoichiometry measurements.

2. Materials and methods

2.1. cDNA expression

Standard molecular biology procedures were carried out according to Sambrook et al. [13] using *Escherichia coli* strain XL1 Blue (Stratagene, La Jolla, CA, USA). cDNA sequences for human core 2 and $E_1\alpha$ were generated using a PCR amplification strategy from a quick-clone human heart cDNA library (Clontech, Palo Alto, CA, USA). Amplification using a 5′ primer incorporates an in-frame N-terminal six histidine tag, also both the 5′ and 3′ primers incorporate restriction sites to facilitate subcloning into the *Nco*I and *Nde*I sites of the *E. coli* expression vector pET15b (Novagen, Madison, WI, USA). Inserts were fully sequenced by automated sequencing. The $E_1\alpha$ se-

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PII: S0014-5793(02)03278-7

quence encoded the known mature form of the protein and the core 2 sequence encoded the entire protein.

2.2. Expression and purification

E. coli strain BL-21 (DE3) (Novagen) was transformed with the pET15b expression vectors containing the appropriate cDNA insert. 200 ml of cells in LB medium containing 50 μg/ml ampicillin were grown to an OD_{600} 0.6. Protein expression was induced by addition of IPTG to a final concentration of 1 mM. Cells were grown at 37°C for 3 h. and then harvested by centrifugation. Cell pellets were resuspended in binding buffer (20 mM Tris–HCl, pH 7.9, 50 mM NaCl, 5 mM imidazole) and sonicated for 5 min. After sonication, the cells were spun at $5000\times g$. In both cases expressed protein was found in the pellet after this low-speed spin, indicating its presence in inclusion bodies. These inclusion bodies were solubilized in binding buffer supplemented with 6 M guanidine and incubated on ice for 1 h. After centrifugation at $20\,000\times g$, the target proteins were found in the supernatant based on SDS–PAGE analysis. This supernatant was applied to a Ni-NTA column that had been pre-equilibrated with binding buffer. The column was washed with 10 volumes of binding buffer followed by 15 volumes of wash buffer (20 mM Tris–HCl, pH 7.9, 50 mM NaCl, 60 mM imidazole). The bound protein was eluted in fractions with 10 volumes of elution buffer (10 mM Tris–HCl, pH 7.9, 25 mM NaCl, 500 mM imidazole).

2.3. Dialysis and protein concentration determination

The elution fractions were analyzed by SDS–PAGE and those containing the highest concentration of pure protein were selected for dialysis. This was done using Spectra/Por dialysis tubing MWCO 3500 (Spectrum, CA, USA) using a sample volume ratio of 1:1000 of dialysis buffer (20 mM Tris–HCl, pH 7.9, 50 mM NaCl, 0.1% SDS) for 5 h; this procedure was performed twice to ensure complete removal of imidazole. Isolated protein concentration was then determined by the method of Gill and von Hippel [14], measuring absorbance at 280 nm as a function of the extinction coefficient of the protein based on its Trp, Tyr and Cys content.

2.4. Sample preparation

Protein concentrations were measured by the BCA method (Pierce, Rockford, IL, USA). Mitochondria from bovine heart were purified according to Smith [15]. Human heart mitochondria were prepared by Analytical Biological Services (Wilmington, DE, USA) also according to Smith. HeLa cells were cultured and counted using a hemocytometer.

2.5. SDS–PAGE and quantitative Western blotting

Gel electrophoresis was performed according to Laemmli in a 15% gel [16]. For Western blotting, samples were transferred in CAPS buffer from gels to polyvinylidene difluoride membranes (0.45 μm pore size, Millipore, Bedford, MA, USA) [17]. All antibodies were diluted to 1 μg/ml. Primary antibodies to core 2, $E_1\alpha$ and the secondary biotinylated goat anti-mouse secondary antibody were obtained from Molecular Probes (Eugene, OR, USA). Reactive secondary antibodies were detected by streptavidin conjugated to the fluorescent probe Alexa Fluor-488 (Molecular Probes). Fluorescence was detected using the STORM scanning system (Molecular Dynamics, Sunnyvale, CA, USA).

2.6. Immunogold transmission electron microscopy (TEM)

Bovine heart tissue was obtained within 90 min post mortem. Tissue was minced and fixed in 0.1% glutaraldehyde/4% paraformaldehyde in 0.1 M sodium phosphate buffer for 2 h. Tissue was washed twice in buffer, 15 min per wash, to remove fixative, and washed once for 15 min in buffer plus 50 mM glycine. Samples were then incubated in 5% gelatin for 15 min at 37°C. Gelatin was solidified at 0°C and blocks were placed in 2.3 M sucrose in buffer overnight at 4°C with rotation. Tissue blocks were then placed on sectioning pins and plunge-frozen in liquid nitrogen.

Cryosections were prepared by the method of Liou et al. [18] using a Reichert-Jung Ultracut microtome with F4E cryo-attachment, placed on 400 mesh nickel grids and incubated on 2% gelatin at 37°C for 20 min. Sections were washed four times with 0.15% glycine in 0.14 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4 (PBS) and then blocked in 5% bovine serum albumin for 15 min. Primary antibodies were diluted to 60 μg/ml in PBS and incubated with sections for 3 h. After washing four times in the same buffer, sections were incubated with 5 or 10 nm gold-conjugated GAM secondary antibodies (Amersham, Buckinghamshire, UK) diluted 1:20 in PBS for 30 min. After four additional washings in PBS, antigen–antibody complexes were stabilized by incubation with 1% glutaraldehyde, 5 min. Sections were washed seven times in double distilled H_2O and counterstained with a 0.4% uranyl acetate–2% methyl cellu-

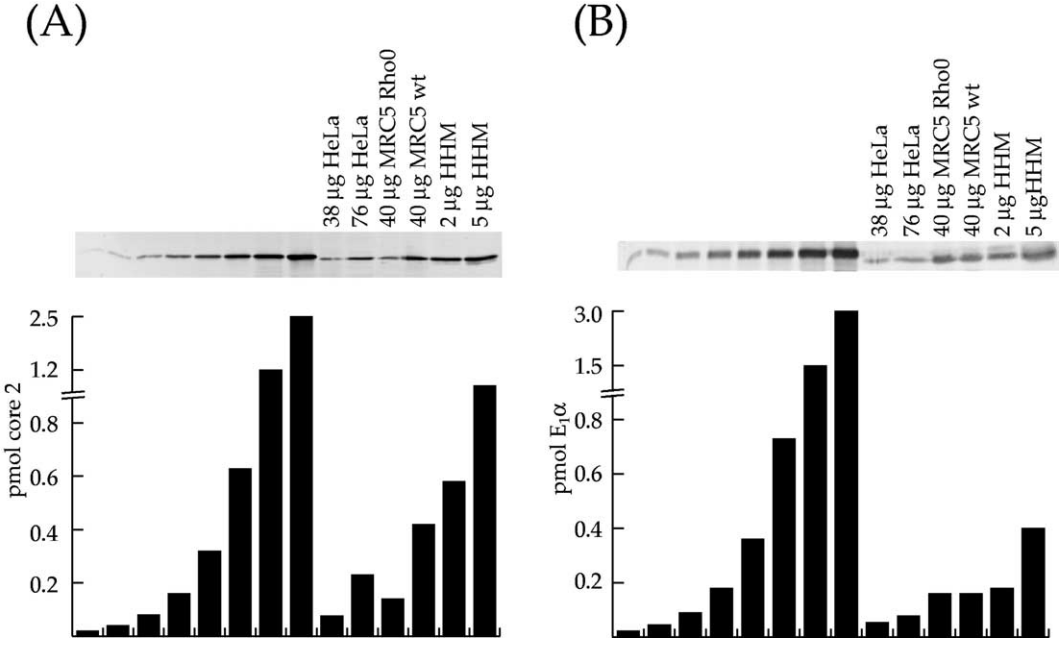

Fig. 1. Western blot quantification of the PDH protein, $E_1\alpha$, and the complex III protein, core 2, in human heart and fibroblast mitochondrial preparations and within a defined number of cultured human cells. The proteins core 2 (A) and $E_1\alpha$ (B) were detected by Western blot in 38 μg, 76 μg of HeLa cells, 40 μg of Rho0 mitochondria, 40 μg of MRC5 wild-type mitochondria, 2 μg and 5 μg of human heart mitochondria. The density of the detected band is then compared to that of increasing amounts of the respective purified recombinant protein (the first eight columns). These band densities were converted to pmol concentrations and are represented as a histogram below.

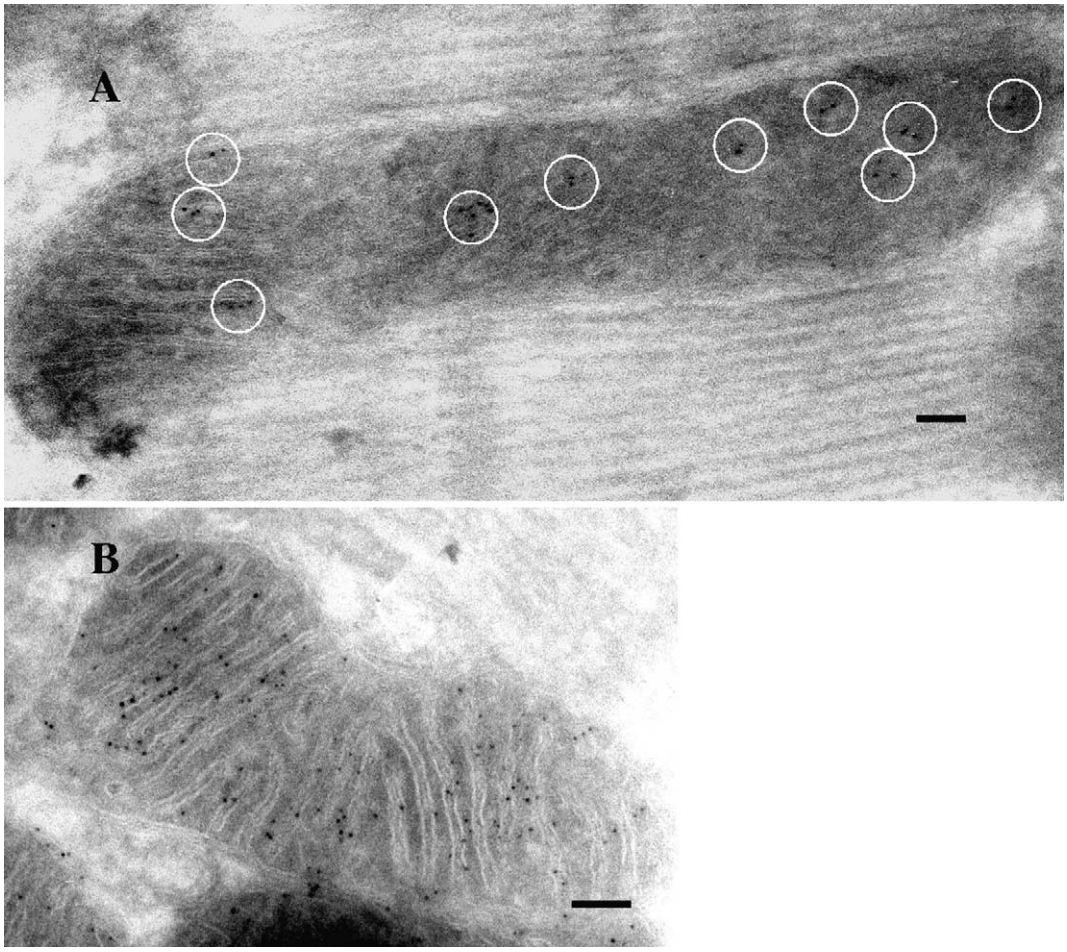

Fig. 2. Immunolabeling of heart ultrathin cryosections. A: Ultrathin cryosection labeled with anti-PDH E_2 mAb and 5 nm Au-conjugated GAM secondary antibody. Note multi-particle label clusters (circled). B: Anti-complex III core 2 mAb, 5 nm Au GAM secondary. All size bars 100 nm.

lose solution, and then viewed on a Philips CM12 TEM at 80 kV. Image analysis of scanned negatives was performed using NIH Image.

2.7. Immunofluorescent microscopy

HeLa cells expressing mitochondrial matrix-targeted green fluorescent protein (GFP) were seeded onto slides and grown to ~50% confluence in Dulbecco's modified Eagle's serum and permeabilized by addition of acetone for 10 min. Cells were then incubated with Alexa Fluor 594-conjugated anti-PDH E_2 for 1 h (Molecular Probes). Slides were viewed on an Axioscope 2 microscope with Attoarc HBO 150 W mercury lamp.

3. Results

The quantitation described here depends on having mAbs highly specific to the proteins of interest. In the cases of PDH and complex III, we have produced several such mAbs, and for Western blotting chose a mAb against the $E_1\alpha$ subunit and core protein 2 respectively. These mAbs show only a single band on SDS–PAGE gels of whole mitochondria from heart, and one band with either mitochondria or whole cell extracts of fibroblast and HeLa cell lines [19]. To complement the chosen mAbs, the two proteins (PDH $E_1\alpha$ and complex III core 2) were purified after over-expression in *E. coli*. Each of these ran as a single band.

3.1. The ratio of complex III to PDH is around 200:1 in human heart mitochondria

Fig. 1 shows a typical experiment in which accurately determined amounts of heart mitochondrial protein were subjected to electrophoresis, and subsequent blotting and detection in the same gel as a range of concentrations of purified $E_1\alpha$ of PDH and core 2 of complex III. In this way, the experimental and control samples were treated equally throughout the entire procedure. Conventional Western blotting methods utilize a catalytic reaction by an enzyme-coupled antibody complex for detection. Here, we incubated the blots with primary mAbs and then in a secondary reaction with an anti-mouse mAb, which was labeled directly with the fluorescent dye Alexa Fluor 488, as a reporter group. In heart mitochondria, values of 80 ± 34 pmol/mg and 270 ± 72 pmol/mg were obtained for the $E_1\alpha$ subunit of PDH and core 2 protein respectively. The large variation in values for each protein is probably due to the variability in purity and integrity of the organelle in different mitochondrial preparations. $E_1\alpha$ is present in 60 copies per PDH complex monomer [8]. Complex III is composed of 11 different subunits with core 2 present in one copy per monomer [10]. Thus, the ratio of PDH to complex III in human heart mitochondria is 1:202, see Table 1.

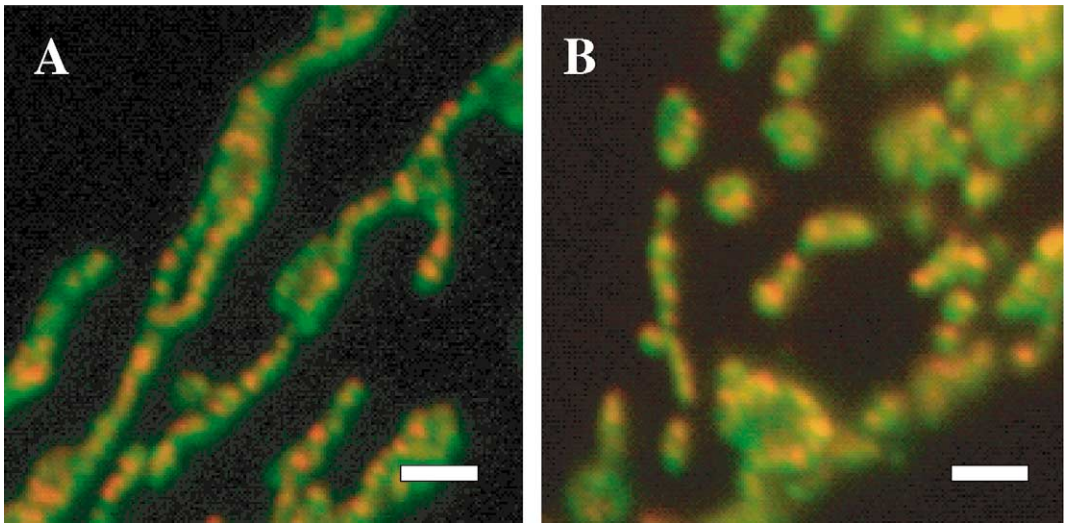

Fig. 3. Fluorescence microscopy of HeLa cells. A: Merged image of HeLa cell mitochondrial reticulum of HeLa cell visualized by mitochondrial matrix-targeted GFP (green) and PDH visualized by immunolabeling with Alexa Fluor 598-conjugated anti-PDH E_2 mAb (yellow). Note punctate PDH labeling. B: Fragmented mitochondria of HeLa cell visualized as in A. Size bars 2 μm.

3.2. *In human cells from tissue culture, the ratio of complex III to PDH is around 100:1*

Also determined were the levels of PDH to complex III in MRC fibroblast mitochondria and in HeLa cells. In both of these cell lines, there are much lower amounts of these two complexes than in heart mitochondria on a per mg protein basis. Thus, fibroblast mitochondria and HeLa whole cell extracts have around 27-fold and 80-fold less complex III respectively than heart tissue.

The ratio of PDH to complex III was approximately 1:128 for fibroblast mitochondria and 1:100 in HeLa cells. In one set of experiments, the levels of complex III and PDH were measured in Rho0 MRC5 fibroblast mitochondria, isolated from cells in which mtDNA had been depleted by EtBr treatment. Such cells are a useful model for mtDNA depletion syndrome, one of many pathological conditions caused by a genetic alteration of mitochondrial biogenesis [20]. In Rho0 cells the level of core 2 was only 35% of untreated cells. However, depletion of mtDNA did not alter the levels of PDH.

3.3. *Immunoelectron microscopy to detect levels of complex III and PDH*

The same mAb used in Western analysis for quantification of core protein 2 gave good immunolabeling of ultrathin heart cryosections as shown in Fig. 2A. For reaction with PDH, a mAb against the E_2 subunit proved superior to the $E_1\alpha$ subunit mAb and was used instead (Fig. 2B). For both complexes, several sections were fixed, reacted with primary mAb and then labeled with secondary antibody with attached 5 nm gold particles.

As evident in Fig. 2B, core 2 protein is distributed relatively evenly through the sections along the clearly observed mitochondrial cristal membrane. By contrast, the E_2 subunit of PDH appears as irregularly distributed clusters of two to six gold particles in the space between these membranes. The density of labeling was 80 particles/μm^2 and 364 particles/μm^2 for PDH E_2 and core 2 respectively. Accounting for 60 copies of E_2 per PDH enzyme, this represents an enzyme ratio of 1:273 for PDH:complex III, which is in the same range found by Western blot quantification in heart mitochondria.

3.4. *Fluorescence imaging of PDH distribution*

HeLa cells expressing a mitochondrial matrix-targeted GFP [21] show fluorescent green labeling throughout the entire mitochondrion, which extends under some conditions as a filamentous network through the cell. As shown in Fig. 3A, PDH labeled red with the E_2-reactive mAb is distributed punctately along this reticulum (yellow spots due to superimposition of the red mAb on the green GFP). Fragmentation of the reticulum, as shown in Fig. 3B, generates a large number of small ovoid mitochondria with at least one, and usually several PDH complexes in each.

4. Discussion

A full description of the protein content of a cell requires both identification of all gene products expressed, including post-translationally modified forms, and estimates of the amounts of each over a range of times and cellular conditions. Here, we have examined the cellular content of two proteins,

Table 1
Quantification of PDH ($E_1\alpha$) and complex III (core 2) in heart and cultured cell mitochondria and also in whole HeLa cell lysate

	PDH $E_1\alpha$ (pmol/mg)	Complex III core 2 (pmol/mg)	Ratio PDH/complex III core 2
Heart mitochondria	80 ± 34 (8)	270 ± 72 (6)	1:202
MRC5 mitochondria	4.7 ± 2.1 (4)	10 ± 1.8 (7)	1:128
MRC5 Rho0 mitochondria	5.7 ± 3.1 (4)	3.6 ± 1.4 (7)	1:38
HeLa cell extract	2.0 ± 1.0 (11)	3.3 ± 1.2 (11)	1:100

The mean concentrations ± S.D. of $E_1\alpha$ and core 2 proteins are shown. The enzyme ratio is determined by this concentration and the stoichiometric subunit composition of these enzymes, i.e. 60 $E_1\alpha$ proteins/PDH enzyme and one core 2 protein/complex III.

PDH and complex III of mitochondria, by a novel Western blotting approach that uses highly specific mAbs to these proteins and purified antigen proteins for calibration. The secondary mAb used in the quantitative detection is conjugated directly with Alexa Fluor dye, which has a high quantum yield and, therefore, does not require enzymatic amplification to generate sufficient signal.

We find that the levels of complex III (and PDH) are 27 times higher in human heart mitochondria on a per mg of total protein basis than in the organelle isolated from the primary cell line MRC lung fibroblasts and 80-fold higher than in whole cell extracts from the transformed cell line HeLa. This is expected because heart mitochondria are specialized for producing high levels of ATP to fuel the contractile function of this tissue and are enriched in cristal membrane [22], the site of OXPHOS enzymes. Cells in tissue culture, in contrast, produce most of their energy by glycolysis rather than OXPHOS [12,23]. Such cells typically show many fewer cristae per unit volume than heart mitochondria [20].

Significantly, there is a change in the ratio of PDH to complex III. In heart cells, this ratio is 1:202, while in fibroblast mitochondria and HeLa cells it is 1:128 and 1:100 respectively. The implication is that the levels of PDH and complex III are differently regulated at the expression or translational level in response to energy substrate supply and cellular ATP needs. When mitochondrial DNA is depleted from fibroblast cells by EtBr, a functional complex III is no longer assembled because the mitochondrially encoded cytochrome *b* is no longer synthesized. As a result, the steady-state levels of core 2 were reduced by two thirds. However, the levels of PDH were unaltered, indicating that expression of PDH is not linked to mtDNA levels.

It is interesting to convert the concentration of various proteins in a cell into copy number, which can be done for HeLa cells, where we measured enzyme concentrations in whole cell extracts obtained from a known number of cells. There are around 15 000 copies of PDH and 1.5 million copies of complex III in these cells. This compares with a copy number of mtDNA of 1000–5000 [24,25] (a value that remains to be determined accurately).

Recent studies have established that mitochondria are pleomorphic [26]. In differentiated heart tissue, there are large individual mitochondria lying along and within the contractile elements. In cell culture, the organelle can exist as a continuous reticulum or as thousands of ovoid mitochondria, depending on the cell cycle stage [26]. These variations of morphology are evident in EM, as well as light microscopy when the organelle is labeled by the fluorescent GFPs. Here, we have examined the distribution of PDH and complex III within mitochondria by these techniques. The two complexes were immunolabeled for EM using specific mAbs and secondary antibodies with 5 nm gold particles attached. In sections of heart tissue, complex III was evenly distributed within the organelle, predominantly on cristal membranes while PDH was clearly in the matrix space between cristae. The labeling efficiency of proteins in immunoelectron microscopy is low due to inaccessibility of epitopes to the primary antibody in the embedded section and also steric hindrance to the reaction of the second antibody [27]. However, the fact that the ratio of PDH to complex III determined by immunogold labeling is 273:1, similar to the 202:1 determined in Western blots, suggests that the accessibility of epitopes for their respective mAbs in the two complexes is nearly identical. The pattern of gold particles is not a true reflection of the density of distribution of complex III for the reasons above. The distribution of PDH seen in the EM pictures may be more representative because each complex has 60 possible antibody binding sites. The circles drawn in Fig. 2 define the size of one PDH complex with mAbs attached (the PDH complex itself has a diameter of 210 Å [28], each mAb is about 100 Å long). It can be seen that the PDH is labeled by clusters of two to six gold particles, indicating that the efficiency of labeling in the EM section is greater than the 1.7% at which each complex will bind one or less mAb. From Fig. 2, the distance between PDH complexes is calculated to be 0.15 μm.

Individual complex III molecules are not sufficiently dispersed to be resolved by light microscopy, and Alexa Fluor 594-labeled mAbs to core protein 2 give a continuous staining of the entire mitochondrial network (result not shown). This is not the case for PDH, which is stained punctately by specific mAbs. Based on data such as in Fig. 3, the average distance between each complex is 0.24 μm. As evident in Fig. 3B, each ovoid organelle would receive multiple PDH complexes under conditions where the mitochondrial reticulum becomes fragmented. From this and other data, the picture is emerging in which the fusion and fission of mitochondria involves a 'repeating unit' that contains at least one nucleoid (one to three copies of mtDNA and associated proteins) along with multiple OXPHOS complexes and several PDH complexes [29]. Whether the mitochondrion is functionally homogeneous or heterogeneous when reticular or fragmented remains to be determined.

Acknowledgements: We thank Kathy Chicas-Cruz for her expertise in cell culture. R.G. was supported by National Institutes of Health Training Grant GM07759.

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