Electrophoretic separation of multiprotein complexes from blood platelets and cell lines: Technique for the analysis of diseases with defects in oxidative phosphorylation

A two-dimensional electrophoretic technique combining blue native polyacrylamide gel electrophoresis (BN-PAGE) with Tricine sodium dodecyl sulfate (SDS)-PAGE was previously used for the localization of oxidative phosphorylation (OXPHOS) defects in human diseases starting from biopsy or autopsy tissues (Schägger, H., *Electrophoresis* 1995, 16, 763–770). In the present work the technique was extended for the resolution of OXPHOS enzymes from platelets and tissue-cultured cells. Silver staining is required to detect the protein subunits of OXPHOS complexes in two-dimensional gels. However, the use of cultured cells has major implications for patients with mitochondrial encephalomyopathies since it will reduce the number of invasive muscle biopsies. The ease of isolating the platelet membrane glycoprotein complex from a few milliliters of blood makes it possible to analyze this complex and its protein subunits in bleeding disorders like Glanzmann's thrombasthenia.

1 Introduction

In the present work a special type of two-dimensional electrophoresis, combining blue native polyacrylamide gel electrophoresis (BN-PAGE) in the first dimension with Tricine-SDS-PAGE in the second dimension, was used. Major principles of BN-PAGE are as follows: Membrane proteins are solubilized from biological membranes by neutral detergents. Coomassie blue dye is added to the cathode buffer and to the protein solution before it is applied to an acrylamide gradient gel buffered at pH 7. Negatively charged Coomassie dye binds to hydrophobic protein surfaces, keeps membrane proteins soluble in the detergent-free gel, drag proteins into the anode, and makes native proteins visible during the run. Protein migration stops when a limiting pore size of the gradient gel is reached. Therefore, a separation of proteins according to their molecular masses is achieved. Tricine-SDS-PAGE, used in the second dimension, then resolves the subunits of protein complexes.

This two-dimensional (2-D) technique has been used for the quantification of oxidative phosphorylation (OXPHOS) complexes in Parkinson’s disease [1], in mitochondrial encephalomyopathies [2], and for the detection of specific complex V deficiencies in Alzheimer’s disease [3]. All these studies used tissue specimens from biopsies (skeletal muscle) and autopsies (brain, liver, heart). Any technique that allows the use of patient cell lines, e.g. skin fibroblasts and lymphoblasts, or the use of the platelet fraction from biopsies would be of great benefit for patients and researchers because less muscle biopsies would be required, and sufficient sample would be available for the analysis of the molecular basis of the defect. The enzymatic analysis of OXPHOS enzymes in fibroblast cell lines and in platelets from patients with encephalomyopathy, Parkinson’s or Alzheimer’s disease, showed already that OXPHOS defects are also often detectable in fibroblasts [4, 5] and platelets [6, 7].

Direct application of the electrophoretic technique developed for skeletal muscle to cultured cells or blood platelets is problematic because these cells have an approximately tenfold lower mitochondrial content compared to skeletal muscle. This would lead to overloading of gels and smearing of protein bands. On the other hand, a preceding preparation of mitochondria from cells is also problematic because the yields of mitochondria obtained by conventional homogenization-centrifugation procedures can vary considerably. Variations in protein quantities would then lead to variations in detergent/protein ratios used for membrane protein extraction, and to considerable variations in the performance of 2-D gels. The major problem to be solved therefore was the development of a protocol for the preparation of mitochondria from cells with reproducible yield.

2 Materials and Methods

2.1 Cell lines

Murine L-929 fibroblasts, human FL amnion cells, HeLa cervix carcinoma epithelial cells and primary fibroblasts from healthy individuals and from one patient with cytochrome oxidase deficiency were grown in Eagle's minimal essential medium in the presence of fetal calf serum and penicillin/streptomycin. Murine NIH/3T3 fibroblasts and human Molt-4 T-cell lymphoblasts were grown in RPMI 1640 medium with fetal calf serum and penicillin/streptomycin.

2.2 Two-dimensional electrophoresis

2.2.1 Preparation of a platelet fraction from blood

Na+/EDTA (1 mg/mL) or Na+/citrate (3.8 mg/mL) was added to fresh human blood from tenfold concentrated...
stocks in 0.9% NaCl, pH 7.4. Four mL of EDTA- or citrate blood, stored at 4°C for maximally two days, was diluted with 2 mL of 0.9% NaCl solution, loaded onto a 6 mL cushion of Histopaque 1.077 (Sigma) in a 14 mL plastic tube, and centrifuged at 20°C for 30 min at 680 g (2500 rpm in a Beckman JS 13.1 swinging bucket rotor). The cells at the interphase, mostly platelets and some lymphocytes, were harvested and diluted with a twofold volume of a buffer containing 150 mM NaCl, 150 mM Na-phosphate, pH 7.2, at 20°C. After centrifugation for 15 min at 1750 g at 20°C the sedimented cells were suspended with 0.5 mL sucrose buffer (250 mM sucrose, 0.5 mM EDTA, 10 mM Tris-HCl, pH 7.4, at 4°C) and centrifuged 15 min at 10000 g. The sediment, usually around 10 mg, was rapidly frozen in liquid nitrogen and stored at −80°C. The subsequent isolation of a mitochondrial fraction, the solubilization of membrane proteins, and the processing for application to BN-PAGE followed the protocol outlined below.

2.2.2 Sample preparation from sedimented cells and protein load to BN-PAGE

Ten milligrams of sedimented cells (wet weight) isolated either from blood or from cell cultures (around 5 × 10⁶ cells) were used for one electrophoretic analysis. Frozen cells were used because direct use of non-frozen cells led to considerable losses. The initial protein amount, determined by a modified Lowry procedure [8], was in the 1.5–1.7 mg range with blood platelets and with cultured cells. The frozen cells were suspended in 500 μL of a hypotonic buffer (83 mM sucrose, 10 mM Na+/Mops, pH 7.2), homogenized extensively in a tightly fitting 1–2 mL glass-Teflon homogenizer (15 cycles 1000 rpm), and collected by rinsing the homogenizer with 500 μL of 250 mM sucrose buffer. After a 15 min centrifugation at 600 g (4°C) a mitochondrial fraction was collected through 15 min centrifugation at 15000 g. The sediment contained 0.10–0.15 mg total protein, i.e., around 6–9% of the starting material, but about 70% of ubiquinol-cytochrome c reductase activity. The mitochondrial-enriched pellet was suspended in 15 μL of 1 mM 6-aminohexanoic acid, 50 mM Bistris/HCl, pH 7.0, before the membrane proteins were solubilized by addition of 5 μL of 10% w/v dodecylmaltoside. After centrifugation for 15 min at 100000 g, 60–70% of ubiquinol-cytochrome c reductase activity was found in the supernatant. After addition of 2.5 μL of 5% w/v Serva Blue G dye in 1 mM 6-aminohexanoic acid to the supernatant, the total volume was applied to 5 × 1.6 mm gel wells in BN-PAGE. The total protein amount applied to the gel could not be determined directly, because protein determinations are disturbed by 6-aminohexanoic acid. Using sodium chloride for solubilization instead of 6-aminohexanoic acid, the total protein was always in the 50–100 μg range. The mitochondrial protein fraction thereof was estimated to be in the 10–20 μg range because the staining intensities of OXPHOS complexes during BN-PAGE (Fig. 1) were lower than those from 28 μg, but higher than those from 9 μg of isolated bovine heart mitochondria (cf. [1], Fig. 1, lanes 6 and 9). Using isolated mitochondria, up to 150 μg protein can be applied to 5 × 1.6 mm gel wells in BN-PAGE without provoking severe overloading effects. However, starting from sedimented cells, not more than 50–100 μg of total protein (isolated from 10 mg cells) should be applied. Increasing the protein load would not help to approach the detection limit of protein subunits by Coomassie staining, but would lead to a further increase of streaking of bands. At a lower protein load, on the other hand, some of the small subunits, e.g., the small subunits of complex II, might not be detectable after silver staining.

2.2.3 Electrophoretic techniques, staining, and quantification

Chemicals, first-dimensional BN-PAGE, second-dimensional Tricine-SDS-PAGE, staining and quantification techniques were used as described recently [1].

3 Results and discussion

3.1 Native resolution of multiprotein complexes by BN-PAGE

Figure 1 shows the native resolution of a mitochondrial-enriched protein fraction isolated from blood platelets (lane 2), compared to bovine heart mitochondrial pro-
1. DIM.: 6% T — BLUE NATIVE PAGE —> 13% T

COMPLEX I  V  III  GP  IV  II

2. DIM.:

TRICINE-SDS-PAGE

Figure 2. 2-D resolution of platelet multiprotein complexes. Lane (2) from Fig. 1 was cut into two equal parts, and the two halves were processed by Tricine-SDS-PAGE using 16% C, 3% T acrylamide gels as described previously [1]. One 2-D gel was silver-stained as shown. The second 2-D gel was used for protein sequencing. Protein subunits of complexes V, III, IV, and two protein spots of the platelet glycoprotein complex (GP) could be identified by their characteristic polypeptide patterns in the silver-stained gel, and by N-terminal protein sequencing, respectively. Complex I and II protein subunits could not be identified unambiguously. Most probable bands compared to the patterns from human heart [2] and from bovine heart mitochondria [10] were also marked.

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6% T — BLUE NATIVE PAGE —> 13% T

COMPLEX V  III  IV  IV  II

SUBUNIT  kDa

I  40

II/III  22

IV  17

Vab  11

VIabc  9.5

VIIabc  9.2

VIII  7.0

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Figure 3. 2-D resolution of OXPHOS complexes starting from 10 mg of sedimented Molt-4 lymphoblasts. First dimension: BN-PAGE using a 6-13% acrylamide gradient gel. The total protein load was in the 50-100 μg range, comprising about 10-20 μg mitochondrial proteins. Second dimension: Tricine-SDS-PAGE using 16.5% T, 3% C gels. Except for complex I, all OXPHOS complexes could be detected after silver staining (cf. Section 3.2). On the left and right sides the apparent molecular masses of complex IV and complex II subunits respectively, were indicated.

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teins (lane 1) and to the proteins from human skeletal muscle (lane 3). Complex I can be detected as a strong band in the lane of bovine heart mitochondrial proteins. Human complex I generally gives rise to a much broader band than the bovine enzyme. It can hardly be detected in human skeletal muscle and not at all in the lane from human platelets. Complexes III, IV, and V from skeletal muscle and also from platelets can be detected on the light screen. The position of complex II, as deduced from two-dimensional gels, is also indicated. The performance of BN-PAGE with human Molt-4 lymphoblasts, HeLa cells, skin fibroblasts, and FL amnion cells was similar to that of platelets, i.e., only faint bands were detectable (not shown). However, only platelets contained a prominent band at an apparent molecular mass of 330 kDa. Further resolution in the second dimension (Fig. 2) identified this band as the human platelet glycoprotein IIb-IIIa complex (see section 3.2.1). This complex is a receptor for adhesive proteins; it binds fibrinogen, providing interplatelet linkages, and the von Willebrand factor as a molecular anchor between the subendothelium and platelets [9]. The blue band of the platelet glycoprotein complex can be excised and electroeluted as described previously [10]. This is an alternative isolation method to the commonly used immunoprecipitation or affinity chromatography with antibodies against single subunits [11]. A protein extract from 200 mL of blood can be applied to one preparative gel (3 mm X 120 mm gel wells) and about 0.5-1.0 mg of electroeluted platelet glycoprotein complex is obtainable, e.g.
for protein chemical work, for studies of glycosylation, and for isolation of antibodies. It can be applicable also in the analysis of diseases with alterations in the platelet glycoprotein complex, e.g., neonatal alloimmune thrombocytopenia [12] and Glanzman's thrombasthenia [13].

### 3.2 Resolution of subunits of multiprotein complexes by SDS-PAGE

For analytical applications 0.5 cm lanes from the first-dimensional BN-PAGE (Fig. 1) can be processed directly by SDS-PAGE in the second dimension. After Coomassie staining of the 2-D gels the largest subunits of complex V and III, and subunit IV of complex IV can be detected and quantified (Coomassie-stained gels not shown). For detection of the characteristic polypeptide patterns of complexes, silver staining is required (Fig. 2). We stopped silver staining only when the small subunits of complex II could be detected, because complex II is the only one of the five OXPHOS complexes that comprises no mitochondrially coded protein subunit. It therefore serves as a mitochondrial marker that is not directly influenced by alterations in the mitochondrial genome. However, this additional information was obtained at the cost of a lower quality of 2-D gels. The prolonged staining led to a high background stain and emphasized the presence of horizontal streaking of bands. Detailed information about the subunit compositions of OXPHOS complexes is given in [14–18].

#### 3.2.1 Two-dimensional platelet protein analysis

A typical 2-D resolution of platelet proteins from a healthy person is shown in Fig. 2. Silver staining revealed the typical polypeptide patterns of OXPHOS complexes III, IV, and V. Subunits of complexes I and II could not be identified unambiguously. This would require the use of specific antibodies. However, a tentative assignment of polypeptide bands similar to the patterns from heart and other tissues [1] is given. The prominent native glycoprotein complex from platelets (lane 2 in Fig. 1) was resolved into two protein bands with molecular masses around 100 kDa and 22 kDa, which were intensely stained by Coomassie dye (not shown), but negatively stained in silver-stained gels (Fig. 2, arrows below GP). Using 10% acrylamide gels the larger band could be resolved into two bands (not shown). After electrophoretic transfer onto PVDF membranes the separated bands were identified by N-terminal protein sequencing (15 amino acids each) as integrins IIb-alpha and IIIa with molecular masses of 114 and 84.5 kDa [19, 20]. The 22 kDa band was not identified by sequencing. We expect that it represents integrin IIb-beta, the third component of the glycoprotein complex, with a molecular mass of 22.5 kDa [19]. Considering the 15% contribution of carbohydrate [11], a total molecular mass of 255 kDa for the holo-glycoprotein complex is calculated. The apparent molecular mass of 330 kDa, determined by BN-PAGE (Fig. 1 and [21]), is in reasonable agreement.
with the molecular mass of 265 kDa determined by analytical ultracentrifugation [11] and confirms the monomeric structure of the glycoprotein complex by an independent method.

3.2.2 Analysis of OXPHOS proteins from cell lines

Figure 3 shows the protein patterns in 2-D gels from Molt-4 lymphoblasts. The patterns from human cell lines, such as FL amnion cells and HeLa cells, look very similar (not shown). Complex I could rarely be identified in silver-stained 2-D gels from human cells, although it could be easily identified in murine cells (not shown). Complex II comprises four different protein subunits in mammals with molecular masses of 70, 27, 13 and 9.0 kDa, although five protein bands can be detected, as indicated by the arrows (Fig. 3). Amino-terminal protein sequencing revealed that the additional 9.3 kDa component is the preprotein to the 9.0 kDa protein that is not yet processed to the mature 9.0 kDa form (Schägger, H., unpublished). Complex IV is present in two forms, the major monomeric form with the normal apparent molecular masses of 200 kDa and a minor IV'-form with an apparent mass of about 150 kDa. Several protein subunits, e.g., the 9.5 kDa subunit V1α and the 7.0 kDa subunit, are missing in the IV'-form. This can be caused by dissociation from the holo-complex at the high detergent/protein ratio required for solubilization. Complex V-monomer and complex III-dimer with apparent molecular masses of 600 kDa and 500 kDa in the first-dimensional BN-PAGE (not shown) seem to be less sensitive to high detergent, and only one form each with the normal polypeptide composition [1—3] is observed in the second-dimensional SDS-PAGE.

3.2.3 Deviations from normal OXPHOS protein patterns in fibroblasts

A normal OXPHOS protein pattern from primary skin fibroblasts of a healthy individual is shown in Fig. 4A. Complex I again is the only complex that is usually not detected in Coomassie and silver-stained 2-D gels. A 2-D gel of a patient with mitochondrial encephalomyopathy is shown in Fig. 4B. Complex IV was hardly detectable in the patient's fibroblasts. This is in agreement with the low complex IV catalytic activity, measured in fibroblasts and muscle of the patient. The most prominent alteration, however, concerned Complex V. The largest subunits of complex V, the alpha and beta subunits, were detected at two different locations, at the normal position of complex V, also called F,F ATP-synthase (600 kDa), and at the position of the free F1, subcomplex (400 kDa). Since BN-PAGE separates proteins according to their molecular masses [21] the F1 subcomplex will be found in the vicinity of complex III, which has an apparent molecular mass of 480 kDa [22]. This complex V abnormality, characterized by the presence of a considerable portion of an F1 subcomplex was detected neither in skeletal muscle samples nor in fibroblasts from control individuals. It was also absent in muscle samples from patients with deletions or individual defects of either complex I or complex IV [2]. However, it was found recently in muscle of patients with Leigh's disease with normal complex IV, and a point mutation (T→G at position 8933) in the mitochondrial ATP 6 gene [23]. The single amino acid exchange in a protein subunit of complex V seemed to selectively influence the structural stability or assembly of this complex. In the present case a point mutation in any gene of a complex V subunit is unlikely, since additionally the amount of assembled complex IV was reduced by 80%. We therefore suspect that a novel point mutation in either one of the mitochondrial tRNAs could be responsible for the simultaneous expression of the complex V abnormality and the complex IV deficiency.

4 Concluding remarks

OXPHOS deficiencies present in skeletal muscle can also be expressed in skin fibroblasts in some forms of mitochondrial encephalomyopathies [24]. If OXPHOS deficiencies are not detectable in fibroblasts of patients with mitochondrial encephalomyopathy, a needle or surgical biopsy of skeletal muscle will still be required. The possibility to use cell lines from control individuals and from patients offers the chance for studies of OXPHOS complexes under varying conditions, i.e., there is more flexibility in probing the influence of inhibitors, hormones, and transcription factors. It seems possible to study the biogenesis, assembly, and turnover of OXPHOS complexes in cell culture and, the influence of thyroid hormones, steroid hormones, and cytokines on the OXPHOS system.

OXPHOS analysis in blood of patients certainly may have the same or even stronger restrictions as the analysis of cell lines. The percentage of the MELAS point mutation in mitochondrial DNA, for example, was found to be 90% in muscle, but only 25% in blood [5]. Similarly, the chronic progressive external ophthalmoplegia (CPEO) and Kears Sayre syndrome (KSS) forms of mitochondrial encephalomyopathies are characterized by deletions in muscle mitochondrial DNA, but these deletions are hardly detectable in blood cells. Therefore, the 2-D electrophoretic approach will not detect OXPHOS alterations in these cases (cf. references in [25]). In Pearson marrow-pancreas syndrome, however, the generalized cytopenia correlates with a high percentage of mtDNA deletions in peripheral blood [26], and enzymatic analyses revealed a complex IV deficiency in fibroblasts and lymphoblastoid cell lines [27]. Future experiments will show if these genetic and enzymatic defects are also expressed by reduced amounts of assembled complexes or by altered ratios of complexes in platelets. We would like to stress the potential use of the 2-D electrophoretic technique for the analysis of Glanzmann's thrombasthenia [13] or other diseases with defects in the platelet glycoprotein complex, e.g. neonatal alloimmune thrombocytopenia [12].

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5 References