CHAPTER NINE

RELIABLE ASSAY FOR MEASURING COMPLEX I ACTIVITY IN HUMAN BLOOD LYMPHOCYTES AND SKIN FIBROBLASTS

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Abstract
Complex I deficiency is probably the most common enzyme defect among the group of OXPHOS disorders. To evaluate a deficiency of complex I activity, biochemical measurements based on estimation of the mitochondrial rotenone-sensitive NADH:ubiquinone oxidoreductase activity are an important tool. Skeletal muscle is the most widely used tissue to examine complex I deficiency. However, obtaining a muscle biopsy requires an invasive surgical operation. It is much easier to obtain blood lymphocytes or skin fibroblasts, and, moreover, these cells can be expanded in number by standard techniques for extensive research on complex I. On the other hand, each of these cell types has disadvantages that hinder its measurement, such as the apparent low enzyme activity of lymphocytes and the highly contaminating nonmitochondrial NADH-quinone oxidoreductase activity of fibroblasts. This chapter describes a method to assay complex I activity reliably in a minute amount of either cell type.

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1. Introduction

The oxidative phosphorylation (OXPHOS) system is located in the inner mitochondrial membrane and is composed of five individual functional enzyme complexes. Complexes I, III, and IV are arranged as supercomplexes (Schagger and Pfeiffer, 2000) containing two additional electron carriers, coenzyme Q₁₀ (ubiquinone), and cytochrome c.

The NADH: ubiquinone oxidoreductase, or complex I, with a total molecular mass of about 900 kDa, is the largest of these five enzyme complexes and is composed of seven subunits encoded by mitochondrial DNA (mtDNA) and 38 nuclear-encoded subunits (Carroll et al., 2006). Although all of its subunits have been identified, how complex I assembles, which proteins aid this process (Lazarou et al., 2008), and how complex I assembles further into higher ordered respirasomes with complex III and IV (Schafer et al., 2006) is only partly understood. Its L-shape configuration contains a water-soluble peripheral arm protruding into the mitochondrial matrix and a water-insoluble hydrophobic arm embedded in the inner mitochondrial membrane (Brandt, 2006; Carroll et al., 2003). The peripheral arm contains the FMN cofactor and several iron-sulfur clusters that provide a bifurcated electron transfer pathway (Verkhovskaya et al., 2008) from NADH to ubiquinone, facilitating a state that might be required for conformational change of the membrane arm of the complex to translocate protons from the mitochondrial matrix to the intermembrane space. The subsequent proton motive force will drive the synthesis of ATP by complex V (Brandt, 2006; Ohnishi and Salerno, 2005).

Isolated complex I deficiency is probably the most common enzymatic defect of the oxidative phosphorylation disorders (Loeffen et al., 2000). Clinical presentation of complex I deficiency starts mostly at birth or early childhood and includes a great variety of clinical presentations, ranging from lethal neonatal disease to adult-onset neurodegenerative disorders that complicate the diagnostic process in individual patients. Mutations or deletions in mitochondrial and nuclear genes appear to account for approximately 50% of the cases of complex I deficiency (Thorburn et al., 2004). At present, it is becoming clear that mutations in assembly factor genes lead to impaired assembly and subsequent dysfunction of complex I as well (Dunning et al., 2007; Ogilvie et al., 2005; Pagliarini et al., 2008; Saada et al., 2008).

Skeletal muscle is the tissue most widely used for respiratory chain enzyme studies. Unfortunately, a biopsy that is large enough for extensive research requires a surgical operation. It would be more convenient to use tissue that can be obtained in a minimally invasive manner and expanded in vitro, such as blood cells or skin fibroblasts. However, determination of complex I in fibroblasts is difficult because of the high activity of
contaminating rotenone-insensitive NADH dehydrogenases (Chretien et al., 1994), and in blood cells the apparent low enzyme activity hampers its proper assay.

This chapter describes a method to measure complex I activity spectrophotometrically in blood lymphocytes and in cultured skin fibroblasts in a limited amount of sample with minimal interference of contaminating rotenone-insensitive NADH dehydrogenases, providing a reliable tool in diagnosing respiratory chain disorders and in studying the relationship between the structure of complex I and its enzyme activity.

2. Complex I Activity Measurement

2.1. Background

Mitochondrial NADH oxidation may proceed by means of two pathways. In one, NADH is oxidized by means of a rotenone-sensitive route by the NADH: ubiquinone oxidoreductase, which is located at the inner side of the mitochondrial inner membrane. In the other, NADH is oxidized in a rotenone-insensitive manner by NADH: cytochrome $b_5$ oxidoreductase (EC 1.6.2.2), which is located in the mitochondrial outer membrane (Sottocasa et al., 1967). The most characteristic enzyme activity of complex I is its rotenone-sensitive NADH: ubiquinone reductase (EC 1.6.99.3). Hence biochemical measurement of complex I is based on estimation of that specific rotenone-sensitive enzyme activity (Fischer et al., 1986). The NADH binding site of the enzyme is located in the peripheral arm of the complex; the ubiquinone-binding site resides in its hydrophobic membrane arm (Brandt, 2006; Carroll et al., 2003; Degli Esposti and Ghelli, 1994; Friedrich et al., 1993; Walker, 1992). The complex I inhibitor rotenone binds to that latter site and its inhibitory effect on the enzyme reaction is generally accepted as a standard for the integrity of complex I.

For the initial steady-state analysis of complex I, artificial electron acceptors such as ferricyanide (Galante and Hatefi, 1978) and 2,6-dichloroindophenol (Dooijewaard and Slater, 1976; Galante and Hatefi, 1978; Janssen et al., 2007; Jewess and Devonshire, 1999; Majander et al., 1991; Saada et al., 2004) have been used. However, because these artificial electron acceptors react with the NADH binding site of those enzymes only, the assay is not rotenone sensitive, and, therefore, discrimination between complex I and other NADH dehydrogenases is not possible. Consequently, the use of such electron acceptors in the assay of complex I will not reveal any defects further down the electron pathway leading to the ubiquinone binding site in the enzyme complex (Ragan et al., 1987; Verkhovskaya et al., 2008). Only by addition of extra ubiquinone will such an assay acquire some rotenone sensitivity, but because fibroblasts contain high activities of rotenone-insensitive
NADH oxidases, complex I is still difficult to detect (De Wit et al., 2008; Janssen et al., 2007). A similar reasoning holds for the recently developed Dipstick Assay (Mitosciences, Eugene, Oregon). In this assay complex I is immunoprecipitated on a dipstick and its activity measured in gel as NADH dehydrogenase activity, which is, of course, not rotenone sensitive. Although this method is easy to carry out and could be of value in obtaining preliminary indication for a deficiency of complex I, to obtain abnormal values, a more specific biochemical measurement should be performed, which is focused on the function of the entire enzyme. To study the biochemical function of complex I, an assay that uses ubiquinone as electron acceptor would be ideal. However, a water-soluble ubiquinone analog would be a more appropriate electron acceptor.

It is important to note that the accumulation of rotenone at its binding site in complex I is not instantaneous (Burgos and Redfearn, 1965; De Vries et al., 1996; De Wit et al., 2007). Therefore, the rotenone-insensitive NADH oxidoreductase should be determined separately from the total NADH oxidase activities to discriminate complex I activity from the total NADH oxidase activities. Because this discrimination requires a substantial amount of material using standard methods, a reliable, miniaturized method is needed.

2.2. Materials

2.2.1. Buffers

Phosphate-buffered saline (PBS-EDTA): 140 mM NaCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 2 mM EDTA, pH 7.4, store at 4 °C
Ammonium chloride solution (NH₄Cl): 155 mM NH₄Cl, 10 mM NaHCO₃, 0.1 mM EDTA, pH 7.4, store at 4 °C
SHE: 250 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.4, store at −20 °C

2.2.2. Other solutions

Lymphoprep™ (Axis-Shield PoC)
Complete protease inhibitors cocktail tablets (Roche Diagnostics)
4-(2-aminoethyl)-benzene sulfonyl-fluoride hydrochloride (AEBSF): 100 mM, store at −20 °C (Pefabloc SC, Roche Diagnostics)
Diisopropyl fluorophosphate (DFP): 0.2 M in dry isopropanol, store at −80 °C (Fluka Chemica)
SHE–PIM: SHE supplemented with two Complete tablets per 50 ml, 1 mM AEBSF and 2 mM DFP
DMEM (BioWhitaker)
Uridine (Sigma)
Digitonin, high purity (Calbiochem): 10 mg/ml heat for 5 min at 95 °C then cool down to room temperature, store at −20 °C
1 M K2HPO4, pH 7.4, store at 4 °C
2 M MgCl₂, store at 4 °C
5% fatty acid–free bovine serum albumin (BSA-FFA), store at −20 °C (Sigma)
5.7 mM NADH, make fresh daily
0.2 M KCN, make fresh daily
7.3 mM antimycin in dimethyl sulfoxide (DMSO), store at −20 °C (Sigma)
2.8 mM coenzyme Q₁ (CoQ₁) in ethanol, store at −20 °C (Sigma)
0.36 mM rotenone in DMSO, store at −20 °C (Sigma)

2.3. Equipment
Cary 300 Bio Spectrophotometer (Varian Inc., Middelburg, The Netherlands)
Cary WinUV software package, version 3.00 (Varian)
Sub-microcell quartz cuvettes, 80 μl, 4 mm × 10 mm (Hellma GmbH, Rijswijk, The Netherlands)

2.4. Methods
2.4.1. Isolation of blood lymphocytes
Lymphocytes are isolated from blood anticoagulated with 0.18% EDTA. Dilute the EDTA-blood by addition of an equal volume of PBS-EDTA at room temperature (RT). Layer two volumes of diluted blood carefully over one volume of Lymphoprep™ in a centrifuge tube and centrifuge at 800g for 20 min at RT in a swing-out rotor. The mononuclear cells form a distinct band at the sample/medium interface. First, remove the upper layer, and then collect the cells from the interface. Dilute the cell fraction with ice-cold PBS-EDTA to reduce the density of the suspension and pellet the cells by centrifugation for 15 min at 140g and 4 °C. Next, lyse the erythrocytes with ice-cold NH₄Cl. Pellet the remaining mononuclear leukocytes by centrifugation for 15 min at 140g and 4 °C, wash once with ice-cold PBS-EDTA and resuspend the cells in ice-cold SHE–PIM. Count the number of lymphocytes and rapidly freeze 50-μl aliquots at a concentration of 100 × 10⁶ cells per ml SHE–PIM in liquid nitrogen and store at −80 °C.

Remarks: the addition of diisofluorophosphate to the buffer is important to effectively inhibit the active serine proteases that may come from any contaminating blood neutrophils (Maianski et al., 2004). By reducing the centrifugal force from the usual 250g to 140g by use of this isolation method, the lymphocyte to platelet ratio increases from 9:15 to 9:1.
2.4.2. Preparation of a mitochondria-enriched fraction from fibroblasts

Human skin fibroblasts are cultured in DMEM supplemented with 10% (vol/vol) fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.2 mM uridine (fibroblasts suffering from a respiratory defect depend on uridine for their growth). Harvest approximately 7 x 10^6 cells, equivalent to about 2 mg protein, from a 175-cm^2 culture flask by trypsinization, wash with PBS at RT, resuspend in ice-cold SHE, rapidly freeze in liquid nitrogen, and keep at −80 °C until use.

The mitochondrial fraction is isolated according to Tiranti et al. (1995) with some small modifications. Thaw the cell suspension and dilute 1:1 with ice-cold 0.2 mg/ml digitonin in SHE. Incubate on ice for 10 min and centrifuge at 750g for 10 min at 4 °C. Save the supernatant and resuspend the pellet in the same volume of ice-cold SHE supplemented with 0.1 mg/ml digitonin. Incubate on ice for another 10 min, and centrifuge again at 750g for 10 min at 4 °C. Combine the supernatants and centrifuge at 8000g for 10 min at 4 °C. Resuspend the pellet in ice-cold 10 mM KPi buffer, pH 7.4, determine the protein concentration, and freeze in liquid nitrogen. If the mitochondrial fraction is not used immediately, store it at −80 °C.

Protein concentration can be determined by the Bio-Rad Protein assay, which only requires 1 to 20 µg protein.

2.4.3. Spectrophotometric assay

The complex I activity is determined by measuring the oxidation of NADH to NAD^+ at 340 nm with 380 nm as the reference wavelength at 37° in an assay mixture of 20 mM K_2HPO_4, pH 7.4, 4 mM MgCl_2, 0.2% BSA, 200 µM NADH, 1.7 mM KCN, 3 µM antimycin, and 100 µM CoQ_1. The difference in activity with and without rotenone at a fully saturating level of 5 µM (Majander et al., 1996; Nakashima et al., 2002) is calculated to differentiate complex I activity from that of the rotenone-insensitive NADH: ubiquinone oxidoreductase (RINQ).

Thaw the frozen lymphocytes; prepare two cell concentrations by dilution with ice-cold SHE-PIM buffer. After two additional cycles of freeze thawing, the permeabilized cells can be used directly in the complex I activity assay.

Dilute the mitochondria-enriched fraction from fibroblasts to 0.2 to 0.6 mg/ml protein with ice-cold 10 mM KPi-buffer, pH 7.4, and freeze–thaw by two additional cycles directly before determination of complex I. Use at least two protein concentrations to determine complex I enzyme activity.

The assay mixture contains 25 mM K_2HPO_4, pH 7.4, 5 mM MgCl_2, 0.25% BSA, 3.7 µM antimycin, and 2 mM KCN.

1. Transfer 120 µl of the assay mixture to a test cuvette and a blanc cuvette.
2. Add 2 µl DMSO plus 5 µl of 5.7 mM NADH to the test cuvette and 2 µl of 0.36 mM rotenone plus 5 µl of 5.7 mM NADH to the blanc cuvette.
3. Add 10 μl of the lymphocytes suspension or of the mitochondria-enriched fibroblasts fraction to each cuvette. Mix with a plastic rod and incubate for 1 min at 37 °C.

4. Start the reaction by adding 5 μl of 2.8 mM CoQ1. Measure the decrease in absorbance for 1.5 min at 340 nm with 380 nm as the reference wavelength.

5. By use of the velocity of the reaction (Δ absorbance/min) and the molar extinction coefficient of NADH (4.8 mM⁻¹ cm⁻¹; at 340 nm with reference wavelength 380 nm (Sherwood and Hirst, 2006), calculate the complex I activity by subtraction of the reaction rates in the presence (RINQ activity only) and absence of rotenone (RINQ and complex I activity).

Important: Clean the cuvettes thoroughly with ethanol after each measurement, because rotenone binds to the quartz surface.

3. Characteristics of the Complex I Assay

The duration of the first-order kinetics decreases in a few minutes even at low protein concentrations for both the blood lymphocytes (Fig. 9.1) and the mitochondrial fraction of the fibroblasts (Fig. 9.2).

![Figure 9.1](image-url) Course of the complex I activity reflected by the decrease in absorbance at 340 nm with 380 nm as the reference wavelength caused by the oxidation of NADH by 0.118 (- ○ -) and 0.234 (- ● -) mg cell protein of blood lymphocytes/ml reaction mixture. The reaction mixture consisted of 20 mM KH₂PO₄, pH 7.4, 4 mM MgCl₂, 0.2% fatty acid free BSA, 1.7 mM KCN, 3 μM antimycin, 200 μM NADH, and 100 μM CoQ₁, with or without 5 μM rotenone. Complex I activity was calculated by subtraction of the reaction rates in the presence (RINQ activity only) and absence of rotenone (RINQ and complex I activity). The duration of the first-order kinetics decreased in a few minutes.
Therefore, it is impossible to determine complex I by adding rotenone to the same cuvette several minutes after the start of the reaction to save material. However, by use of sub-microcell cuvettes only minute amounts of material are required, for example, for the determination of the complex I activity in two cell protein concentrations of lymphocytes only 1 to 2 ml of anticoagulated blood is needed. The relationship between protein concentration and complex I activity of the lymphocytes is linear up to at least 0.234 mg/ml reaction mixture (y = −0.439 + 13.7x; R² = 0.797, P < 0.0001; where y represents the initial rate expressed as nmol NADH min⁻¹ ml⁻¹, and x, the cell protein concentration in mg/ml reaction mixture).

The amount of fibroblasts needed to determine the complex I activity in two mitochondrial protein concentrations by this assay is 250,000 to 750,000 cells. The activity is linearly related to the mitochondrial protein concentration up to at least 0.060 mg/ml reaction mixture (y = 0.554 + 76.9x; R² = 0.978, P < 0.0001; where y represents the initial rate expressed as nmol NADH min⁻¹ ml⁻¹, and x the protein concentration of the mitochondrial fraction in mg/ml reaction mixture).

With both cell types, the intraassay imprecision (CV) has been determined at three protein concentrations in triplicate on the same day and varied between 8 and 15% (mean: 11.3%). The interassay imprecision was between 5 and 27% (mean: 15.9%) and was determined at three protein concentrations on three different days (Table 9.1). The normal donor

![Figure 9.2](image-url)  
**Figure 9.2** Course of the complex I activity reflected by the decrease in absorbance at 340 nm with 380 nm as the reference wavelength caused by the oxidation of NADH by 0.018 (- ○ -) and 0.035 mg (- ● -) fibroblast mitochondrial protein/ml reaction mixture. The reaction mixture and the calculation of complex I activity was as described in the legend of Fig. 9.1. Again, the duration of the first-order kinetics decreased in a few minutes.
Table 9.1  Assay imprecisions for complex I in PBMC’s and mitochondrial fractions of cultured fibroblasts

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(continued)
The mitochondrial fractions were isolated on three different days from one batch of cultured skin fibroblasts, and complex I activity was measured on the day of isolation.
variation in the determination of complex I of blood lymphocytes amounted to 41.1% (De Wit et al., 2007), which was there mistakenly held for the interassay imprecision. To determine the effect of the isolation procedure of the mitochondrial fraction of fibroblasts on the complex I assay, mitochondrial fractions were isolated on three different days from the same batch of cultured fibroblasts. Complex I activities were measured in three cell concentrations on the day of isolation. The imprecision of 19% in the measured complex activities lay in the range of the interassay imprecision (Table 9.1).

REFERENCES


