Functional characterization of mitochondrial respiratory complex I by delayed luminescence spectroscopy

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Abstract - Delayed luminescence (DL) spectroscopy proved successful in several clinical applications, but its cellular origin is still unclear. Our results obtained with human leukemia Jurkat T cells challenged with Complex I targeting agents like rotenone, menadione and quercetin support the notion that DL is produced primarily within the mitochondrial electron transfer system at the level of Complex I. The data provide novel insights into the structural and functional organization of respiratory Complex I, which appears to function predominantly as a dimer and less frequently as a tetramer in this cell type. States emitting blue or green/yellow light are most likely excited states of Complex I-associated NADH or FMN, respectively, which are produced after a series of forward and backward electron transfer steps involving inner Fe/S clusters of Complex I. Furthermore, the timescales of various electron transfer steps involving the formation of flavin and ubisemiquinone radicals with subsequent production of superoxide can be estimated from delayed red-light emission.

INTRODUCTION

Complex I (NADH:ubiquinone oxidoreductase), the largest enzyme of the mitochondrial respiratory chain, catalyzes the transfer of two electrons from NADH, the reduced form of nicotinamide adenine dinucleotide (NAD^+), to ubiquinone (Q), in a process coupled to the translocation of four protons to the intermembrane space. The first stage in the overall enzymatic reaction is a one-step transfer of two electrons from NADH to the prosthetic group of the complex, flavine mononucleotide (FMN), situated in the remotest hydrophilic subunit, denoted as FMN-b. Subsequently, reduced FMN-b transfers individually the two electrons to a wire of eight consecutive iron-sulfur (Fe/S) clusters which span the extramembranous domain of the complex. The reduction of Complex I-associated ubiquinone by the first electron from the terminal Fe/S cluster (N2) produces the ubisemiquinone radical ($Q^\bullet$) which can then receive the second incoming electron to become fully reduced ubiquinone (ubiquinol, QH_2). It is currently considered that Q reduction by N2 occurs in a large cleft formed by the two subunits that contact the first subunit (ND1) of the membranous domain of Complex I. This wide hydrophobic cavity formed at the junction between the two arms of Complex I is also the place where hydrophobic inhibitors of Complex I like rotenone (ROT) or artificial electron acceptors like menadione (vitamin K_3; MD) also appear to bind.

Here we performed a detailed analysis of the properties of ultra-weak photon-induced delayed photon emission (Delayed Luminescence, DL) of Jurkat cells by employing treatments with rotenone, as well as with menadione and quercetin (QC). All the three drugs share net structural similarities with ubiquinone and therefore can bind Complex I, inside or close to the Q-binding pocket. In view of the common effects of these drugs at the level of Complex I, we could investigate in more detail the connection between delayed luminescence and the mitochondrial metabolism. At present, despite the fact that DL spectroscopy proved successful in some clinical applications, the origin of delayed luminescence of living cells is still under debate [1-6]. DL, also called "delayed fluorescence", represents a very weak light emission elicited upon light- or UV-irradiation, which differs from ordinary ("prompt") fluorescence by an unusual long decay time. Our current results demonstrate that the ultra-weak photon-induced delayed photon emission of Jurkat cells is quantitatively related to the mitochondrial level of NADH and that of oxidized FMN, supporting the notion that DL is mainly produced within the mitochondrial electron transfer system at the level of Complex I.

MATERIALS AND METHODS

Human leukemia Jurkat lymphoblasts were cultured in MegaCell RPMI 1640 medium as described [1,2]. Cell density and viability were examined by microscopy. For *Delayed Luminescence spectroscopy*, an improved version of the ARETUSA set-up was used [6]. Cell samples were excited by a Nitrogen Laser source (Laser

Photonics LN 230C; wavelength 337 nm, pulse-width 5 ns, energy 100±5 μJ/pulse). A multi-alkali photomultiplier tube (Hamamatsu R-7602-1/Q) was used as a detector. The detected signals were acquired by a Multi-channel Scaler (Ortec MCS PCI) with a minimum dwell-time of 200 ns. DL measurements were done on at least 3 different drops from each cell sample (drop volume 15-25 μl) at room temperature (20±1ºC). For s*pectrofluorimetry*, cells were washed and resuspended in a standard saline solution. Fluorescence was recorded at 37ºC in a Horiba Jobin Yvon spectrofluorimeter [1,2]. The cell sample was excited sequentially at 360 and 400 nm and emission was collected at 600 nm and 435 nm. The level of NADH and FMN was estimated as the fluorescence intensity at Ex. 360/Em. 435 and Ex. 450/Em. 510, respectively.

RESULTS AND DISCUSSION

DL spectroscopy and NADH/FMN_{ox} fluorimetric data were collected after treatments with ROT, QC, MD and H_2O_2, or QC in combination with MD or H_2O_2. Consistent with our previous reports [1,2], QC and MD inhibited DL significantly, in a dose-dependent manner. H_2O_2 elicited as well an inhibitory effect on DL, but to a much lesser extent. Combination of QC and MD at high dose also decreased consistently the VIS-DL yield, whereas combination of quercetin with a high dose of H_2O_2 exerted a more reduced effect. The DL yield in the visible domain depended in a Hill-like fashion on the level of NADH as well as that of oxidized flavine mononucleotide (FMN_{ox}), with apparent Hill coefficients of 4.3 (Fig. 1). In addition, spectral DL analysis indicated different degrees of cooperativity of both NADH and FMN_{ox}, reflected in differing Hill coefficients associated with blue, green/yellow and red light emission (3.9, 7.2 and 2.8, respectively). In addition, measurements of NADH and FMN_{ox} levels indicated that ROT binding exhibits an apparent Hill coefficient of 2.0-2.4, suggesting that Complex I functions mainly as a dimer in intact Jurkat cells. Based on these findings, we propose that blue and red DL is produced predominantly by dimeric Complex I, whereas green/yellow DL arises mainly from tetrameric Complex I. Thus, the DL data indicate that green/yellow light emission (presumably associated with tetrameric Complex I) contributes 39%, whereas blue- and red-light emission (associated with dimeric Complex I) contributes 61% to total DL. So, our results suggest that about 60% and 40% of Complex I oligomers in Jurkat cells are in dimeric and tetrameric form, respectively. In conclusion, based on the main findings that: 1) DL is closely related to the level of oxidized FMN, which is found primarily in the mitochondria, 2) DL is also linked to the level of NADH, the substrate of mitochondrial Complex I, 3) ROT, a specific inhibitor of Complex I, affected DL considerably, and 4) MD and QC, which interact robustly with Complex I, also affected DL significantly, all our results reinforce the idea [1] that mitochondrial Complex I plays a major role in delayed luminescence.

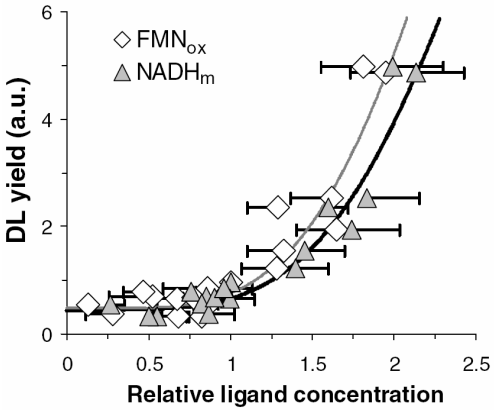

Figure 1: The dependence of the VIS total DL yield on the relative concentration of NADH or FMN_{ox}, obtained after treatments of Jurkat cells with ROT, MD, QC or H_2O_2. Data were fitted to a Hill-type equation.

To summarize, our studies provide evidence that the ultra-weak photon-induced delayed photoemission of intact Jurkat cells originates mainly from mitochondrial Complex I, which appears to function predominantly as a dimer and less frequently as a tetramer in this cell type. Complex I oligomers appear to exhibit cooperative interaction between monomers at the level of the rotenone site 1, NADH/NADPH sites and both FMN sites. Moreover, in individual monomers, both pairs of pyridine nucleotide (NADH/NADPH) binding sites and flavin (FMN-b/FMN-a) binding sites, which are situated at the two extremities of the extramembranous domain of Complex I, display strong cooperativity in the binding of their specific ligands. In addition, the timescales of various electron transfer steps involving the formation of flavin and ubisemiquinone radicals with subsequent production of superoxide can be estimated from delayed red-light emission. All these findings raise the attractive possibility that DL spectroscopy could be used as a reliable, sensitive and robust technique to probe electron flow within Complex I and gain valuable insights into the structural and functional organization of this respiratory complex in situ.

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