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Conformation-specific crosslinking of mitochondrial complex I

Margherita Ciano [a,d], Matthew Fuszard [b], Heinrich Heide [c], Catherine H. Botting [b], Alexander Galkin [a,*]

[a] Queen's University Belfast, School of Biological Sciences, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL, UK
[b] School of Chemistry, Biomedical Sciences Research Complex, BMS Annexe, University of St. Andrews, St. Andrews KY16 9ST, UK
[c] Molecular Bioenergetics Group, Faculty of Medicine, Goethe University Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany
[d] Department of Experimental Evolutionary Biology, University of Bologna, via Selmi 3, 40126 Bologna, Italy

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ABSTRACT

Complex I is the only component of the eukaryotic respiratory chain of which no high-resolution structure is yet available. A notable feature of mitochondrial complex I is the so-called active/de-active conformational transition of the idle enzyme from the active (A) to the de-active, (D) form.

Using an amine- and sulfhydryl-reactive crosslinker of 6.8 Å length (SPDP) we found that in the D-form of complex I the ND3 subunit crosslinked to the 39 kDa (NDUFA9) subunit. These proteins could not be crosslinked in the A-form. Most likely, both subunits are closely located in the critical junction region connecting the peripheral hydrophilic domain to the membrane arm of the enzyme where the entrance path for substrate ubiquinone is and where energy transduction takes place.

Structured summary of protein interactions:
ND3 and **NDUFA9** physically interact by cross-linking study (View interaction)

1. Introduction

Mitochondrial complex I (EC 1.6.5.3, proton-translocating NADH:ubiquinone oxidoreductase) is responsible for oxidation of matrix NADH by membrane-bound ubiquinone and most likely, is also a major source of reactive oxygen species (ROS) generated by the mitochondrial respiratory chain [1–3]. This enzyme is the largest component of the mitochondrial and bacterial respiratory chains [4]. Structural information about mitochondrial complex I is still very limited, although recently the atomic structure of the 14 subunits of the prokaryotic homologue enzyme was determined at 4.5 Å resolution [5]. Only a low resolution structure is available for the eukaryotic enzyme [6]. Unlike the 14 subunit bacterial enzyme, the mammalian complex I is composed of at least 44 different subunits and the function of more than 30 of the so-called accessory subunits is not completely understood [7,8].

A notable feature of mitochondrial complex I is the so-called active/de-active conformational transition of the idle enzyme from the fully competent, "active" (A) to the dormant, "de-activated" D-form. These catalytically and structurally distinct forms of the enzyme have been identified in purified preparations in vitro, in cells, as well as in rat heart ex vivo during lack of oxygen [9–11] (see [12] for detailed review). Only the A-form catalyses rotenone-sensitive oxidation of NADH with a fast rate of 10^4 min^{-1}. Incubation of idle enzyme at 30–37 °C results in its transition to the D-form. In presence of NADH and ubiquinone the D-form of complex I can be converted back to the A-form during slow (4 min^{-1}) catalytic turnover(s). It can be seen as a lag phase during continuous assay of the NADH-oxidase reaction catalysed by the D-form. This lag phase represents a slow activation of the D-form of the enzyme during the time of the measurement [10]. Several hypotheses for the function of the D-form of complex I accumulated in tissues in conditions of lack of oxygen were suggested: prevention of proton leakage through the idle enzyme [13], fine tuning of mitochondrial activity [14,15], interaction with nitric oxide metabolites [16], alleviating respiratory burst during reperfusion [17] and potential Na^+/H^+ antiporter activity [18].

The molecular mechanism of A/D transition is not known at present. Sensitivity of NADH-oxidation by submitochondrial particles (SMP) to SH-reagents after incubation at 37 °C was observed many years ago [19]. Recently the thiol group exposed to the

Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; HAR, hexaammineruthenium(III)-chloride; NEM, N-ethylmaleimide; nLC-ESI-MSMS, nano-HPLC electrospray ionization multistage tandem mass spectrometry; ROS, reactive oxygen species; SMP, submitochondrial particles; SPDP, N-succinimidyl 3-(2-pyridyldithio) propionate

* Corresponding author. Fax: +44 (0)28 9097 5877.
E-mail address: a.galkin@qub.ac.uk (A. Galkin).

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outside and rendering sensitivity to SH-reagent of the D-form was identified as cysteine-39 of mitochondrial ND3 subunit [20]. This is the only known structural difference between two forms of the enzyme. However, the high activation energy of the A–D transition (270 kJ/mol [9]) indicates that major conformational changes take place during the de-activation process and, therefore, other subunits are most likely involved. It has been suggested that more than one protein is involved in complex I A/D transitions in *Neurospora crassa* complex I and 29.9 kDa protein (B13 in bovine enzyme) may modulate the process of de-activation [21].

Here we explored subunit proximity around the cysteine-39 residue of ND3 subunit in order to gain further information on conformational differences between two forms. Treatment of the D-form of complex I by the amine- and sulfhydryl-reactive heterobifunctional crosslinker N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, length 6.8 Å) resulted in covalent binding of ND3 to the 39 kDa (NDUFA9) subunit indicating that these subunits are very closely located in the enzyme molecule. These subunits could not be crosslinked in the A-form. The implications of this finding with regard to the changes in the complex I molecule upon deactivation are discussed.

2. Materials and methods

2.1. SMP and activity measurements

Bovine heart SMP were prepared according to the standard procedure [9] and stored in liquid nitrogen. In order to prepare SMP in which complex I is present almost entirely in the D-form, an aliquot of frozen membranes was thawed, diluted to 5 mg/ml with standard medium (comprising 0.25 M sucrose, 50 mM Tris–HCl (pH 7.5), 0.2 mM EDTA) and incubated at 35 °C for 1 h and 30 min. This treatment resulted in almost complete deactivation of complex I. For complex I activation, SMP (5 mg/ml) were incubated aerobically for 30 min at room temperature with 1% ethanol and 400 μM NADH and 0.1 mg/ml alcohol dehydrogenase from *Saccharomyces cerevisiae* (Sigma). During incubation with alcohol dehydrogenase, the sub-stoichiometric amounts of NADH produced by the regeneration system were oxidized by complex I; resulting in full activation of the enzyme. The diagnostic test for determination of the A/D ratio at the end of the procedure, based on the fact that in the presence of divalent cations and at alkaline pH the rate of re-activation of complex I is very slow was performed as previously described [11].

Oxidation of NADH was determined spectrophotometrically (Varian Cary 4000) as a decrease in absorption at 340 nm (ε_{340} - = 6.22 mM^{-1} cm^{-1}) with 165 μM NADH in 1 ml of standard assay medium (0.25 M sucrose, 50 mM Tris–HCl (pH 7.5), 0.2 mM EDTA) containing 10–50 μg protein/ml. Additional measurements of NADH:Q_1 or NADH:HAR reductase were assayed in the presence of 1 mM cyanide with the addition of 45 μM Q_1 or 1 mM HAR, respectively (Sigma). In all inhibition studies the D-form was treated with an effector and activity was assessed only after activation by 10 μM NADH.

2.2. SPDP crosslinker treatment

SMP containing either the A or the D complex I were diluted to 50 μg/ml with PBS (pH 8.0), 8 μM SPDP dissolved in DMF was added and samples were incubated on ice under constant stirring. A sample of the A-form of the enzyme treated with an equal volume of DMF was used as a control. Residual NADH-oxidase activity was checked, every 10 min for both samples and once it reached its final level the reaction was stopped by adding 50 mM Tris–HCl (pH 7.0).

2.3. Protein electrophoresis

Native mitochondrial respiratory complexes were separated using Blue native gel electrophoresis in accordance to published protocols [22]. SMP samples were solubilized by 3 mg of DDM per mg protein as described previously [20]. Due to the formation of large intercomplex oligomers, careful titration of SMP activity by SPDP was performed before upscaling of labelling experiments in order to isolate complex I by BN PAGE. The band containing complex I was cut out, treated with 1% SDS and placed on top of a 10% acrylamide gel containing 6 M urea and subjected to Tricine/SDS–PAGE for the first dimension. For the second dimension lane strips of 10% gels were cut out treated with DTT (20 mM, 20 min, 40 °C) and mounted on the top of a 16% acrylamide gel. Tricine–SDS–PAGE in the absence or in the presence of 20 mM DTT was performed as described before [20,23]. Gels were stained with silver using a mass spectrometry compatible protocol [23] or by Sypro Ruby fluorescent dye (Sigma).

2.4. Mass spectrometric analysis

The gel band was excised and cut into 1 mm cubes. These were then subjected to in-gel digestion, using a ProGest Investigator in-gel digestion robot (Genomic Solutions, Ann Arbor, MI) using standard protocols [24]. Briefly the gel cubes were destained by washing with a 1:1 mixture of 30 mM potassium ferricyanide and 100 mM sodium thiosulphate for the silver stained bands [25] and washing with acetonitrile for the Sypro Ruby stained bands and subjected to DTT reduction and alkylation by iodoacetamide before digestion with trypsin at 37 °C. The peptides were extracted with 10% formic acid and concentrated down to 20 μl using a SpeedVac (ThermoSavant).

The peptides were then separated using a nanoLC Ultra 2D plus loading pump and nanoLC AS2 autosampler equipped with a nanoflex cHiPLC chip-based chromatography system (Eksigent), using a 200 μm × 0.5 mm ChromXP C18-CL 3 μm 120 Å trap and 75 μm × 15 cm ChromXP C18-CL 3 μm 120 Å column (Eksigent). The peptides were eluted at 300 nl/min with a gradient of increasing acetonitrile, containing 0.1% formic acid (15–40% acetonitrile in 5 min, 40–95% in a further 1 min, followed by 95% acetonitrile for 3 min to clean the column, before reequilibration to 5% acetonitrile for 10 min). The eluent was sprayed into a TripleTOF 5600 electrospray tandem mass spectrometer (ABSciex, Foster City, CA) fitted with a NANOSpray® III ion source (ABSciex), running in positive ion mode, and analysed in Information Dependent Acquisition (IDA) mode, performing 250 ms of MS followed by 20 × 150 ms MS/MS analyses on the 20 most intense MS peaks with charge states +2 to +5 which exceed 150 cps. Target ions were subsequently excluded from analysis for 13 s. The MS ions tolerance was set at 50 mDa. LINAC® Collision Induced Dissociation (CID) was performed with a collision energy of 45 and spread of 15 at high sensitivity and unit resolution. The MS/MS data file generated was analysed using the ProteinPilot Beta 4.1.46 Paragon algorithm (ABSciex) against the UniProt/SwissProt database (accessed Nov 2011) in 'Thorough' mode with the 'Biological Modifications' ID focus specified. The search was performed with no species restriction, trypsin as the cleavage enzyme and 'Gel-Based ID' as a special factor. Specific, bespoke, modification parameterizations were created to accommodate both a SPDP [$C_5H_8O_2NS$] artifact (a covalently bound residual of crosslinking and DTT cleavage) within ProteinPilot's ParameterTranslation file with prior probabilities of 0.01.

All proteins were identified with >99% confidence (Prot Score > 2.0) with False Discovery Rates of: Local FDR < 5%, Global FDR < 1%.

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