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Conformational Change of Mitochondrial Complex I Increases ROS Sensitivity During Ischemia

Natalia Gorenkova,1 Emma Robinson,2 David J. Grieve,2 and Alexander Galkin1

Abstract

Aims: Myocardial ischemia/reperfusion (I/R) is associated with mitochondrial dysfunction and subsequent cardiomyocyte death. The generation of excessive quantities of reactive oxygen species (ROS) and resultant damage to mitochondrial enzymes is considered an important mechanism underlying reperfusion injury. Mitochondrial complex I can exist in two interconvertible states: active (A) and deactive or dormant (D). We have studied the active/deactive (A/D) equilibrium in several tissues under ischemic conditions in vivo and investigated the sensitivity of both forms of the heart enzyme to ROS. Results: We found that in the heart, t½ of complex I deactivation during ischemia was 10 min, and that reperfusion resulted in the return of A/D equilibrium to its initial level. The rate of superoxide generation by complex I was higher in ischemic samples where content of the D-form was higher. Only the D-form was susceptible to inhibition by H2O2 or superoxide, whereas turnover-dependent activation of the enzyme resulted in formation of the A-form, which was much less sensitive to ROS. The mitochondrial-encoded subunit ND3, most likely responsible for the sensitivity of the D-form to ROS, was identified by redox difference gel electrophoresis. Innovation: A combined in vivo and biochemical approach suggests that sensitivity of the mitochondrial system to ROS during myocardial I/R can be significantly affected by the conformational state of complex I, which may therefore represent a new therapeutic target in this setting. Conclusion: The presented data suggest that transition of complex I into the D-form in the absence of oxygen may represent a key event in promoting cardiac injury during I/R.

Introduction

The effects of a reduction in the oxygen level involves a rapid response from the mitochondrial system as this is the major consumer of oxygen in a cell. Hypoxia can be followed by recovery of the oxygen supply (reoxygenation), which augments tissue damage. Ischemia/reperfusion (I/R) injury and its therapeutic reduction have become increasingly important issues in clinical medicine. It is well established that cardiac ischemia leads to a decline in the activity of several mitochondrial components, which is intensified by reperfusion. This results in myocardial dysfunction, most likely due to the production of excessive quantities of reactive oxygen species (ROS), which is considered as one of the major mechanisms underlying I/R injury (3, 5, 28, 38, 51).

Mitochondrial complex I (EC 1.6.5.3) oxidizes NADH, contributing to the formation of membrane potential and consequently ATP synthesis, and therefore occupies a key position in cellular metabolism. Complex I is also an important source of superoxide and, most likely, it is responsible for the majority of ROS produced by the respiratory chain in vivo (10, 33). Mitochondrial complex I responds rapidly to lack of

1Medical Biology Centre, School of Biological Sciences, Queen’s University Belfast, Belfast, United Kingdom.
2Centre for Vision & Vascular Science, Institute of Clinical Science A, Royal Victoria Hospital, Belfast, United Kingdom.
oxygen and is damaged by subsequent reoxygenation (5, 24, 34, 37, 45). The altered activity of complex I can have a significant effect on mitochondrial ROS generation. Moreover, this enzyme is not only a major source of ROS, but is also susceptible to damage during I/R, including that caused by oxidative and nitrosative stress (6, 12, 51).

Reversible conversion of the active, A-form of complex I into the dormant, D-form has been described in vitro (31), in rat heart ex vivo (39), and recently, in studies of cultured cells (14). If idle at physiological temperatures, the enzyme undergoes conversion into the D-form, which is characterized by a 10,000-fold lower catalytic activity compared to the catalytically competent A-form (53). In contrast to irreversibly inactive enzyme, the D-form is potentially capable of catalyzing a fast reaction and can be converted to the A-form after slow catalytic turnover(s) when substrates become available. Despite recent progress made in the resolution of the bacterial enzyme (9), very little is known about the eukaryotic complex I, so it is not yet possible to suggest the nature of the gross structural changes in the enzyme during activation/deactivation.

Deactivation of the enzyme in the absence of oxygen (14, 39) is an intrinsic property of complex I and it would be expected to play a functional role. However, in the time frame of ischemic conditioning, prolonged accumulation of the D-form may have severe pathophysiological consequences, depending on the duration of exposure, type of tissue, and the presence of natural effectors of the active/deactive (A/D) transition and of the ROS/antioxidant balance.

We have previously investigated the effects of lack of oxygen on the conformational state of mitochondria complex I and its sensitivity to nitric oxide-metabolites in isolated mitochondrial membranes and cultured cells (14, 17). Taking into account the role of complex I in generating ROS in I/R (13, 22, 42) and subsequent damage to oxidative phosphorylation, we sought to characterize changes in complex I states during I/R in an established experimental model. Here we present data showing that reversible deactivation of mitochondrial complex I takes place in situ under ischemic conditions and report the differential sensitivity of the two forms of the enzyme to superoxide anion and peroxide. We found that 50% deactivation of complex I occurs within 10 min of cardiac arrest, while reperfusion resulted in the return of complex I A/D equilibrium to its initial level. The D-form of complex I isolated from the ischemic samples was found to be sensitive to ROS treatment, and that sensitivity was eliminated by activation. Furthermore, oxidative modification of the D-form of the enzyme in vitro resulted in a decrease in the rate of NADH oxidation indicating functional damage of the enzyme. Subunits responsible for functional modification of the D-form of the enzyme have also been identified.

Results

A/D transition in vivo in different tissues

First, the effect of induced cardiac arrest on the A/D ratio of complex I was investigated in different tissues 20 min after cardiac arrest (Fig. 1). The greatest degree of deactivation was observed in highly metabolizing tissues such as the heart and brain.

FIG. 1. Percentage (%) of the D-form of mitochondrial complex I in different tissues determined as described in Materials and Methods. Tissues were extracted immediately after cardiac arrest (black bars) or 20 min later (white bars). Each column represents mean ± SEM, n = 3 animals per group, two experiments, triplicate measurements. *p < 0.05; **p < 0.01, compared to corresponding control samples.

Cardiac I/R and activation state of complex I

Next, reversibility of complex I deactivation was assessed in situ. The effect of local myocardial ischemia with or without reperfusion, on complex I-catalyzed activities and on the A/D ratio is shown in Figure 2. There was almost twice as much
complex I in the D-form in samples from ischemic tissues as there was in control tissues. In samples taken after reperfusion of the ischemic area, the D-form content was similar to that in the control samples. As judged by the NADH-oxidase and hexaammineruthenium (III) chloride (HAR)-reductase activity, the complex I activity and content in ischemic and reperfused samples was not different from that in the control samples. There was no significant difference between NADH- and succinate-supported generation of superoxide in any of the samples (data not shown).

Time course of complex I deactivation in heart

To determine the time course of complex I deactivation after cardiac arrest in situ, heart mitochondrial membranes were isolated at different periods after cardiac arrest, taking care to preserve the A/D ratio. The time course of myocardial complex I deactivation after cardiac arrest is shown in Figure 3. The t1/2 of deactivation was around 10 min. However, it should be noted that neither the total NADH-oxidase nor cytochrome c oxidase activity was significantly altered by ischemia.

Myocardial superoxide production by the mitochondrial respiratory chain after cardiac arrest

The percentage of the D-form of complex I in mitochondrial membranes obtained from mouse hearts from the control group and 20 min after cardiac arrest was 10% and 65%, respectively (Table 1). The rate of NADH-supported superoxide generation was significantly higher in mitochondrial samples obtained 20 min after cardiac arrest than in control samples. Turnover-dependent activation eliminated that difference between these samples (data not shown). Rate of succinate-supported superoxide generation was found to be similar in control and ischemic samples (Table 1).

Sensitivity of the A- and the D-forms of complex I to H2O2 and O2−

Mitochondrial membranes isolated from heart at 0 and 20 min after cardiac arrest (containing 8% and 67% of complex I in the D-form, respectively), were subjected to incubation for 30 min in the presence of the xanthine/xanthine oxidase superoxide generating system. After addition of 50 μM xanthine, the system is able to generate superoxide at an initial rate of 50 nmol/min/mg protein, as assessed by superoxide dismutase (SOD)-sensitive reduction of acetylated cytochrome c.

As shown in Table 2, incubation of mitochondria with this O2−-generating system resulted in a significant decrease in the NADH-oxidase activity in heart tissue samples obtained 20 min after cardiac arrest. This effect was abolished when all the D-form is converted into the A-form. Addition of 50 U/ml catalase to the incubation medium did not alter the observed effect, while the presence of 50 U/ml SOD completely prevented the inhibitory actions of the O2−-generating system in all samples. There was no significant effect on the succinate-oxidase activity of mitochondrial membranes from either sample.

Figure 4A demonstrates a dramatic difference in the sensitivity of the A- and the D-form of complex I to hydrogen peroxide. Incubation in the presence of 1.0 mM H2O2 led to the inhibition of the NADH-oxidase reaction of the D-form, but not the A-form. The time course of inhibition of the NADH-Q1 reductase activity was similar to that of

![Graphs showing time course of complex I deactivation in heart tissue](image-url)

**Table 1. Generation of Superoxide by Myocardial Mitochondrial Membrane Preparations from a Control Group and 20 Min After Cardiac Arrest**

<table>
<thead>
<tr>
<th>Sample</th>
<th>D-form content (%)</th>
<th>NADH dependent (nmol × min−1 × mg−1)</th>
<th>Succinate dependent (nmol × min−1 × mg−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10±3</td>
<td>0.55±0.08</td>
<td>1.35±0.35</td>
</tr>
<tr>
<td>20 min</td>
<td>65±5b</td>
<td>1.02±0.21b</td>
<td>1.21±0.29b</td>
</tr>
</tbody>
</table>

Values represent means of quadruplicate measurements in two experiments.

*Activity was measured in the presence of 1 μM antimycin A.

b n=3 animals per group, two experiments, quadruplicate measurements p<0.05 versus control.
Succinate oxidase Control — 0.45
NADH-oxidase Control — 0.43

Table 2. Sensitivity to Superoxide of Myocardial Complex I Obtained from Control Group and 20 min After Cardiac Arrest

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial percentage of the D-form</th>
<th>Initial activity</th>
<th>After superoxide treatment</th>
<th>Percentage of the D-form after activation</th>
<th>Superoxide treatment after reactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH-oxidase</td>
<td>Control 10 ± 3</td>
<td>0.54 ± 0.14</td>
<td>0.56 ± 0.15</td>
<td>2.0 ± 1.1</td>
<td>0.51 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>20 min 65 ± 3</td>
<td>0.50 ± 0.18</td>
<td>0.11 ± 0.03b</td>
<td>4.5 ± 1.4</td>
<td>0.45 ± 0.14</td>
</tr>
<tr>
<td>Succinate oxidase</td>
<td>Control —</td>
<td>0.45 ± 0.05</td>
<td>0.43 ± 0.10</td>
<td>—</td>
<td>0.45 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>20 min —</td>
<td>0.51 ± 0.04</td>
<td>0.44 ± 0.09</td>
<td>—</td>
<td>0.38 ± 0.12</td>
</tr>
</tbody>
</table>

*The heart mitochondrial fractions were diluted to 1 mg/ml with a medium containing a 50 mM phosphate buffer pH 7.2, 20 mM KCl, 0.1 mM EDTA, and components of the superoxide-generating system (50 μM hypoxanthine and 5 μg/ml xanthine oxidase from bovine milk). Incubation was carried out at 20°C. Superoxide dismutase 50 U/ml was used to scavenge the superoxide generated. To activate complex I, NADPH was used as described previously (17). All activities are given in μmol substrate·min⁻¹·mg⁻¹.

**Discussion**

I/R injury has been associated with many types of surgery and vascular interventions. This phenomenon is particularly relevant to cardiac operations in which reperfusion of coronary flow is necessary to resuscitate the myocardium after a period of ischemia, and to percutaneous coronary intervention after myocardial infarction. If performed in a controlled fashion and within a short time postischemia, reperfusion may facilitate cardiomyocyte survival, reduced cardiac damage, and improved post-traumatic recovery. However, the resultant increase in ROS generation in I/R poses significant risks and may mediate irreversible tissue damage. It is therefore essential to understand underlying mechanisms to inform novel therapeutic strategies to prevent I/R injury in such clinical situations.

Two catalytically and structurally distinct forms of complex I have been shown to be present in mitochondrial membranes: one the fully competent, active A-form and the other the dormant, deactivated, D-form.

In the present study, we performed analysis of the A/D ratio of mitochondrial complex I from various tissues after the onset of cardiac arrest. In the absence of oxygen, the respiratory chain is over-reduced, resulting in the lack of second complex I substrate ubiquinone, decrease of complex I catalytic turnover, and eventual deactivation of the enzyme. In highly metabolic tissues such as the brain and heart, 20 min of global ischemia...
significantly shifted the A/D equilibrium toward formation of
the D-form. This form exhibits a much lower catalytic activity
than the A-form, but unlike the irreversibly inactivated or
denatured enzyme, the D-form can be converted to the A-form
during that slow catalytic turnover(s) and is potentially able to
catalyze a rapid physiological reaction.

To measure the full complex I activity, enzyme prepara-
tions should be activated before the measurements as carried out in the present study and described previously (17). It is
important to stress that accumulation of the D-form during
the ischemic period observed here is fully reversible, but
could easily be mistaken for enzyme inactivation when the
NADH-oxidase activity is assessed by conventional methods.
Preparations composed of a mixture of the A- and the D-form
catalyze oxidation of NADH with a lag phase during con-
tinuous assay. This lag phase represents slow activation of the
D-fraction of the enzyme during the time of the measurement
(14) and can be easily interpreted for a linear initial rate when
an assay buffer of pH > 7.5 is used or divalent cations are present (53). If the preparation composed mostly of the D-
form (i.e., postischemic mitochondrial samples) is assessed
without activation, the observed initial rate is low and rep-
resents only the contribution of the A-form fraction. In our
experiments, total activated NADH-oxidase did not change
with the time of ischemic treatment.

The time course of complex I deactivation in situ revealed
that the half-time (t1/2) for the heart was 12 min after the onset
of global ischemia. Using an in vivo mouse infarction model,
we showed that local cardiac ischemia results in accumulation
of the D-form, while reactivation occurs in situ after reperfu-
sion. At the same time, the D-form of complex I isolated from
the ischemic area can be reactivated in vitro during turnover-
dependent reactivation when substrates are added (14, 31, 39).
The difference in the D-form content in mitochondria derived
from the samples subjected to 20-min global ischemia (< 60%)
and local infarction (40%) most likely reflects possible oxy-
genation of the border regions of the ischemic zone during
post-treatment surgery as well as some inaccuracy in the ex-
cision of the ischemic area of the heart.

In our I/R experiments, the complex I activity and content,
estimated from the NADH-oxidase and NADH:HAR reduc-
tase activity, was not significantly different in mitochondrial
membranes from the control and ischemic samples. A similar
oxidase/reductase rates ratio in all of the samples indicates
that neither the complex I content nor its turnover were
affected by ischemia or following reperfusion. Since the

FIG. 4. Effect of hydrogen peroxide on the A- and the D-
form of complex I. (A) NADH-oxidase activity of the A-form
(solid symbols) and the D-form (open symbols) of complex I
from heart mitochondrial membranes. (B) Effect of hydrogen
peroxide on the NADH-Q1-reductase activity of the D-form
of complex I (circles) in the presence of 50 U/ml superoxide
dismutase (triangles), 100 U/ml catalase (squares), or 1.5 mM
glutathione (diamonds). Mitochondrial membranes contain-
ing either the A- or the D-form, prepared as described in the
Materials and Methods section (17), were diluted with the
standard buffer pH 8.0 to 2.5 mg/ml and treated with 1 mM
H2O2 at 20°C. Aliquots were taken during incubation and the
activity was assayed. (C) Dose dependence of the inhibition
of NADH-oxidase activity of the D-form by H2O2. Mem-
branes (20 μg/ml) were incubated for 1 h at 30°C in the
presence of various concentration of H2O2 in the standard
buffer (pH = 8.0) supplemented with 6 mM 3-amino-1,2,4-
triazole, 15 μg/ml alamethicin, and 2 mM MgCl2. At the
end of the incubation period, 10 μM NADH was added and
after activation, the reaction was started by addition of
150 μM NADH and 5 μM cytochrome c. Values represent
mean ± SEM for duplicate measurements in three experi-
ments. Q1, 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-
1,4-benzoquinone.
complex I full activity could be restored after turnover-dependent reactivation, the enzyme did not undergo significant covalent modifications affecting its activity. It is therefore likely that our chosen conditions of 20-min ischemia, with or without 10-min reperfusion, were not sufficient to cause the significant damage to complex I observed by others using a longer ischemic treatment (23, 24). Nonetheless, complex I catalyzed activities in mitochondria obtained from control, ischemic, or reperfused samples in our experiments, in agreement with the findings of several other studies (51).

Oxidative stress is considered as an important mechanism of I/R injury. Complex I-dependent production of ROS is increased during reperfusion, although the underlying mechanism remains unclear (2, 38, 43, 54). We have shown that the complex I-dependent superoxide anion generation was significantly higher in mitochondrial membranes isolated from the ischemic heart, most likely, due to the presence of the D-form accumulated during the ischemic period. This is in agreement with the earlier observation of higher capacity for superoxide generation by the D-form (20).

We also determined the sensitivity of the A- and the D-forms of complex I to superoxide anion and hydrogen peroxide. In heart mitochondrial membranes, exogenously added H$_2$O$_2$ inhibits only the D-form of the enzyme, but not the A-form. This has been further tested on control samples and those obtained 20 min after ischemia, using a superoxide generating system able to produce 0.25 nM/min superoxide. As expected, incubation of mitochondrial membranes from the ischemic tissue in the presence of a low steady-state concentration of superoxide resulted in a dramatic inhibition of the NADH-oxidase activity in comparison with control. Activation of complex I completely abolished that inhibition, rendering the A-form of the enzyme less sensitive to superoxide.

It is not clear what level of sensitivity of the enzyme to ROS may be expected in situ, where the enzyme is exposed to additional factors such as high osmolarity and electrical field, and is surrounded by other proteins [including enzymes mediating H$_2$O$_2$ oxidative action (21)]. Prolonged exposure of the D-form of the enzyme to low steady-state levels of endogenous ROS at physiological temperature would inevitably result in irreversible damage to some fraction of the enzyme population leading to accumulation of irreversibly modified enzymes over time. Therefore, taking into account the high degree of flux control of complex I over oxidative phosphorylation (18, 26, 32), a slight decrease in the NADH:ubiquinone reductase activity, even if this is not sufficient to induce an acute effect on apparent respiration, may lead to a significant decrease of the ATP production by mitochondria (52).

Together, our observations suggest that accumulation of the D-form of the enzyme takes place during ischemia (i), this accumulation increases ROS production (ii); the presence of complex I in the D-form may potentially increase susceptibility of mitochondria to oxidative damage (iii), so could result in the so-called vicious cycle of damage during I/R (5, 8, 14, 48).

The link between inhibition of complex I in I/R and the nature of the oxidative modification of the enzyme is not

**Table 3. Proteins Involved in Oxidative Modification of the D-Form of Mitochondrial Complex I**

<table>
<thead>
<tr>
<th>Number</th>
<th>Subunit name</th>
<th>UniProt accession number</th>
<th>Molecular mass</th>
<th>A/D intensity ratio</th>
<th>Number of interhelical Cys/total Cys</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ND4</td>
<td>P03911</td>
<td>51 882</td>
<td>1.942 ± 0.537</td>
<td>1/3$^a$</td>
</tr>
<tr>
<td>2</td>
<td>B12</td>
<td>Q9CQZ6</td>
<td>11 692</td>
<td>2.183 ± 0.697</td>
<td>0/0</td>
</tr>
<tr>
<td>3</td>
<td>ND3</td>
<td>P03899</td>
<td>13 219</td>
<td>6.12 ± 1.961</td>
<td>1/1$^a$</td>
</tr>
</tbody>
</table>

$^a$Number of cysteins located in the interhelical region was determined from the high-resolution structure of prokaryotic complex I (9). A/D, active/deactive.
completely understood, but it is likely to involve oxidation, nitration, nitration, glutathionylation, or disulfide formation of cysteine thiols (41). There have been a number of reports on conformational modifications of complex I in I/R: thiol oxidation of NDUF1, NDUF2, NDUFV1, NDUFV2, NDUFV3, and NDUFV4 (35), and tyrosine nitration of NDUF1, NDUF2, NDUF3, and NDUF4 (35) in the heart and nitration of GRIM-19 in brain mitochondria (6). Our redox difference gel electrophoresis (DIGE) results suggest three subunits that may be involved in the functional modification of the D-form by H2O2: ND3, B12, and ND4. However, the subunit B12 (NDUFB3) has no cysteine residues in its primary sequence and can therefore be excluded. The highest difference in Cy-dye labeling of the H2O2-treated A- and D-form makes ND3 the most likely candidate. Moreover, identification of peptide, thiol labeled by Cy-dye identifies cysteine-39 as the exact region of modification. This cysteine is exposed only in the D-form of the enzyme as shown before (16). Most likely, if Cys-39 of the ND3 subunit complex I is modified, the enzyme does not catalyze the physiological NADH:ubiquinone reaction, making it an early mitochondrial target for oxidative/nitrosative stress during I/R.

Modification of the FeS clusters of complex I can be a major factor for complex I inhibition (44, 50). In our experiments, the possibility of FeS cluster damage cannot be excluded, however, in vitro studies indicate that the accessibility of all clusters to the outside environment is the same in the A- and D-form (29).

The combined processes of accumulation of mitochondrial fatty acid (27) and an increase in the matrix Mg2+ and Ca2+ concentration (11, 19, 47) would shift the A/D equilibrium of complex I toward the D-form during an ischemic episode affecting myocardial recovery. Moreover, after reoxygenation, the possible opening of the mitochondrial permeability transition pore could also result in Ca2+ overload (1, 7, 28, 40). Together with the release of cytochrome c (4), this would significantly delay complex I reactivation. At that stage, the exposure of Cys-39 of the ND3 subunit of the D-form of complex I (16, 17) may be an important factor determining specific inhibition of the NADH:ubiquinone reductase activity by an as yet unknown mechanism. As shown in our experimental settings, functional modification of the D-form may give a rise to a population of mitochondria with a decreased respiration rate, an over-reduced pool of matrix NAD(P) nucleotides, and a low ATP-synthesizing capacity. Such a population would delay or significantly retard the functional recovery of the cardiomyocytes in I/R. Our results suggest that the deactivation of mitochondrial complex I and increased susceptibility of the enzyme to ROS or nitric oxide metabolites (17) after the ischemic period may represent one of the important contributory mechanisms involved in cardiac injury during acute I/R. Combined classical therapy and interventions for fine tuning of the A/D ratio in mitochondria during the reperfusion process may provide new avenues for ischemic treatment.

**Materials and Methods**

**Experimental animals**

Female C57BL/6J mice (8–12 weeks, Charles River) were employed for all studies and were fasted overnight before experimentation. Animals were housed under constant climatic conditions with free access to food and water. All experiments were performed in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (UK).

**Cardiac arrest and tissue extraction**

Cardiac and respiratory arrest was initiated by cervical dislocation, carcasses were placed in a portable 37°C incubator to maintain physiological body temperature. After specific time periods (2–30 min), organs or tissues were rapidly (within 90 s) extracted, washed in an ice-cold phosphate saline buffer, and snap-frozen in liquid nitrogen.

**In vivo ischemia reperfusion**

Mice were subjected to acute myocardial ischemia by ligation of the left anterior descending coronary artery under 2% isofluorane/oxygen anesthesia as previously described (36). This procedure has been shown to produce reliable and reproducible myocardial infarcts of ~40%. Animals were sacrificed by cervical dislocation either after 20 min of ischemia or after a further 10-min period of reperfusion. Hearts were then rapidly excised and separated into ischemic or nonischemic regions (control) before being frozen in liquid nitrogen and stored at –80°C for further analysis.

**Mitochondrial membrane isolation**

Isolation of the mitochondrial membranes preserving of the A/D ratio was performed essentially as previously described, with minor modifications (39). Pieces of frozen tissue were then placed in a liquid nitrogen precooled metal mortar and pulverized by striking with a mallet. The resulting powder was added to 10 ml of the isolation medium (200 mM Tris-HCl, pH 8.8, 0.5 mM EDTA 1 mM K3Fe(CN)6, and 2 mM potassium malonate) and homogenized in a IKA tissue disruptor (2 min × 6000 rpm). Particular care was taken to cool down all the mediums, glassware, and centrifuge rotors. An alkaline buffer and 1 mM ferricyanide were used to allow rapid oxidation of reduced matrix pyridine nucleotides to prevent turnover-dependent complex I activation. Tissue debris was discarded after brief centrifugation for 10 min at 16,000 g at 0°C. The supernatant was diluted to 40 ml with the same medium and the membranes were collected by centrifugation for 25 min at 48,000 g. The membrane pellet was rinsed with 20 mM Tris-HCl (pH 8.0), 0.25 M sucrose, and 0.2 mM EDTA and resuspended in 400 μl of the same buffer. The membranes were then frozen in liquid nitrogen and stored at –80°C until use. The protein content was determined by bicinchoninic acid (BCA) assay (Sigma).

**Activity measurements**

Oxidation of NADH was determined spectrophotometrically (Varian Cary 4000) as a decrease in absorbance at 340 nm with 150 μM NADH in 1 ml of the standard assay medium (0.25 M sucrose, 50 mM Tris-HCl pH 7.0, 0.2 mM EDTA) supplemented with 5 μM cytochrome c and containing 10–25 μg protein/ml mitochondrial membranes. Additional measurements of NADHQ2, or NADH:HAR oxidoreductase reductase were assayed in the presence of 1 mM cyanide with the addition of 80 μM Q2, or 1 mM HAR, respectively (Sigma). The formation of superoxide radicals was monitored as the SOD-sensitive reduction of acetylated cytochrome
c [ε_{550-539nm} = 21.5, (15)] in the same assay medium, pH 8.0, supplemented with 20 μM acetylated cytochrome c, substrates (50 μM NADH or 5 mM succinate) and containing 0.4–0.5 mg/ml mitochondrial membranes. Acetylated cytochrome c was prepared as described previously (15).

Determination of A/D ratio. The diagnostic test for determination of the A/D ratio is based on the fact that in the presence of divalent cations such as Mg"\(^{2+}\) or Ca"\(^{2+}\) and at alkaline pH (8.5), the rate of reactivation of complex I is very slow (39) (see also (30) for the details). The total amount of the enzyme (A + D) was estimated after full activation of complex I by preincubation of the sample in 0.1 ml of a standard medium (pH 7.0) with 20 μM NADH for 30 s before the addition of 0.9 ml of the standard medium (pH 8.8), 5.5 mM MgCl2, and 165 μM NADH. For estimation of the A-form fraction, 20 μM NADH was omitted from the initial preincubation in the pH 7.0 medium. In these conditions, the initial rate of NADH-oxidase accurately corresponds to the activity contributed only by the A-form, since the activation of the D-form is significantly slower than the time of the assay. In all inhibition studies, the D-form was treated with an effector and activity assessed only after activation by NADH.

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 a standard assay medium (pH 8.5), and incubated at 35 °C for 1 h. To obtain a fully active enzyme, after thermal deactivation, membranes were incubated aerobically for 10–20 min at room temperature with 1% ethanol, 400 mM I by preincubation of the sample in 0.1 ml of a standard medium supplemented with 20 mM NEM was added and samples were incubated at 10°C for 15 min. Membranes were pelleted by centrifugation and washed three times with the same buffer, resuspended at 1 mg/ml, and treated with 30 μM Cy3- or Cy5-maleimide. Each sample was treated with both dyes. After 30 min, the reaction was quenched with 10 mM DTT and washed twice before pooling of labeled samples.

Complex I subunits were separated as described previously (16). The gels were scanned using the Fujifilm FLA fluorescent scanner and stained with silver (49). Images were quantitatively analyzed using Aida Image Analyzer software (Raytest).

Spots of interest were excised, the proteins in-gel digested, and identification was performed through mass spectrometric analysis at the BSRC Mass Spectrometry and Proteomics Facility, University of St Andrews (See Supplementary Data). All proteins were identified with >99% confidence (Prot Score >2.0) with False Discovery Rates of Local FDR<5%, Global FDR<1%.

**Statistical analysis**

All results are expressed as mean±SEM unless specifically indicated and were analyzed by one-way ANOVA to determine statistically significant differences of means among groups.

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**Author Disclosure Statement**

No competing financial interests exist.

**References**


Address correspondence to:
Dr. Alexander Galkin
Medical Biology Centre
School of Biological Sciences
Queen’s University Belfast
97 Lisburn Road
Belfast
BT9 7BL
United Kingdom
E-mail: a.galkin@qub.ac.uk

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### Abbreviations Used

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A/D transition</td>
<td>active/deactive transition</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
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<tr>
<td>DIGE</td>
<td>difference gel electrophoresis</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>HAR</td>
<td>hexaammineruthenium (III) chloride</td>
</tr>
<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>Qt</td>
<td>2,3-dimethoxy-5-methyl-6-(3-methyl-2-buteryl)-1,4-benzoquinone</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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