Effect of oxygen on activation state of complex I and lack of oxaloacetate inhibition of complex II in Langendorff perfused rat heart

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Abstract Two main entry points for electrons into the mitochondrial respiratory chain are NADH:ubiquinone oxidoreductase (complex I) and succinate:ubiquinone oxidoreductase (complex II). Metabolic regulation of these two respiratory complexes is not understood in detail. It has been suggested that the Krebs cycle metabolic intermediate oxaloacetate (OAA) inhibits complex II in vivo, whereas complex I undergoes a reversible active/de-active transition. In normoxic and anoxic hearts it has been shown that the proportion of complex I in the active and de-active states is different suggesting a possible mode of regulation of the enzyme by oxygen concentration. In the current studies rapid isolation of mitochondrial membranes in a state that preserves the activity of both complex I and complex II has been achieved using Langendorff perfused rat hearts. The findings indicate that the state of activation of complex I is controlled by the oxygen saturation in the perfusate. In addition, these studies show that complex II is fully active in the mitochondrion and not inhibited by OAA regardless of the oxygen concentration.

Key words: NADH:ubiquinone oxidoreductase; Complex I; Active/de-active transition; Succinate dehydrogenase; Complex II; Krebs cycle; regulation; Oxygen; Mitochondrion

1. Introduction

When tissue is supplied with glucose as a primary energy source, mitochondrial complex I (NADH:ubiquinone oxidoreductase) and complex II (succinate:ubiquinone oxidoreductase) (succinate dehydrogenase, SDH) are two entry points into the respiratory chain for electrons provided by operation of the Krebs cycle. One turnover of the Krebs cycle results in three NAD(P)H molecules and one ubiquinone molecule reduced by succinate. Regulation of these two enzyme activities is important for the coupling of the Krebs cycle to the electron transport chain.

Since isolated bovine heart submitochondrial particles (SMP) always show significant inhibition of SDH activity by oxaloacetate (OAA), it was suggested that OAA regulates complex II activity and the overall Krebs cycle [1,2]. It is also known that mammalian complex I exists in equilibrium between interchangeable active (A-) and de-active (D-) forms, where only the A-form is capable of ubiquinone reduction by NADH [3,4]. Recently it was shown in Langendorff perfused rat hearts that modulation of normoxia/anoxia affects the complex I equilibrium from the almost fully A-form in normoxia to a predominately D-form in anoxia [5]. Oxygen as a final electron acceptor controls the rate of mitochondrial respiration and utilization of reducing equivalents formed by operation of the Krebs cycle. Thus, metabolic changes due to alterations in tissue oxygenation may control the equilibrium of the A/D-forms of complex I and the extent of SDH inhibition by OAA.

In the present study we used a Langendorff rat heart model to ask two questions: (i) what is the relation between oxygen level on the activation state of complex I over a wide range of oxygen concentrations; and (ii) does OAA inhibit complex II in vivo? To answer these questions mitochondrial membranes were rapidly isolated from perfused rat hearts and the proportion of the A/D-forms of complex I and levels of OAA inhibition of SDH were determined. We observed that varying the levels of oxygen in the perfusate profoundly influenced the A/D ratio of complex I and found that in vivo complex II is fully active and not inhibited by OAA.

2. Materials and methods

2.1. Langendorff perfused rat hearts

Male Sprague–Dawley rats (250–300 g) were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal (i.p.)) and anticoagulated with heparin (5000 USP units/kg, i.p.). The thorax was opened and the heart excised and rapidly transferred to an ice-cold solution containing 120 mM NaCl and 30 mM KCl. The heart was then cannulated via the aorta and perfusion started within 1 min in the Langendorff apparatus with a constant pressure of 70 mm Hg and temperature of 37°C. The perfusion solution was Krebs–Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$, 11 mM glucose, pH 7.4) that was vigorously bubbled with O_2/CO_2 (95:5, normoxia) as previously described [6]. The heart was immersed in a temperature controlled chamber and equilibrated for 10 min by perfusion with the above normoxic buffer. Hearts were then perfused for different times with

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Abbreviations: BTP, bis-Tris–propane; Complex I, NADH:ubiquinone oxidoreductase; Complex II, succinate:ubiquinone oxidoreductase; SMP, submitochondrial particles; OAA, oxaloacetate; SDH, succinate dehydrogenase; A-form, active form; D-form, de-active form; PES, phenazine ethosulfate

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buffer saturated with varying gas mixtures that had O_2/N_2 ratios of 0/95, 5/90, 40/55, and 70/25 with a constant CO_2 content of 5%. Oxygen concentration in air-saturated buffer at 37°C was taken as 19%. At the end of the perfusion the hearts were immediately frozen in liquid nitrogen.

2.2. Modified Keilin–Hartree mitochondrial membrane preparation from single rat hearts

The method used has been adapted from the Keilin–Hartree preparation as previously described [7,8]. Each frozen heart was powdered in a pre-cooled (−20°C) porcelain mortar (12.5 cm diameter). The frozen tissue was ground with 2 g of quartz sand and 10 ml of isolation medium (200 mM bis-Tris–propane (BTP, pH 8.8), 0.5 mM ethylenediamine tetraacetic acid (EDTA), 1 mM $K_3Fe(CN)_6$, and 2 mM potassium malonate) for 15 min until the frozen powder started to melt. Sand and tissue debris were discarded after brief centrifugation for 2 min at $16\,000 \times g$ at 4°C. The supernatant was diluted to 40 ml with the same medium and the membranes were collected by centrifugation for 25 min at $48\,000 \times g$. The membrane pellet was rinsed with 20 mM Tris–HCl (pH 8.0), 0.2 mM EDTA, 1 mg/ml bovine serum albumin (BSA), and resuspended in 1 ml of the same buffer. The membranes were then frozen in liquid nitrogen and stored at −80°C until use.

2.3. Activation state of complex I

Activation state was determined spectrophotometrically at 25°C by comparing the NADH oxidase activity of isolated membranes to the same sample in which complex I was fully activated [5]. Activity of each isolated sample (20 μg protein/ml) was assayed in 0.2 M BTP (pH 8.8), 1 mg/ml BSA, 5 mM $CaCl_2$. Complete activation of complex I was achieved by preincubation of the sample (20 μg protein/ml) in 0.1 ml of 2 mM BTP (pH 6.8) with 100 μM NADH for 2 min prior to the addition of 0.9 ml of 0.2 M BTP (pH 8.8), 1 mg/ml BSA with 5.5 mM $CaCl_2$ and initiation of the reaction with 150 μM NADH ($\varepsilon^{340} = 6.22\ mM^{-1}\ cm^{-1}$).

2.4. Inhibition of complex II by OAA

Complex II activity was determined at 25°C by comparison of isolated membranes to the same sample with OAA-free complex II. Activity of isolated membranes (5 μg/ml) was assayed in 50 mM BTP (pH 8.0), 0.2 mM EDTA, 1 mM phenazine ethosulfate (PES), 50 μM dichloro-phenolindophenol (DCIP) ($\varepsilon^{600} = 21.8\ mM^{-1}\ cm^{-1}$) with 20 mM succinate. The initial rate of the reaction after addition of the membranes was calculated. To completely remove OAA from the active site of complex II, membranes (5 mg/ml) were incubated with 1 mM malonate for 20 min at 30°C and then assayed in the above buffer. Succinate oxidase activity was determined by oxygen consumption using an oxygraph equipped with a Clark type electrode.

2.5. Other assays

NADH–$K_3Fe(CN)_6$ reductase [3] and NADH:Q_1 reductase activities [5] of complex I were assayed as previously described. Horse heart cytochrome *c* was used to stimulate NADH oxidase activity. Protein concentration was determined by the Biuret method.

3. Results

Initially a protocol was designed and tested for preparation of mitochondrial membranes that would simultaneously preserve the activation status of complex I and permit measure of OAA-induced inhibition of complex II during heart perfusion. The membrane preparation was judged to be free of intact mitochondria by treating the preparation with the channel-forming antibiotic alamethicin [9] as intact mitochondria are impermeable to NADH. No change in NADH oxidase activity was found whether or not alamethicin was present (data not shown). The kinetic properties of the membrane preparation are summarized in Table 1. It is known that interconversion between the A- and D-forms of complex I is extremely temperature dependent [4]. Deactivation (A to D transition) is a spontaneous process which occurs within minutes at 37°C and hours at 4°C [4]. Activation (D to A transition) requires reduction of the D-form by NADH followed by oxidation by quinone [3,4]. This process occurs within tens of seconds at 37°C but takes minutes at 4°C. In the case of complex II, release of OAA from the enzyme is also highly temperature dependent and occurs within minutes at 37°C but takes hours at 4°C [1]. Since temperature is such an important factor in preserving the activation state of complex I and the OAA-induced inhibition of complex II, a temperature of 0–2°C was maintained throughout the isolation procedure (Section 2). Additional considerations taken into account in designing the protocol are as follows. Activation of complex I was suppressed by raising the pH of the isolation medium to 8.8, a condition known to inhibit activation [10]. To eliminate NADH from the homogenate and completely block activation, 1 mM potassium ferricyanide which causes fast oxidation of NADH was included in the isolation medium. The NADH–ferricyanide reductase reaction is temperature independent (~4 kJ/mol) whereas the NADH oxidase activity is highly temperature dependent (~65 kJ/mol). Thus at the low temperature (0–2°C) used for isolation of membranes the NADH dehydrogenase fragment of complex I would be largely responsible for NADH oxidation, minimizing the possibility that complex I could be activated through the distal portion of the respiratory chain. To protect complex II from binding OAA during SMP isolation, malonate was added to the isolation medium. Malonate reversibly binds to complex II and at mM concentration prevents OAA binding to the enzyme in the homogenate [1,2].

Table 1
Catalytic properties of Keilin–Hartree membranes isolated from rat heart

Assay	Activity, μmol/min/mg protein[a]
NADH oxidase	0.42
+cyt *c* (10 μM)	0.64
+rotenone (5 μM)	0
NADH:Q_1 reductase[b]	0.4
+rotenone (5 μM)	0.01
NADH–ferricyanide (2e)	2.6
Succinate–PES–DCIP	0.22

[a]The assay conditions were 50 mM BTP (pH 8.0), 0.2 mM EDTA, 1 mg/ml BSA, temperature was 25°C. + indicates addition of horse heart cytochrome *c* or rotenone to stimulate or inhibit activity respectively.
[b]Activity was measured with 50 μM Q_1 in the presence of 2 mM potassium cyanide.

Table 2 shows the NADH oxidase and SDH activity of mitochondrial membranes isolated from perfused rat hearts. When hearts were perfused with normoxic buffer (95% O_2) for 30 min 95% of complex I was in the A-form and complex II was fully active, i.e. in the OAA-free form. Anoxic perfusion for 15 min drastically decreased the proportion of the A-form of complex I to about 28%. When this was followed by normoxic perfusion, however, complex I activity was restored to about 85%, confirming that A/D transitions are reversible in ex vivo conditions consistent with previous observations [5]. By contrast, no change in SDH activities was evident in the membrane samples after 15 and 30 min of anoxic perfusion. Incubation of the membranes with malonate did not increase SDH activity indicating that the enzyme existed completely in the OAA-free form. Membranes from a control heart, freshly taken from the rat, contained a completely active complex I and complex II, in agreement with the data obtained during

Table 2
Activation status of complex I and OAA inhibition of complex II in perfused rat hearts[a]

Samples	Complex I A-form (%)	SDH OAA-free enzyme (%)
30 min normoxic perfusion	95 ± 0.5	100
15 min anoxic perfusion	28 ± 1.5	100
+20 min normoxic perfusion	85 ± 2.5	100
30 min anoxic perfusion	18 ± 2	100
60 min anoxic perfusion	16 ± 2	100
Control heart[b]		
2 mM malonate in isolation medium	100 ± 1.5	100
no malonate in isolation medium	100 ± 1.5	25 ± 3

[a]The perfusion, membrane isolation, and assay are described in Section 2. Each data point is expressed as a mean from three separate experiments.
[b]Activity in mitochondrial membranes from freshly isolated rat heart. 100% activity corresponds to 0.42 and 0.22 μmol/min per mg protein for the NADH oxidase and SDH reactions, respectively.

normoxic perfusion. However, when 2 mM malonate was omitted from the isolation medium, about 75% of the initial rate of SDH was inhibited which could be subsequently reactivated upon incubation with malonate at 30°C. This indicates that OAA inhibition of SDH routinely occurs during membrane isolation unless malonate is supplied. Thus, metabolic changes caused by anoxic perfusion and changing the A/D complex I equilibrium do not affect SDH inhibition by OAA.

Since anoxic perfusion caused a dramatic decrease in the activation state of complex I the question remained whether different oxygen concentrations during heart perfusion would result in different steady-state levels between the A- and D-forms. The steady-state levels of the A/D equilibrium in rat hearts perfused with N_2 (Table 2) and air-saturated buffer (data not shown) were established by 30 min of perfusion. Fig. 1 shows the effect of 30 min perfusion at different levels of oxygen saturation on the steady-state levels of the A-form of complex I. A steep rise in the proportion of the A-form of complex I was observed (from 18 to 75%) when the oxygen saturation was increased to 40%. At higher than 70% oxygen saturation complex I was found to be almost completely in its A-form. The level of the A-form of complex I in membrane preparations obtained from hearts after 60 min of anoxic perfusion was 16%. However, these membranes when incubated for 10 min at 37°C become completely deactivated (data not shown). This observation raises the question whether the residual amount of A-form is due to uncontrolled oxygen contamination of the perfusion system. To test this possibility, the hearts were perfused anaerobically in the presence of 2 mM KCN to completely block the respiratory chain. Fig. 2 shows that there is no difference in the activation state of complex I after the perfusion of hearts with or without KCN, indicating that oxygen contamination is not the reason for the residual amount of the A-form of complex I.

In this study it has been found that complex II remained OAA-free regardless of the level of oxygenation. Mammalian complex II is capable of catalyzing the quinol:fumarate reductase reaction [11], thus allowing an alternative pathway for quinol reoxidation. Indeed, when 2 mM fumarate was introduced into the anoxic perfusion buffer, the steady-state level

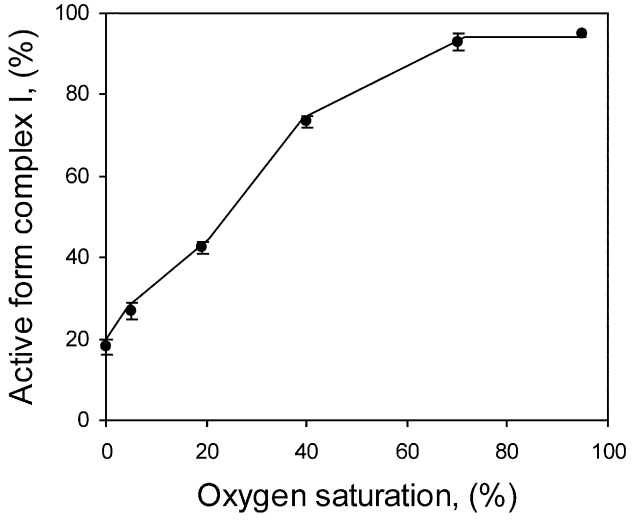

Fig. 1. A-form complex I equilibrium established during 30 min of perfusion with different oxygen saturation in the perfusate. Perfusion conditions, membrane isolation, and enzyme assay are described in Section 2. Fully active NADH oxidase activity corresponds to 0.6 μmol NADH per min per mg protein. Each data point is a mean of three separate independent experiments.

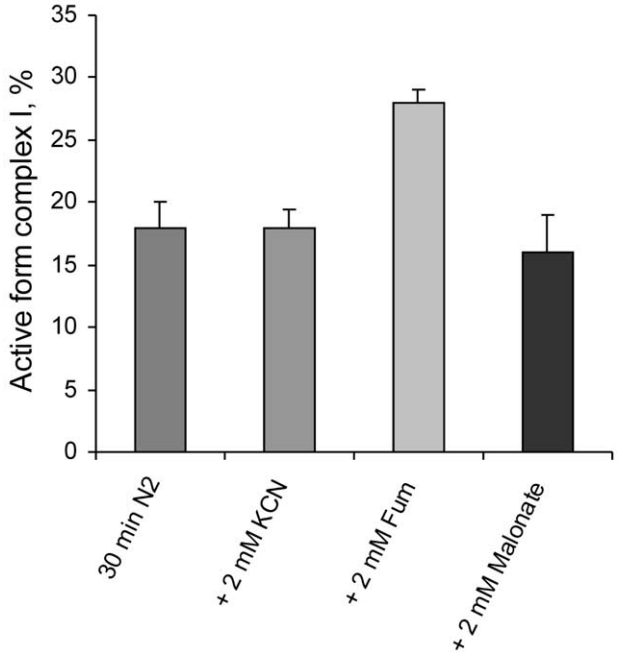

Fig. 2. Effect of KCN, fumarate, and malonate on the A-form complex I equilibrium established during 30 min of anoxic perfusion. The concentrations of KCN, fumarate, and malonate added to the perfusate are indicated on the figure. Perfusion conditions, membrane isolation, and enzyme assay conditions are described in Section 2. Each bar represents a mean of three to four separate independent experiments.

of the A-form of complex I increased to 28% (Fig. 2). To determine if quinol:fumarate reductase activity of complex II may be responsible for the residual levels of the A-form of complex I during anoxic perfusion with glucose, anoxic perfusion with 2 mM malonate was performed. However, as seen in Fig. 2, malonate had no effect on the A/D equilibrium of complex I.

4. Discussion

Complexes I and II in perfused rat hearts responded differently to hypoxia. While oxygen controlled the steady-state levels of the A-form of complex I during heart perfusion, complex II remained in an OAA-free form regardless of the oxygen content of the perfusate. Earlier experiments with bovine SMP suggested that the decrease of NADH oxidation by the respiratory chain resulted in a lower steady-state level of the A-form of complex I [12]. This is in agreement with the data presented here, where variation of the levels of oxygen saturation used during rat heart perfusion controlled the activation state of complex I. Complex I was completely in the A-form during normoxia and lowering the oxygen saturation in the perfusate resulted in a decrease of steady-state levels of active complex I to about 20%.

The data in Table 2 show that complex II appears to be in the OAA-free form after either prolonged normoxic or anoxic perfusion. During normoxic metabolism with glucose as a primary energy source the intramitochondrial OAA concentration is believed to be very low, as thermodynamics would poise OAA for citrate synthesis. Anoxia causes inhibition of NADH oxidation which results in a significant increase of mitochondrial NADH [13]. Increased concentration of NADH is a situation where reversal of malate dehydrogenase activity is thermodynamically favored which would also reduce OAA levels in the mitochondrion. Moreover, hypoxia should cause significant reduction of the quinone pool. Redox equilibration of the quinol pool with SDH resulted in more than a 10-fold increase in the dissociation for OAA with reduced SDH [14]. The experimental protocol used to isolate membranes in the current studies suggests that OAA-inhibited SDH is likely due to OAA binding during the isolation procedure and is not characteristic of the state of activation of complex II in the intact mitochondrion.

These studies have also shown that the residual 20% of the A-form of complex I found in isolated membranes after prolonged anoxic perfusion of rat hearts can be fully deactivated by incubation of membranes at 37°C. The question remains therefore as to why complex I can not be completely deactivated in the whole organ. The possibility of an oxygen leak contaminating the perfusion system can be discounted since KCN did not affect the residual portion of the A-form of complex I. The significant improvements in membrane isolation by modification of the Keilin–Hartree preparation allow one to follow the activation state of complex I in the membranes in parallel to the actual A/D equilibrium that occurs in perfused hearts. As the rate of quinol reoxidation through the respiratory chain determines the A/D equilibrium of complex I [12], it can be hypothesized that alternative pathways for quinol reoxidation may also affect the activation state of complex I.

Another result of this study is the finding that complex II remains fully active during anoxia. High levels of ubiquinol and the availability of fumarate may allow the quinol–fumarate reductase reaction under anoxic conditions. Indeed, anoxic perfusion with fumarate shifted the A/D complex I equilibrium from 18 to 28% of A-form. This is the same level as that occurred during perfusion with 5% oxygen. These data correlate with observations that SDH shows low rates of fumarate reduction [11]. To understand whether the anoxic A/D equilibrium is a result of the NADH–fumarate reductase reaction in tissue supplied with glucose, anoxic perfusion with malonate was performed. Even though malonate did not decrease the A/D equilibrium, one cannot completely exclude the possibility of NADH–fumarate reductase activity under anaerobic conditions as SDH is less sensitive to dicarboxylate inhibitors when reduced. Another possible alternative pathway for quinol oxidation may include reversible operation of mitochondrial glycerol-3-phosphate dehydrogenase, an enzyme in the inner mitochondrial membrane that oxidizes glycerol-3-phosphate in the cytoplasm and reduces the quinone pool in the mitochondrial membrane. Finally, we can not exclude the presence of other effectors in intact cells that may modulate the A/D equilibrium.

Several experimental observations suggest that complex I is a prime source for superoxide production from the respiratory chain [15,16] especially when a quinone site inhibitor such as rotenone is present [17,18]. The D-form of complex I functionally resembles the A-form when the latter is blocked with rotenone as the D-form is reduced by NADH and able to interact with different acceptors [10]. Anoxia resulted in an increased fraction of the D-form and a decrease of the A-form (Table 2). It is presumed that the first minutes of reoxygenation are the most damaging to the heart following periods of ischemia. During this period the remaining A-form of complex I would supply the respiratory chain with reducing equivalents, while activation of the D-form is not instantaneous and takes several minutes in the Langendorff rat model [5]. It seems possible that although complex I remains in the deactive state during the initial period of oxygen reperfusion, deactive complex I may contribute to increased levels of superoxide production and subsequent cardiac damage.

In summary the data presented here suggest that when a rat heart is supplied with highly oxygenated arterial blood complex I is fully active. However, the data also show that the activation status of complex I is determined by oxygen concentration over a wide range of saturations. Since the A/D phenomenology of complex I is not tissue specific [19], other organs such as liver can also become significantly hypoxic. In such instances different steady-state A/D complex I equilibria may be established in various tissues.

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