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The topology of superoxide production by complex III and glycerol 3-phosphate dehydrogenase in *Drosophila* mitochondria

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Abstract

The topology of superoxide generation by *sn*-glycerol 3-phosphate dehydrogenase and complex III in intact *Drosophila* mitochondria was studied using aconitase inactivation to measure superoxide production in the matrix, and hydrogen peroxide formation in the presence of superoxide dismutase to measure superoxide production from both sides of the membrane. Aconitase inactivation was calibrated using the known rate of matrix superoxide production from complex I. Glycerol phosphate dehydrogenase generated superoxide about equally to each side of the membrane, whereas centre *o* of complex III in the presence of antimycin A generated superoxide about 30% on the cytosolic side and 70% on the matrix side.

Keywords: *Drosophila*; Mitochondria; Superoxide; Glycerol phosphate dehydrogenase; Complex III

1. Introduction

While reactive oxygen species (ROS) have important biological functions, such as cell signalling [1], their overproduction is associated with various pathologies [2]. ROS are capable of triggering molecular damage to cellular components, and accumulation of such damage is proposed to be responsible for the development of various diseases and to be a causal factor of ageing [3–5]. Therefore, understanding the nature of ROS production in biological systems is of great importance. Mitochondria were first shown to produce ROS in the 1970s [6–8], since then, they have been the object of intense investigation as a major source of cellular ROS [9,10]. The primary ROS product of mitochondria is superoxide, which in turn can give rise to hydrogen peroxide (H_2O_2), hydroxyl radicals and other species [11,12].

Despite decades of intensive investigation, the exact mechanisms of superoxide production by mitochondria are not understood. Most authors agree that the major sites of superoxide production in mitochondria are complex I and complex III of the electron transport chain [10,13–16], and *sn*-glycerol 3-phosphate dehydrogenase in brown adipose tissue and in insect flight muscles [17–19]. However, the exact location of superoxide formation within these enzymes is the object of debate.

Within complex I, each of the electron transferring components (FMN, Fe–S centres and ubiquinone) has been proposed at one time or another to be the major superoxide-producing site by different research groups [20–25]. Nevertheless, there is agreement that superoxide from complex I is generated exclusively on the matrix side of the mitochondrial inner membrane [16,19,26,27].

Superoxide production by complex III is better characterized. It is well established that complex III produces superoxide in the presence of antimycin, an inhibitor of centre *i* of complex III [28]. This effect is suppressed by centre *o* inhibitors such as myxothiazol and stigmatellin (e.g., [28,29]). Myxothiazol can also induce superoxide production at centre *o* when the quinone pool is highly reduced, albeit at smaller rates than antimycin [29–31]. Employing myxothiazol, it was demonstrated that centre *o* semiquinone is the locus of superoxide production at complex III [28]. Centre *o* is located in the hydrophobic domain on the cytosolic side of the

Abbreviations: ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; FMN, flavin mononucleotide; Fe–S, iron sulphur cluster; FAD, flavin adenine dinucleotide; Mn-SOD, manganese superoxide dismutase; KCN, potassium cyanide; $HO_2^{\bullet}$, hydroperoxyl

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membrane, leading to the prediction that superoxide from this site is most likely to be released on the cytosolic side [32,33]. Superoxide production from complex III in the presence of antimycin was originally detected on the matrix side (in submitochondrial particles [10,34]), but it has also been detected on the cytosolic side of the membrane [16,19,35,36]. Up to now, the proportion of superoxide released by complex III to each side of the membrane has not been intensively studied, although a recent report found that equal amounts of superoxide were produced in the matrix and in the intermembrane space [27].

The mechanism of superoxide production by *sn*-glycerol 3-phosphate dehydrogenase is poorly understood. The flavin, Fe–S or semiquinone sites could be responsible. The enzyme is located on the outer side of the inner membrane [37,38], with the FAD and presumably the Q-binding site in the membrane [39]. In *Drosophila* mitochondria, it produces superoxide partly and perhaps mostly on the cytosolic side of the membrane [19]. However, as with complex III, the proportion of superoxide production to each side of the membrane is not yet fully clear.

We previously measured mitochondrial H_2O_2 production by *Drosophila* mitochondria fluorometrically (with homovanillic acid) to identify sites responsible for superoxide generation [19]. Superoxide production in the matrix was measured as the rate of H_2O_2 production in the absence of exogenous superoxide dismutase, since superoxide in the matrix is readily converted to H_2O_2 by Mn-SOD, and H_2O_2 freely diffuses out to the cytosolic side, becoming accessible to the fluorometric probe [16,19,33]. Superoxide production on the cytosolic side of the membrane was measured as the increase in the rate of H_2O_2 production on addition of exogenous superoxide dismutase, since only external superoxide will be converted to H_2O_2 by added enzyme. However, both of these assays can overestimate or underestimate superoxide production in a particular compartment [19,36], and an independent method of determining the topology of superoxide production in intact mitochondria is required for more confident conclusions.

The matrix enzyme, aconitase, can be used to measure the steady state concentration of superoxide in the matrix [40]. Aconitase catalyses the conversion of citrate to isocitrate. It contains cubane [4Fe–4S] centres with three iron atoms interacting with cysteine residues, while the fourth iron, Fe_α, is exposed to the solvent to allow the catalytic dehydration of citrate to form the intermediate *cis*-aconitate, and the subsequent hydration of *cis*-aconitate to form isocitrate. However, this renders Fe_α exposed to attack by superoxide, so aconitase is highly susceptible to oxidative inactivation by superoxide [40–42]. Other reactive species, such as H_2O_2, can inactivate aconitase, but superoxide reacts with aconitase several orders of magnitude faster than H_2O_2 [40]. Therefore, the rate of decay of aconitase activity can be used to infer the relative concentration of matrix superoxide in a very sensitive way.

Hence, the hypothesis that glycerol phosphate dehydrogenase and centre *o* of complex III produce superoxide in the matrix can be addressed by measuring aconitase activity in the appropriate experimental conditions. The rate of aconitase inactivation will be a function of the steady-state superoxide concentration in the matrix, and it can be calibrated using the rate of superoxide production from complex I, measured fluorometrically.

The aim of the present study was to further characterize superoxide production by *Drosophila* mitochondria by using the aconitase method (i) to determine if glycerol phosphate dehydrogenase and complex III produce superoxide to the matrix side of the inner membrane and, if so, (ii) to determine the proportion of superoxide released to each side of the membrane from each of these two enzymes.

2. Materials and methods

2.1. Isolation of mitochondria

Wild type *Drosophila melanogaster*, Dahomey stock (from the Department of Biology, University College London), around 10 days old were used. Mitochondria were prepared as described previously [19], using a one-step fly crushing process. Briefly, about 200 flies were immobilized by chilling on ice, and gently pressed with a pestle in a chilled mortar containing a little isolation medium (250 mM sucrose, 5 mM Tris–HCl, 2 mM EGTA, 1% (w/v) bovine serum albumin, adjusted to pH 7.4 at 4 °C) then passed through two layers of absorbent muslin (adding extra isolation medium as needed) and collected into a centrifuge tube and immediately centrifuged at 150×*g* for 3 min in a Sorvall SS-34 rotor at 4 °C. The supernatant was passed through one layer of muslin and recentrifuged at 9000×g for 10 min. The supernatant was discarded and the pellet was carefully resuspended with a little more isolation medium to give about 30 mg protein/ml (Bio-Rad *Dc* protein assay kit, Bio-Rad, Richmond, CA, USA). Yield of mitochondria was about 8–9 mg/ g wet weight flies. This protocol was optimized for mitochondrial coupling and yield at the expense of purity; however, effects of cellular contaminants on mitochondrial superoxide production appeared to be minimal [19]. The detailed characteristics of mitochondria prepared in this way were reported in Miwa et al. [19]; the respiratory control ratio (above 3.0 with glycerol 3-phosphate) was found to be stable for 3 h [19]. However, the present study used only freshly prepared mitochondria, and all the experiments were started within 10 min after the isolation.

2.2. Incubation of mitochondria

The incubation medium consisted of 120 mM KCl, 5 mM KH_2PO_4, 3 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, and 0.2% (w/v) bovine serum albumin, adjusted to pH 7.2 at room temperature, together with 50 U/ml superoxide dismutase (from bovine liver) in order to eliminate extramitochondrial superoxide. Time courses of aconitase inactivation were measured in four different conditions: (a) with *sn*-glycerol 3-phosphate (20 mM), (b) with *sn*-glycerol 3-phosphate plus rotenone (5 μM), (c) with *sn*-glycerol 3-phosphate plus rotenone plus myxothiazol (3 μM) and (d) with *sn*-glycerol 3-phosphate plus rotenone plus antimycin A (3 μM). Condition (a) measures overall native superoxide production. Rotenone blocks reverse electron flow and superoxide formation by complex I. Therefore, superoxide arising in condition (b) is due to forward electron flow, and comes mostly from glycerol phosphate dehydrogenase [19]. The difference between (a) and (b) reports superoxide production from complex I. The maximum capacity to produce superoxide by glycerol phosphate dehydrogenase is monitored in condition (c) and that of centre *o* of complex III is calculated as (d) minus (c) [19]. The justification for this centre *o* calculation is that in *Drosophila* mitochondria, superoxide production when complex III was reduced in the presence of KCN in the absence of complex III inhibitors was no greater than that in the presence of myxothiazol, which binds at centre *o*, suggesting that no site except centre *o* in complex III produces superoxide [19]. All experiments were supported by paired, parallel controls omitting glycerol phosphate, that is, in unenergized mitochondria. Inclusion of catalase did not affect aconitase inactivation (data not shown), showing that inactivation was not caused by externally-generated H_2O_2.