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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.

Time-course experiments were started by adding mitochondria (about 0.5 mg/ml) to medium in 3 ml chambers fitted with magnetic stirrers and maintained at 25 °C. Samples at each time point were snap frozen in pre-chilled Eppendorf tubes in an aluminium block on dry ice, then stored at −80 °C until assay.

2.3. Aconitase assay

Aconitase activity was measured spectrophotometrically in 96-well microplates on a plate reader (Biotek Instruments) as NADPH formation monitored at 340 nm using the coupled assay of Rose and O'Connell [43], as outlined by Gardner [40]. The frozen samples were thawed rapidly immediately prior to assay and 10 μl of sample were added to 190 μl of assay buffer (50 mM Tris–HCl pH 7.4, 0.6 mM $MnCl_2$, 5 mM sodium citrate, 0.2 mM $NADP^+$, 0.1% v/v Triton X-100 and 0.4 units/ml isocitrate dehydrogenase (Sigma) pre-equilibrated to 30 °C). Each sample was assayed in quadruplicate, readings were taken at 15-s intervals over 7 min, and the resulting linear slopes were averaged to give a measurement of aconitase activity for that sample. Aconitase activity (i.e., NADPH formation) had linear dependence on protein concentration over the range of concentrations used (results not shown).

3. Results and discussion

3.1. Decay in aconitase activity in mitochondria

Fig. 1 shows the decay of aconitase activity with time in *Drosophila* mitochondria incubated under different conditions. Fig. 1a shows that there was a gradual decrease in aconitase activity in unenergized mitochondria in the presence of respiratory inhibitors. Fig. 1b shows that there were much stronger decreases in aconitase activity in mitochondria following the addition of glycerol phosphate. All the changes in activity were first order processes, as plotting these curves on a logarithmic scale resulted in linearization (Figs. 1c, d). The slopes of these lines represent the rates of aconitase inactivation in each condition, and are a function of the steady-state concentration of superoxide in the matrix, and hence of the rate of matrix superoxide production provided that superoxide scavenging remains the same.

The rates of aconitase inactivation due to matrix superoxide produced using electrons derived from glycerol phosphate in each experimental condition were calculated by subtracting the slopes in Fig. 1c from the paired slopes in Fig. 1d. The resulting rates in each experimental condition are shown in Table 1. These values were used to calculate the rates attributable to complex I (reverse electron flow-linked; the difference between with and without rotenone), glycerol phosphate dehydrogenase native (forward electron flow; in the presence of rotenone) and maximum (in the presence of rotenone and myxothiazol) and centre *o* of complex III (the difference between antimycin and myxothiazol, in the presence

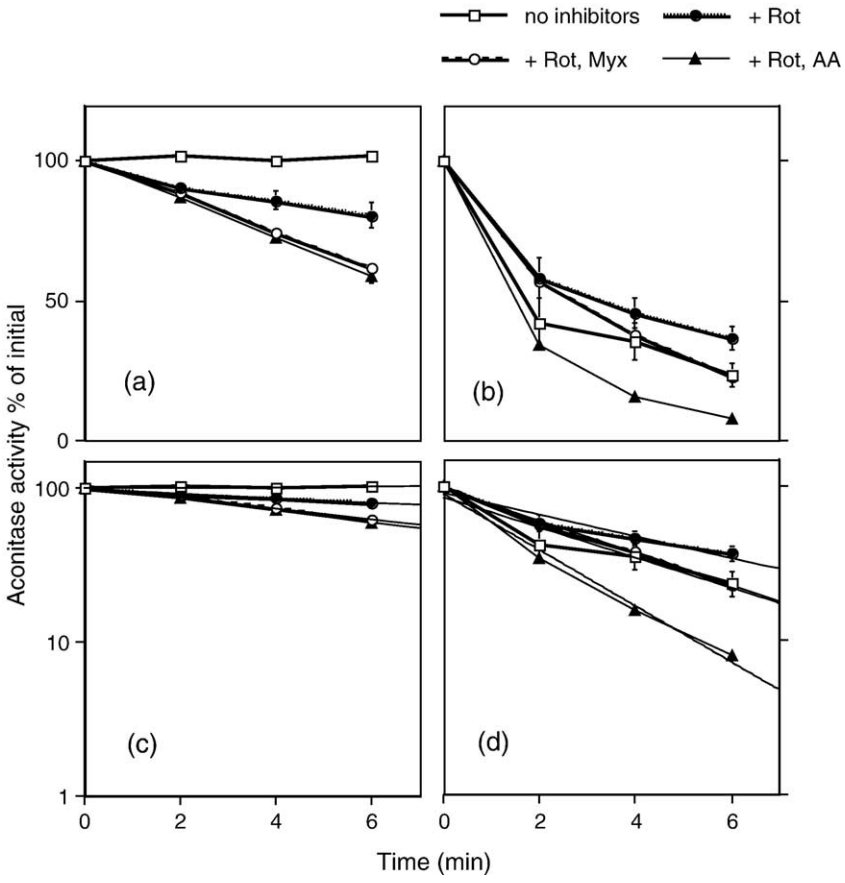

Fig. 1. Decay in aconitase activity in *Drosophila* mitochondria under different conditions. For details, see text. (a), (c), no substrate; (b), (d), plus substrate (20 mM glycerol 3-phosphate). Open squares, substrate 20 mM glycerol 3-phosphate (for b, d) only; closed circles, substrate and 5 μM rotenone; open circles, substrate, rotenone and 3 μM myxothiazol; closed triangles, substrate, rotenone and 3 μM antimycin A. (c) and (d) are logarithmic plots of (a) and (b) respectively. Values are means±S.E.M. of four independent experiments. Error bars which are not visible were smaller than the symbol size.

Table 1
Rates of superoxide production from different sites in *Drosophila* mitochondria measured by aconitase inactivation and by fluorometric assay of H_2O_2 production

	Rate in different experimental conditions				Calculated rate for each component			
	GP	GP, Rot	GP, Rot, Myx	GP, Rot, AA	Comp I	GPdh (native)	GPdh (max)	Centre *o* (max)
Rate of aconitase inactivation (min^{-1})	0.239	0.130	0.161	0.331	0.108	0.130	0.161	0.170
Rate of matrix superoxide production (calculated from aconitase inactivation) (nmol H_2O_2 equivalents/min per mg protein)	2.21	1.21	1.49	3.06	1.0	1.21	1.49	1.57
Rate of matrix H_2O_2 production assayed fluorometrically (no SOD added) (nmol/min per mg protein)	2.58	1.60	2.04	3.57	0.94	1.60	2.04	1.53
Overall rate of H_2O_2 production assayed fluorometrically (SOD added) (nmol/min per mg protein)	3.72	2.56	3.09	5.26	1.04	2.56	3.09	2.17

The left hand column shows measured rates, the right hand column shows the rates from different components of the electron transport chain calculated from the measured rates as described in Materials and methods (Comp I = {GP} − {GP, Rot}; GPdh (native) = {GP, Rot}; GPdh (max) = {GP, Rot, Myx}; Centre *o* = {GP, Rot, AA} − {GP, Rot, Myx}). Rates of aconitase inactivation (row 1) were calculated from data in Figs. 1c, d. All calculations were made using paired experiments, not averaged data (which are shown in Fig. 1). The equivalent rates of H_2O_2 production (row 2) were calculated from the data underlying row 1 using the calibration factor of H_2O_2 rate/aconitase inactivation rate = 1.0/0.108 (the calibration against matrix H_2O_2 production from complex I (shaded in the table) as described in the text). Data for fluorometric assays (rows 3 and 4) are from [19]. GP-substrate (20 mM glycerol 3-phosphate); GP, Rot-substrate and 5 μM rotenone; GP, Rot, Myx-substrate, rotenone and 3 μM myxothiazol; GP, Rot, AA-substrate, rotenone and 3 μM Antimycin A; GPdh-Glycerol 3-phosphate dehydrogenase; SOD-superoxide dismutase.

of rotenone) as described in Materials and methods (Table 1). The calculations show that there was considerable superoxide release on the matrix side from both glycerol phosphate dehydrogenase and centre *o* of complex III.

3.2. Calibration of aconitase inactivation rates

We have previously shown that complex I of *Drosophila* mitochondria produces superoxide exclusively to the matrix side of the membrane [19]. Therefore, the aconitase inactivation rate of 0.108 min^{-1} by complex I (Table 1) corresponds directly to the measured rate of H_2O_2 production from this site under the same conditions (1.0 nmol H_2O_2/min per mg protein; [19]). Using this calibration factor of 1.0/0.108, the rate of matrix superoxide production by the other sites can be converted from aconitase inactivation rates to nmol H_2O_2 equivalents/min per mg protein (Table 1).

The relative rates of aconitase inactivation under different conditions in the present work are very similar to the relative rates of H_2O_2 production in the absence of exogenous superoxide dismutase we found previously [19], so there is good agreement between the two sets of values after calibration. See Table 1, compare the row 'Rate of matrix superoxide production (calculated from aconitase inactivation)' with the row 'Rate of matrix H_2O_2 production assayed fluorometrically (no SOD added)'; there was no statistical difference between the two members of any pair (unpaired Student's *t*-test). It was originally thought that the rate of mitochondrial H_2O_2 production in the absence of added superoxide dismutase may overestimate matrix superoxide production because some superoxide produced on the cytosolic side of the membrane may be dismutated to H_2O_2 spontaneously or by contaminating or native external superoxide dismutases [16,19,36] such as Cu,Zn-SOD in the intermembrane space. The similarity of the relative rates across a range of values using the two independent methods of estimating matrix superoxide production suggests that any such overestimation is negligible.

3.3. Proportion of total superoxide produced in the matrix from different sites

The rate of H_2O_2 production by mitochondria measured fluorometrically in the presence of superoxide dismutase ([19], Table 1) represents overall superoxide production from both sides of the membrane. By comparing this rate to the rate of matrix superoxide production estimated by the calibrated aconitase assay, the proportions of superoxide production on the matrix side and on the cytosolic side of the membrane can be calculated (Fig. 2). Glycerol phosphate dehydrogenase appears to produce superoxide on each side of the membrane at about equal rates (47% of native, 48% of maximum rate on the matrix side). This was surprising considering the known location of the active site of this enzyme, which is on the outer side of the inner membrane [37,38], although the FAD [39] and, presumably, the quinone-reducing site [39] are within the membrane. However, the sites where superoxide is formed within this enzyme are not known.

Matrix superoxide production accounted for about 70% of the total from centre *o* of complex III (Fig. 2). Considering the fact that this centre is within but faces the cytosolic side of the membrane, it was rather unexpected that it should generate more superoxide to the matrix than to the cytosolic side.

For both glycerol phosphate dehydrogenase and centre *o* of complex III, we cannot exclude the possibility that we have underestimated external superoxide production and therefore overestimated the proportion produced in the matrix. For example, some of the externally-produced superoxide may have reacted with cytochrome *c* rather than being dismutated to H_2O_2 by the added superoxide dismutase, although the rate for Cu,Zn-SOD ($k = 0.62 \times 10^9$/M/s) is much faster than that for cytochrome *c* ($k = 2.5 \times 10^5$/M/s) [26,36].

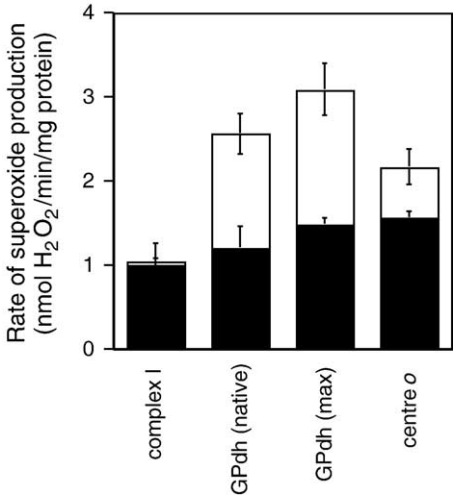

Fig. 2. Topology of superoxide production by different components of the electron transport chain. Data from Table 1. Black bars, rate of matrix superoxide production calculated from aconitase inactivation (nmol H_2O_2 equivalents/min per mg protein); combined bars, overall rate of matrix H_2O_2 production assayed fluorometrically with superoxide dismutase added; white bars, by difference, superoxide production on the cytosolic side of the membrane. GPdh, glycerol phosphate dehydrogenase. Values are means±S.E.M. of four (aconitase) or 8 independent experiments (fluorometric assay).

Few other studies have addressed the proportion of superoxide released on the matrix and cytosolic sides of the membrane by complex III. Han et al. suggested that complex III in antimycin-treated mitochondria produced superoxide mostly on the matrix side [26]. They measured superoxide production from the cytosolic side of the membrane using EPR, and superoxide from the matrix side as H_2O_2 production in the absence of exogenous superoxide dismutase. However, they pointed out that superoxide on the cytosolic side might have been underestimated due to interaction of superoxide with cytochrome *c*, while matrix H_2O_2 production might have been overestimated due to dismutation of external superoxide to H_2O_2. A recent report investigated the sidedness of superoxide release in rat skeletal muscle mitochondria, employing three different methods: fluorometric H_2O_2 detection, chemiluminescent superoxide detection and aconitase inactivation. It was concluded that complex III released superoxide to each side of the membrane in about equal proportions [27]. Our results suggest that 70% of total superoxide production from centre *o* of complex III in *Drosophila* mitochondria is released to the matrix side. Thus, different sources of mitochondria may have slightly different sidedness of superoxide release.

Earlier studies suggested that superoxide can penetrate lipid membranes [44,45]. To ensure that inactivation of matrix aconitase in our experiments was by superoxide produced in the matrix, and not by superoxide produced on the cytosolic side of the membrane followed by diffusion of this superoxide through the membrane, we added superoxide dismutase to all of the experimental media. Added superoxide dismutase will convert externally-produced superoxide to H_2O_2 and eliminate this possibility. H_2O_2 may also inactivate aconitase, albeit weakly compared to superoxide. However, added catalase did not affect the results reported here, so any aconitase inactivation by H_2O_2 was by matrix H_2O_2 (derived from matrix superoxide) and not by H_2O_2 produced outside the mitochondria and diffusing in.

Given that the reactions between semiquinone and oxygen at complex III and glycerol phosphate dehydrogenase may occur within the membrane bilayer, how can superoxide reach both the inner and the outer surface of the membrane? Some mechanistic speculations have been proposed for how superoxide might move within the membrane. Neutral $HO_2^{\bullet}$ may be formed at centre *o* and then dissociate to superoxide (and H^+) when it contacts the aqueous phase [46], or neutral semiquinone may escape from centre *o* to reach either side of the membrane and react with oxygen to form superoxide [31]. By analogy, these explanations could also be applied to glycerol phosphate dehydrogenase if the reaction site(s) are in the hydrophobic phase of the membrane.

To summarise, we have shown that *sn*-glycerol 3-phosphate dehydrogenase and centre *o* of complex III in *Drosophila* mitochondria produce superoxide at significant rates on the matrix side, as well as on the cytosolic side of the inner membrane. Glycerol phosphate dehydrogenase generates superoxide to both sides of the membrane at roughly equal rates, while centre *o* of complex III (in the presence of antimycin) generates about 70% of the total on the matrix side. Thus, in the electron transport chain as a whole, more superoxide appears to be released on the matrix side (particularly from complex I, complex III and glycerol phosphate dehydrogenase) than on the cytosolic side of the inner membrane.

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