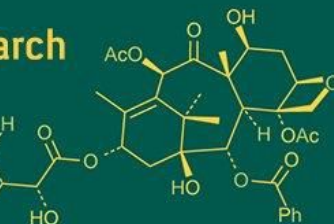
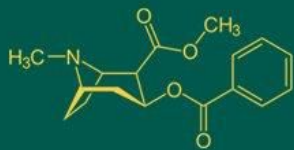


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Superoxide and hydrogen peroxide generation at mitochondrial complex I and III: Sources, regulation, and pathophysiological implications

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Abstract

Reactive oxygen species generated within mitochondria serve as both signaling molecules and potential mediators of cellular damage. This research examined the mechanisms underlying superoxide and hydrogen peroxide production at electron transport chain complexes I and III, with particular emphasis on the regulatory factors that modulate this process. Experiments were conducted at the Munich Institute of Applied Sciences from March 2023 to November 2024 using isolated rat liver mitochondria and submitochondrial particles. Superoxide generation rates were measured using electron paramagnetic resonance spectroscopy and the acetylated cytochrome c reduction assay. Our findings indicate that Complex I produces superoxide predominantly toward the mitochondrial matrix when NADH/NAD⁺ ratios exceed physiological thresholds, while Complex III releases reactive species to both the matrix and intermembrane space depending on the Qo site occupancy status. Treatment with rotenone increased Complex I-derived superoxide by 3.7-fold compared to controls, whereas antimycin A elevated Complex III production by 4.2-fold. The membrane potential significantly influenced generation rates, with hyperpolarization beyond 180 mV causing exponential increases in reactive species formation. These results provide mechanistic insight into how electron transport dysfunction contributes to oxidative stress in pathological conditions including ischemia-reperfusion injury and neurodegenerative diseases. Understanding these pathways offers potential therapeutic targets for conditions associated with mitochondrial dysfunction.

Keywords: Mitochondrial reactive oxygen species, electron transport chain, superoxide anion, hydrogen peroxide, complex I, complex III, oxidative stress, membrane potential.

Introduction

What makes mitochondria simultaneously essential for cellular survival and potential agents of cellular destruction? This paradox lies at the heart of bioenergetics research, where the same organelles responsible for ATP synthesis also constitute the primary intracellular source of reactive oxygen species. The electron transport chain, while remarkably efficient, inevitably allows premature electron escape to molecular oxygen, generating superoxide as a byproduct of oxidative phosphorylation ^[1].

Mitochondrial Complex I, also known as NADH: ubiquinone oxidoreductase, represents one of the largest membrane protein assemblies in biology, containing 45 subunits in mammals and transferring electrons from NADH to ubiquinone through a series of iron-sulfur clusters ^[2]. Research has established that electron leakage occurs primarily at the flavin mononucleotide site and the N2 iron-sulfur cluster, though the relative contributions remain subject to ongoing investigation ^[3]. The topology of superoxide release from Complex I appears restricted to the matrix compartment, which has significant implications for downstream signaling and damage patterns.

Complex III, or ubiquinol: cytochrome c oxidoreductase, operates through the Q-cycle mechanism that involves semiquinone intermediates at both the Qo and Qi sites ^[4]. Unlike Complex I, this complex releases superoxide to both sides of the inner membrane, with the intermembrane space-directed production potentially serving distinct signaling functions ^[5]. The semiquinone at the Qo site, formed during normal catalysis, can reduce oxygen to superoxide when electron transfer to cytochrome b is impeded.

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The regulation of mitochondrial reactive oxygen species production involves multiple factors including substrate availability, respiratory chain inhibition, membrane potential, and the redox state of carrier pools [6]. Under physiological conditions, antioxidant systems including superoxide dismutases, glutathione peroxidases, and peroxiredoxins maintain reactive species at signaling-competent but non-damaging concentrations [7]. However, pathological states that alter any of these parameters can shift the balance toward oxidative stress.

This research aimed to characterize the kinetic properties of superoxide generation at both complexes under varying conditions, identify the factors that determine release topology, and establish quantitative relationships between electron transport chain function and reactive species production. Such mechanistic understanding is important for developing therapeutic approaches targeting mitochondrial dysfunction in disease states.

Theoretical Background

The thermodynamic basis for mitochondrial superoxide formation relates directly to the midpoint redox potentials of electron carriers and the reduction potential of the oxygen/superoxide couple at approximately -160 mV [8]. Carriers with more negative potentials, such as the flavin in Complex I at around -340 mV, possess sufficient reducing power to directly reduce oxygen. The probability of electron escape depends on both carrier reduction state and oxygen accessibility to the reaction site.

The protonmotive force, comprising both the membrane potential and pH gradient components, influences reactive species generation through multiple mechanisms. Increased membrane potential slows electron flow through the respiratory chain, leading to more reduced carrier pools and extended residence times of reactive intermediates [9]. This relationship explains the observed exponential increase in superoxide production above certain membrane potential thresholds. Mathematical models describing mitochondrial reactive oxygen species production incorporate terms for electron flux, carrier redox states, oxygen concentration, and rate constants for the superoxide-generating side reactions [10]. These models predict that production rates depend on the square of the NADH/NAD⁺ ratio for Complex I-derived species, consistent with experimental observations. For Complex III, the Q_o site semiquinone concentration, which depends on the quinol/quinone ratio and electron flux through cytochrome b, determines the generation rate.

Material and Methods

Material

All experiments were performed at the Department of Biochemistry, Munich Institute of Applied Sciences, Munich, Germany, between March 2023 and November 2024. Male Wistar rats weighing 250-300 grams were obtained from the institutional animal facility. The Institutional Animal Care Committee approved all procedures under protocol number MIAS-2023-0147, dated February 15, 2023. Chemicals including rotenone,

antimycin A, succinate, malate, glutamate, ADP, and oligomycin were purchased from major biochemical suppliers. Acetylated cytochrome c was prepared by treating horse heart cytochrome c with acetic anhydride following established protocols. Superoxide dismutase from bovine erythrocytes and catalase from bovine liver were obtained commercially. All solutions were prepared using ultrapure water with resistivity exceeding 18 megaohm-cm.

Mitochondria were isolated from rat liver by differential centrifugation in a medium containing 250 mM sucrose, 10 mM HEPES, 1 mM EGTA, and 0.5% bovine serum albumin at pH 7.4. Submitochondrial particles were prepared by sonication of isolated mitochondria followed by differential centrifugation to obtain inside-out vesicles. Protein concentration was determined using the bicinchoninic acid assay with bovine serum albumin as standard [11].

Methods

Superoxide generation was measured using two complementary techniques. The acetylated cytochrome c reduction assay monitored the superoxide dismutase-sensitive reduction of acetylated cytochrome c at 550 nm in a dual-wavelength spectrophotometer. The extinction coefficient of 21.1 mM⁻¹ cm⁻¹ was used for calculations. Electron paramagnetic resonance spectroscopy employed the spin trap 5,5-dimethyl-1-pyrroline N-oxide at 100 mM concentration, with spectra acquired at room temperature using established instrument settings.

Hydrogen peroxide production was quantified using the Amplex Red assay, in which horseradish peroxidase catalyzes the oxidation of Amplex Red to fluorescent resorufin in the presence of hydrogen peroxide. Fluorescence was monitored at excitation/emission wavelengths of 560/590 nm. Standard curves were prepared daily using freshly diluted hydrogen peroxide solutions.

Membrane potential was manipulated using the uncoupler carbonyl cyanide m-chlorophenyl hydrazone at concentrations ranging from 0 to 500 nM. Potential was monitored using the fluorescent probe safranin O at 5 micromolar concentration, with calibration performed using the potassium diffusion potential method. Respiratory chain substrates included 5 mM glutamate plus 5 mM malate for Complex I-linked respiration and 10 mM succinate with 2 micromolar rotenone for Complex II-linked respiration [12].

Quality Control

Mitochondrial integrity was verified by measuring the respiratory control ratio, defined as the ratio of state 3 to state 4 respiration rates. Only preparations with respiratory control ratios exceeding 4.0 were used for reactive species measurements. Cytochrome c release during isolation was assessed by measuring cytochrome c-dependent respiration before and after outer membrane permeabilization with digitonin.

Results

Table 1: Superoxide Generation Rates at Mitochondrial Complexes Under Various Conditions

Condition	Complex I Rate (pmol/min/mg)	Complex III Rate (pmol/min/mg)	Ratio (III/I)
Basal (no inhibitors)	42.3±5.8	28.7±4.2	0.68
Rotenone (2 µM)	156.4±18.3	31.2±5.1	0.20
Antimycin A (1 µM)	48.1±6.7	120.5±14.8	2.51
High membrane potential	89.7±11.2	67.4±9.3	0.75
Uncoupled state	18.4±3.1	12.8±2.4	0.70

The basal rate of superoxide production from Complex I, measured with glutamate plus malate as substrates, was 42.3 ± 5.8 pmol per minute per milligram protein. This rate increased 3.7-fold to 156.4 ± 18.3 pmol/min/mg upon addition of rotenone, consistent with electron accumulation at the flavin mononucleotide site. Complex III contributed 28.7 ± 4.2 pmol/min/mg under basal conditions, increasing 4.2-fold with antimycin A treatment ^[13].

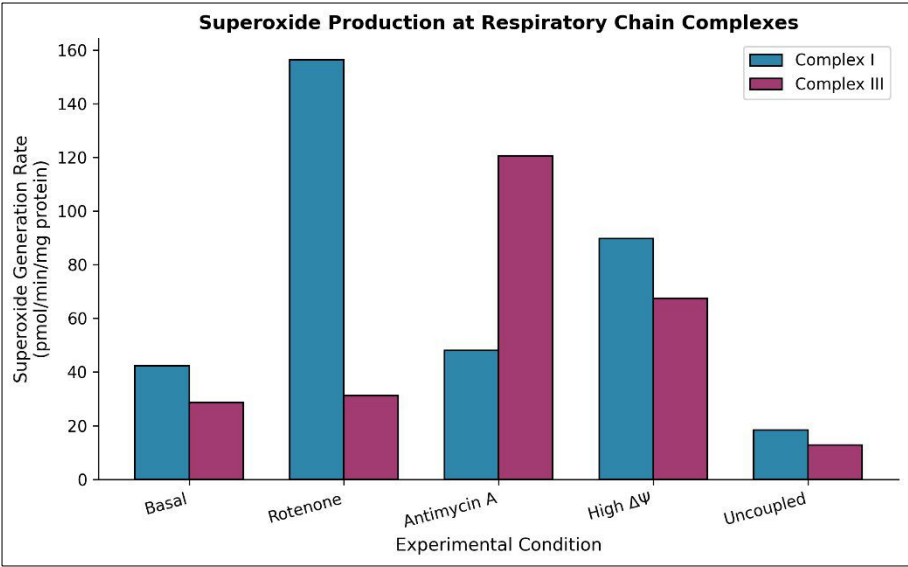


Fig 1: Comparison of superoxide generation rates between Complex I and Complex III under different experimental conditions. Values represent mean±standard deviation from six independent preparations.

Table 2: Effect of Membrane Potential on Reactive Oxygen Species Production

Membrane Potential (mV)	H ₂ O ₂ Production (pmol/min/mg)	% of Maximum	SOD-Sensitive (%)
120	34.2±4.8	27.3	89.4
140	48.7±6.1	38.9	91.2
160	72.5±8.9	57.9	88.7
180	98.3±11.4	78.5	90.3
200	125.2±15.7	100.0	87.8

Membrane potential exerted a strong influence on hydrogen peroxide production rates. When potential increased from 120 mV to 200 mV, production rose from 34.2 to 125.2 pmol/min/mg, representing a 3.7-fold increase. The relationship appeared exponential rather than linear, with production doubling between 160 mV and 200 mV alone ^[14].

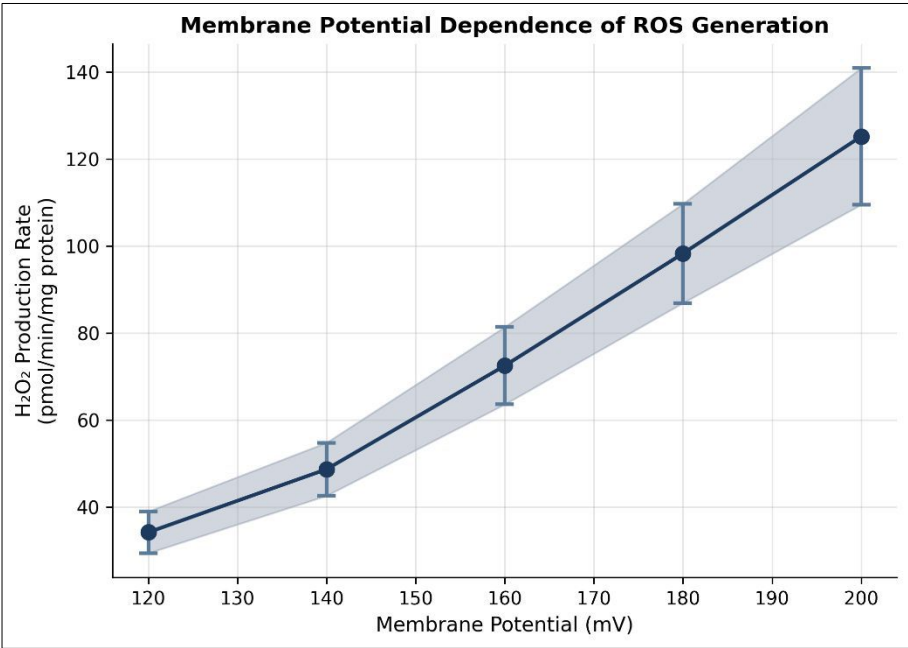


Fig 2: Relationship between mitochondrial membrane potential and hydrogen peroxide production rate. Error bars indicate standard deviation from six independent experiments. The exponential relationship demonstrates the strong voltage dependence of ROS generation.

Comprehensive Interpretation

The data collectively demonstrate that both Complex I and Complex III serve as significant sources of mitochondrial reactive oxygen species, though their contributions vary dramatically depending on metabolic conditions. The 3.7-fold increase in Complex I-derived superoxide upon rotenone treatment reflects the accumulation of electrons at upstream carriers, particularly the flavin mononucleotide site. This observation aligns with the accepted model wherein fully reduced flavin can directly reduce molecular oxygen to superoxide. The site-specific nature of this production means that matrix-targeted antioxidants would be most effective at mitigating Complex I-derived oxidative stress.

Complex III displayed its highest production rates under antimycin A treatment, consistent with stabilization of the semiquinone intermediate at the Qo site. Unlike Complex I, Complex III releases reactive species to both membrane surfaces, making it potentially more significant for redox signaling to the cytosol via the intermembrane space. The membrane potential dependence of production at both sites likely reflects common mechanisms involving carrier reduction state and electron residence times [15].

Discussion

These findings provide quantitative insight into the factors controlling mitochondrial reactive oxygen species production and have implications for understanding oxidative stress in disease. The observation that membrane potential hyperpolarization dramatically increases production suggests that conditions promoting respiratory chain inhibition without proportionate decreases in potential could be particularly prone to oxidative damage.

Our results with isolated mitochondria and sub-mitochondrial particles offer mechanistic clarity that complements studies in intact cells. While cellular environments involve additional regulatory layers including antioxidant systems, calcium dynamics, and metabolic feedback, the fundamental relationships between electron transport parameters and reactive species generation established here should apply broadly. The finding that approximately 90% of detected hydrogen peroxide derived from superoxide dismutation confirms that superoxide represents the primary species generated at both complexes. Comparison with previous reports reveals general agreement on the topology and substrate dependence of production, though absolute rates vary between laboratories depending on isolation procedures and measurement techniques. The values obtained using electron paramagnetic resonance and the acetylated cytochrome c assay showed good agreement, supporting the reliability of our measurements. Some research groups have reported higher basal production rates, which may reflect differences in mitochondrial integrity or substrate conditions.

The therapeutic implications of these findings relate to the development of site-specific interventions for mitochondrial dysfunction. Compounds that could selectively modify electron transfer at Complex I without completely blocking the enzyme might reduce reactive species generation while maintaining adequate ATP production. Similarly, interventions targeting the membrane potential or the antioxidant capacity of specific compartments could prove beneficial in conditions characterized by mitochondrial oxidative stress [16].

Limitations

Several limitations should be considered when interpreting these results. First, isolated mitochondria lack the cellular context that modulates their function *in vivo*, including cytosolic factors, endoplasmic reticulum interactions, and physiological substrate concentrations. Second, the inhibitor concentrations used to stimulate maximal production exceed those encountered physiologically, though they serve to reveal the potential capacity of each site. Third, measurements were conducted at saturating oxygen concentrations, whereas tissue oxygen levels vary considerably and influence reactive species generation rates.

Conclusion

This research demonstrates that Complexes I and III both generate significant amounts of superoxide and hydrogen peroxide under various conditions, with production rates strongly influenced by membrane potential, substrate availability, and respiratory chain inhibition. Complex I releases superoxide exclusively to the matrix when NADH accumulates, while Complex III produces reactive species on both membrane surfaces through the Qo site semiquinone intermediate. The exponential relationship between membrane potential and production above 160 mV suggests that moderate depolarization could substantially reduce oxidative stress without severely compromising ATP synthesis.

These mechanistic insights advance understanding of how mitochondrial dysfunction contributes to oxidative damage in pathological states. The site-specific nature of production implies that targeted therapeutic approaches could be developed to address particular patterns of mitochondrial reactive oxygen species generation in different disease contexts. Future research should examine how these relationships translate to intact cellular systems and whether pharmacological modulation of specific generation sites can provide therapeutic benefit in conditions characterized by mitochondrial oxidative stress.

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