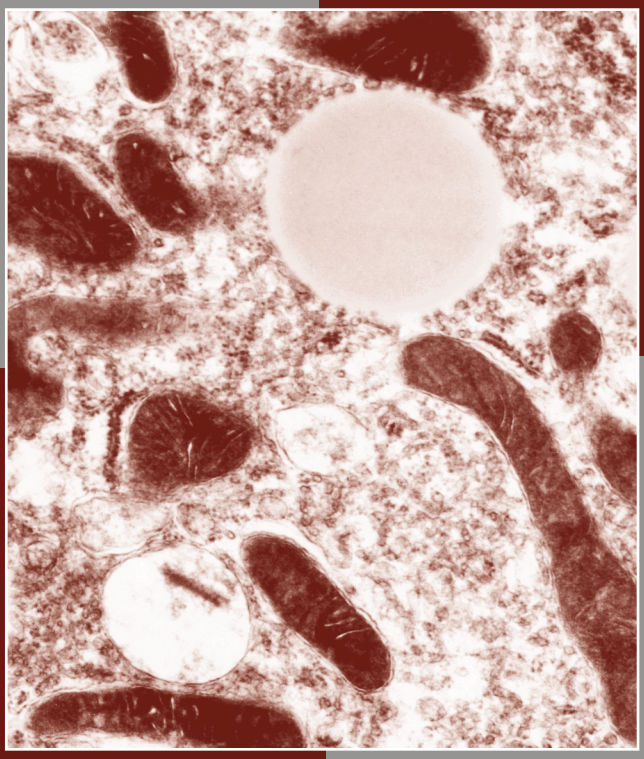
MITOCHONDRIA IN LIVER DISEASE



EDITED BY

DERICK HAN
NEIL KAPLOWITZ

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MITOCHONDRIA IN LIVER DISEASE

OXIDATIVE STRESS AND DISEASE

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MITOCHONDRIA IN LIVER DISEASE

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Series Preface

Oxidative stress is an underlying factor in health and disease. In this series, the significance of oxidative stress and disease associated with organ systems is highlighted by exploring the scientific evidence and the clinical applications of this knowledge. This series is intended for clinicians and researchers in the basic biomedical sciences. The potential of such knowledge on healthy aging and disease prevention warrants further knowledge about how oxidants and antioxidants modulate cell and tissue function, a theme explored in this book edited by Derick Han and Neil Kaplowitz.

The first section of the book, "Overview of Mitochondria," consisting of seven chapters, offers an excellent overview of mitochondria metabolism and their function in energy-dependent redox reactions, as well as the role of superoxide, hydrogen peroxide, and hydrogen sulfide in signal transduction and transcription and mitochondrial biogenesis. The second section of the book, "The Role of Mitochondria in Liver Diseases," focuses on, among other things, the role of mitophagy, epigenetic mitochondrial DNA modifications, mitochondrial dynamics, and sirtuins in several liver diseases, such as nonalcoholic fatty liver, viral hepatitis, diabetes, and ceramide and drug toxicity.

Derick Han and Neil Kaplowitz are congratulated for producing this excellent, well-organized, and timely book in the expanding field of mitochondrial metabolism and, especially, how liver energy metabolism—centered on diverse aspects of mitochondrial function—is affected by drug toxicity and disease states.

Lester Packer
Enrique Cadenas

Preface

The liver is a vital organ that is responsible for a wide range of functions, most of which are essential for survival. The multitude of functions the liver performs makes it vulnerable to a wide range of diseases. The liver is also responsible for metabolism and clearance of xenobiotic compounds and, thus, is susceptible to injury caused by drugs, alcohol, and herbal compounds. The liver is a major site for fatty acid synthesis, thus, is susceptible to developing fatty liver disease. In most of these diseases, mitochondria appear to play a central role. *Mitochondria in Liver Disease* gathers the most current knowledge regarding the role of mitochondria in liver diseases. This book is divided into two sections. The first section highlights the latest exciting developments in mitochondrial research. Cutting-edge topics such as the regulation of mitochondrial respiration by hydrogen sulfide and mitochondrial biogenesis are covered. In the second section, the most current research regarding the role of mitochondria on a wide range of liver diseases are reviewed. This book, written by experts in the field, provides a comprehensive overview of the latest research on mitochondria and its central role in mediating liver diseases.

Editors

Derick Han, PhD, is assistant professor in the Department of Biopharmaceutical Sciences at the School of Pharmacy, Keck Graduate Institute (Claremont, California), and a member of The Claremont Colleges. Dr. Han earned his PhD in molecular pharmacology and toxicology from the School of Pharmacy at the University of Southern California and a BA in sociology and biochemistry from the University of California, Berkeley. His research is focused on mitochondrial remodeling in the liver caused by metabolic stress, such as alcohol. He is also interested in mitochondrial reprogramming that occurs in cancer cells. He has published more than 70 research publications, reviews, and book chapters.

Neil Kaplowitz, MD, is director of the University of Southern California–National Institute of Diabetes and Digestive and Kidney Diseases (USC NIDDK)–sponsored Research Center for Liver Diseases (Los Angeles, California). He holds two endowed chairs, the Brem Professor of Medicine and the Budnick Chair in Liver Diseases, and is chief of the Division of Gastrointestinal and Liver Diseases. He is also professor of physiology and biophysics and pharmacology and pharmaceutical sciences at the Keck School of Medicine at the University of Southern California.

Dr. Kaplowitz has received a number of important honors and distinctions, including election to membership in the American Society for Clinical Investigation and the Association of American Physicians. He is the recipient of the Western Gastroenterology Research Prize, the William S. Middleton Award, the Solomon A. Berson Medical Alumni Achievement Award in Clinical Science from his alma mater, the Merit Award from the National Institutes of Health, the Mayo Soley Award from the Western Society for Clinical Investigation, the American Association for the Study of Liver Diseases (AASLD) Distinguished Achievement Award, and the American Liver Foundation (ALF) Distinguished Scientific Achievement Award. He has served as the president of the American Association for the Study of Liver Diseases and as vice chair for research at the American Liver Foundation. He has also served as associate editor of leading medical and scientific journals such as *Hepatology*, *Gastroenterology*, and the *American Journal of Physiology*.

In recent years, he has focused on the role of signal transduction, endoplasmic reticulum, and mitochondrial stress in the pathogenesis of liver injury. He has published more than 195 peer-reviewed scientific articles and 150 scholarly reviews and has edited 10 books related to liver diseases.

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Abbreviations

ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
AOAA	Aminooxyacetic acid
APAP	Acetaminophen
AST	Aspartate aminotransferase
AVG	Aminoethoxyvinylglycine
Bag4	Bcl2-associated athanogene 4
BCA	β-Cyano-L-alanine
Bcl-2	B-cell lymphoma 2
Bcl2L1	Bcl-2 like 1
BDL	Bile duct ligation
BH3	Bcl-2 homology 3 domain 3
BNIP3	Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3
CAT	Cysteine aminotransferase
CBS	Cystathionine β-synthase
CCCP	*m*-Chloro phenyl hydrazine
CI	Complex I
CII	Complex II
CIII	Complex III
CIV	Complex IV
CK2	Casein-kinase II
Clec16a	C-type lectin domain family 16, member A
CLP	Cecal ligation and puncture
CO	Carbon monoxide
CPTI	Carnitine palmitoyltransferase 1
CREB	cAMP-response element binding
CSE	Cystathionine γ-lyase
CV	Complex V
Cyp	Cytochrome p450
DAMP	Damage-associated molecular pattern
DAO	D-Amino acid oxidase
DNMTs	DNA methyltransferases
Dr4/5	Death receptors 4 and 5
Drp1	Dynamin-related protein 1
ETHE1	Persulfide dioxygenase
FAO	Fatty acid oxidation
FasL	Fas ligand
Fis1	Mitochondrial fission 1
FUNDC1	FUN14 domain-containing protein 1
GSH	Glutathione
GSK	Glycogen synthase kinase
GST	Glutathione *S*-transferase

HA	Hydroxylamine
H_2S	Hydrogen sulfide
HFD	High fat diet
Hif-1	Hypoxia-inducing factor 1
HMGB1	High mobility group box 1
HRE	Hif-1 responsive element
HSPA1L	Heat shock 70 kDa protein 1-like
HSPB1	Heat shock protein β-1
IR	Insulin resistance
IRS	Insulin receptor substrate
LC3	Microtubule-associated protein1 light-chain 3
LIR	LC3-interacting region
LPS	Bacterial lipopolysaccharide
MCD	Methionine-choline-deficient diet
Mff	Mitochondria fission factor
MFN1/2	Mitofusin 1 and 2
MFV	Mitochondria-derived vesicles
MPT	Membrane permeability transition
MPTP	Mitochondrial permeability transition pore
MST	3-Mercaptopyruvate sulfur transferase
mtDNA	Mitochondrial DNA
NAFLD	Nonalcoholic fatty liver disease
NAPQI	*N*-acetyl-*p*-benzoquinone imine
NASH	Nonalcoholic steatohepatitis
NMR	Nuclear magnetic resonance
NO	Nitric oxide
Nrdp1	Neuregulin receptor degradation protein 1
Nrf1/2	Nuclear respiratory factors 1 and 2
OPA1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
PAAT	Periaortic adipose tissue
PAG	DL-Propargylglycine
PARL	Presenilin-associated rhomboid-like
PARP	Poly (ADP-ribose) polymerase
PDEs	Phosphodiesterases
PGAM5	Phosphoglycerate mutase family member 5
PGC-1α	Peroxisome proliferator-activated receptor γ, coactivator 1 α
PINK1	PTEN-induced putative kinase 1α
PKA	cAMP-dependent protein kinase/protein kinase A
PPARGC1A	Peroxisome proliferator-activated γ coactivator-1α
RC	Respiratory chain
ROS	Reactive oxygen species
SIRT1	Sirtuin 1
Smurf1	Smad-specific E3 ubiquitin protein ligase 1
S-OPA1 and L-OPA1	Short and long form OPA1

SQR	Sulfide: quinone oxidoreductase
SQSTM1	Sequestosome 1
STS	Sodium thiosulfate
T1D	Type 1 diabetes
T2DM	Type 2 diabetes mellitus
tBid	Truncated bid
TCA	Tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor A
TNF-R1	TNF-α receptor 1
TNF-α	Tumor necrosis factor-α
TOMM7	Translocase of outer mitochondrial membrane 7
TOMM20	Translocase of outer mitochondrial membrane 20
TPP^+	Triphenylphosphonium
TRAIL	TNF-related apoptosis-inducing ligand
UBA	Ubiquitin-associated
UCP	Uncoupling protein
ULK1	Unc-51 like autophagy activating kinase 1
Usp30	Ubiquitin-specific peptidase 30
VDAC	Voltage-dependent anion channel

Section I

Overview of Mitochondria

1 Metabolism of Superoxide Radicals and Hydrogen Peroxide in Mitochondria

H. Susana Marinho and Fernando Antunes

CONTENTS

ABSTRACT

We review the mechanisms of superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) formation in and removal from mitochondria. The formation of $O_2^{\cdot-}$/H_2O_2 in the mitochondrial inner membrane occurs through three respiratory chain complexes: I, II, and III—glycerol 3-phosphate dehydrogenase, the electron-transferring flavoprotein/electron-transferring flavoprotein ubiquinone oxidoreductase system, and dihydroorotate dehydrogenase. In the mitochondrial outer membrane, monoamine oxidase and cytochrome b_5 reductase also form $O_2^{\cdot-}$/H_2O_2. Recently described is H_2O_2-forming NADPH oxidase isoform (NOX4) whose mitochondrial topology is uncertain. Finally, we briefly mention enzymatic systems present in the mitochondrial matrix that produce $O_2^{\cdot-}$/H_2O_2. Concerning H_2O_2 removal, we compare the relative contribution of peroxiredoxins (Prx3 and Prx5) and glutathione peroxidases (Gpx1 and Gpx4) to the removal of H_2O_2. Calculations show that either glutathione peroxidases or peroxiredoxins may be the predominant enzymes depending on the rate of production of H_2O_2 and on the reduction state of thioredoxin, the electron donor for peroxiredoxins. At low H_2O_2 production rates, near 0.2 nmol/min/mg of protein, most H_2O_2 (estimated steady state near 1–2 nM) will be removed through Prx3 (nearly 90%). For 10-fold higher H_2O_2 production, both H_2O_2 removal and steady-state levels are strongly dependent on reduced thioredoxin concentrations: for 10 μM reduced thioredoxin, Prx3 reduces nearly 74% of the H_2O_2 formed, attaining a 40 nM steady state; for a 1 μM reduced thioredoxin, Prx3 reduces only 15% of the H_2O_2 formed, resulting in a 130 nM H_2O_2 steady state. Finally, the role of pyruvate as a metabolic sink for H_2O_2 is analyzed.

INTRODUCTION

The physiological production of H_2O_2 by mitochondrial membranes was first observed in 1966 by Jensen [1] who found that the antimycin-insensitive oxidation of reduced nicotinamide adenine dinucleotide (NADH) and succinate by bovine heart submitochondrial particles was coupled with the formation of H_2O_2. Soon after, Loschen et al. [2] demonstrated, for the first time, that isolated mitochondria produce H_2O_2 at rates mainly dependent on the metabolic state. Further studies made by Britton Chance's group [3] established that H_2O_2 production in intact mitochondria from rat liver or pigeon heart (about 0.3–0.6 nmol H_2O_2/min mg^{-1} protein) is maximal in state 4 [2–4], that is, when adenosine diphosphate (ADP) is depleted and the redox components of the respiratory chain are reduced, accounting for about 2% of the total oxygen utilization under these conditions [5]. Later, it was shown that this H_2O_2 arose from the dismutation of $O_2^{\cdot-}$ generated within the mitochondria [6,7]. In this review, we describe the enzymatic systems that mediate the production of $O_2^{\cdot-}$ and H_2O_2 as well as the antioxidant systems responsible for their removal.

GENERATION OF $O_2^{\cdot-}$ AND H_2O_2 IN MITOCHONDRIA

Two of the respiratory electron transport complexes, complex I and complex III, have been frequently cited as being the most important sites of $O_2^{\cdot-}$ formation in the mitochondria [8,9]. However, for mammalian cells, the use of inhibitors to both

manipulate the redox states of particular $O_2^{\cdot-}/H_2O_2$-producing sites and prevent $O_2^{\cdot-}/H_2O_2$ formation from others allowed the identification of at least 12 enzymes involved in $O_2^{\cdot-}/H_2O_2$ production in the mitochondria at measurable rates (reviewed in [8–13]) (Figure 1.1). All these enzymes are present ubiquitously in mammalian mitochondria, but their capacity in producing $O_2^{\cdot-}/H_2O_2$, as well as their expression, varies greatly among tissues and species [10,11,14] (Figure 1.2). Moreover, the production of $O_2^{\cdot-}/H_2O_2$ in isolated mitochondria is strongly dependent on the substrates being oxidized and on the metabolic state [14–19] (Figure 1.3). The overall rates of $O_2^{\cdot-}/H_2O_2$ production can differ by an order of magnitude between substrates and the relative contribution of each site can also be very different with different substrates [19].

Generation of $O_2^{\cdot-}$ and H_2O_2 by Mitochondrial Inner Membrane Enzymes

The electron transport chain has been extensively studied regarding the formation of $O_2^{\cdot-}/H_2O_2$ (reviewed in [8,9,28,29]). With the exception of complex IV that is able to bind tightly partially reduced intermediates during oxygen reduction without significant release of reactive oxygen species [9,28], respiratory complexes form $O_2^{\cdot-}/H_2O_2$. Studies using isolated mitochondria from skeletal muscle showed that complex III (at site Q_o) has the greatest capacity to form $O_2^{\cdot-}/H_2O_2$ followed by complex I (at the ubiquinone-binding site) and complex II (at the flavin) [21–23,30] (Figure 1.1). In the mitochondrial inner membrane, $O_2^{\cdot-}/H_2O_2$ can also be formed by enzymes involved in electron transfer to the electron transport chain such as the electron-transferring flavoprotein (ETF) and electron-transferring flavoprotein ubiquinone oxidoreductase (ETFQOR) system or glycerol 3-phosphate dehydrogenase and also by dihydroorotate dehydrogenase (DHODH).

Complex I

Complex I (NADH–ubiquinone oxidoreductase, EC 1.6.5.3) catalyzes the first step in the respiratory electron transport chain in the mitochondria, the reduction of ubiquinone to ubiquinol by NADH. The free energy from this redox reaction is used to translocate four protons across the mitochondrial inner membrane, contributing to the protonmotive force (Δp) that is used to drive adenosine triphosphate (ATP) synthesis [31]:

$$NADH + H^+ + Q + 4H^+_{in} \rightarrow NAD^+ + QH_2 + 4H^+_{out} \quad (1.1)$$

In complex I from bovine heart mitochondria, which is a close model for the human enzyme, 45 different subunits have been identified [32]. Complex I contains at least 10 redox components, namely, flavin mononucleotide (FMN), 8 iron–sulfur clusters, and bound ubiquinone, all of which are present in the hydrophilic part of the complex exposed to the matrix [31].

Formation of $O_2^{\cdot-}$ by complex I has been shown to occur using the isolated complex [33,34], submitochondrial particles [35–37], and intact mitochondria isolated from different sources [14,15,38].

Complex I has a major role in $O_2^{\cdot-}/H_2O_2$ formation in the mitochondria. It has been estimated that complex I may account for about half of the total NADH-supported H_2O_2 formation in the mitochondria [39] and most of the $O_2^{\cdot-}$ produced by the respiratory chain complexes using succinate as a substrate [19].

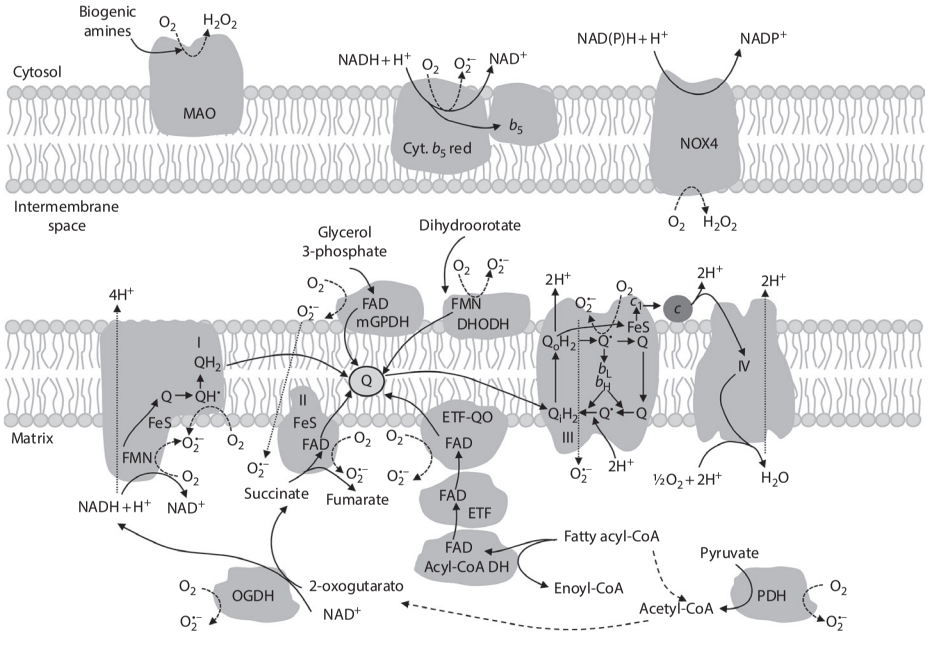

FIGURE 1.1 Enzymes involved in mitochondrial $O_2^{\cdot-}/H_2O_2$ formation in mammalian cells. Three respiratory chain complexes form $O_2^{\cdot-}/H_2O_2$. Complex I forms $O_2^{\cdot-}$ at the flavin site and ubiquinone-binding site releasing it into the matrix. Complex II forms $O_2^{\cdot-}$ into the matrix at its flavin site. Complex III forms $O_2^{\cdot-}$ at the ubiquinone Qo site releasing it into both the matrix and intermembrane space. Mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH) forms $O_2^{\cdot-}$ to both sides of the mitochondrial inner membrane. The ETF and ETFQOR are involved in the final steps of β-oxidation and ETFQOR is the main candidate for $O_2^{\cdot-}/H_2O_2$ formation. DHODH also produces $O_2^{\cdot-}$ during pyrimidine biosynthesis. NOX4 forms H_2O_2, but its mitochondrial topology and location is not known and it has been tentatively located in the mitochondrial outer membrane. In the mitochondrial outer membrane, MAO forms H_2O_2 while cytochrome b_5 reductase (Cyt. b_5 Red) forms $O_2^{\cdot-}$, and both are released into the cytosol. In the matrix, the enzyme complexes OGDH, PDH, and BCOADH (not shown) contain the DLDH subunit that produces $O_2^{\cdot-}/H_2O_2$. *Note*: FMN, flavin mononucleotide; FAD, falvin adenine dinucleotide.

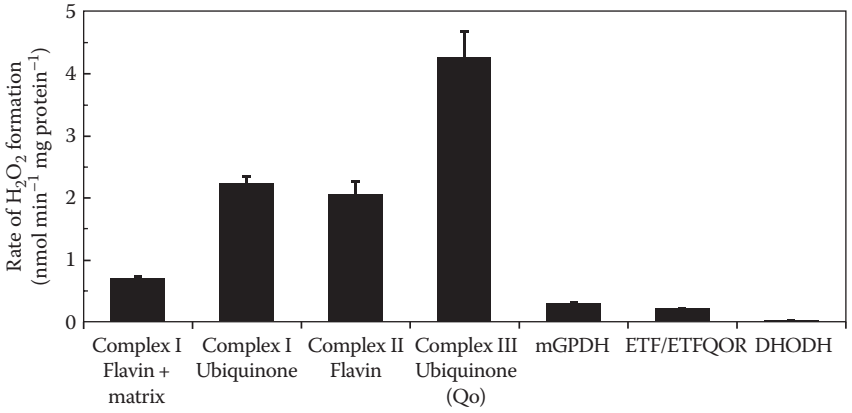

FIGURE 1.2 Maximum rates of $O_2^{\cdot-}/H_2O_2$ formation from different sites in isolated muscle mitochondria. Different combinations of substrates and inhibitors were used to obtain the maximum rates from each site and all rates were corrected for H_2O_2 consumption in the matrix through GSH peroxidases [20]. DHODH, dihydroorotate dehydrogenase; ETF/ETFQOR, electron-transferring flavoprotein and electron-transferring flavoprotein ubiquinone oxidoreductase; mGPDH, mitochondrial glycerol 3-phosphate dehydrogenase. Data are the mean ± SEM ($n \geq 3$) and rates were obtained for complex I from Treberg et al. [20,21], complex II from Quinlan et al. [22], complex III from Quinlan et al. [23], mGPDH from Orr et al. [24], DHODH from Hey-Mogensen et al. [25], and ETF/ETFQOR from Perevoshchikova et al. [26].

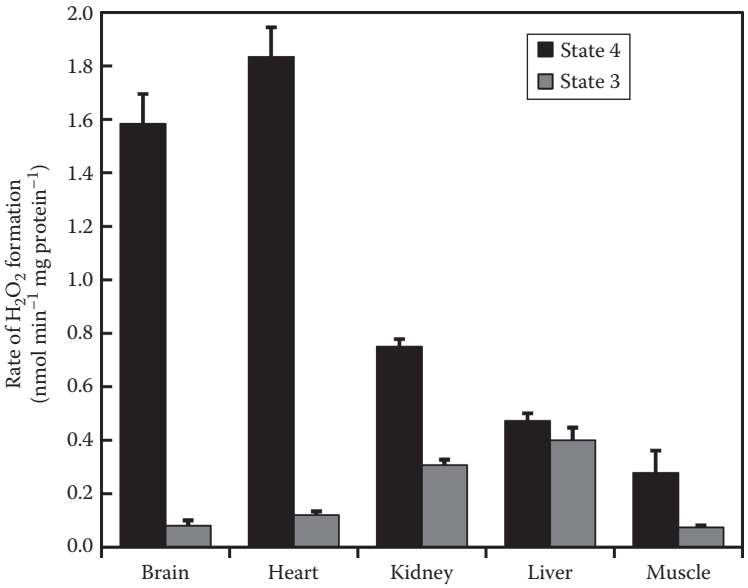

FIGURE 1.3 H_2O_2 formation in isolated mitochondria is tissue and state dependent. Data were obtained from Tahara et al. [27] and are the mean ± SEM ($n \geq 3$). Formation of H_2O_2 was measured in isolated mitochondria using 1 mM succinate in the presence of 1–6.3 μg of oligomycin (state 4) or 1 mM ADP (state 3).

Considering the complexity of complex I, it is not surprising that there is some debate regarding which of its redox components interacts with O_2 to generate $O_2^{\cdot-}$. Thus, the enzyme-bound $NAD^{\cdot}$ radical [35], FMN [40,41], the iron–sulfur clusters N-1a and N-2 [38,42], and ubisemiquinone [17,43] have all been suggested as being involved in $O_2^{\cdot-}$ production. Results supporting the presence of two separate sites of $O_2^{\cdot-}$ production (ubisemiquinone and FMN) were obtained by Treberg et al. [21] using rat skeletal muscle intact mitochondria. Recently, Grivennikova and Vinogradov [11] proposed that both the purified bovine heart complex I and also the membrane-bound complex I produce not just $O_2^{\cdot-}$ but also H_2O_2 at two different sites. According to Grivennikova and Vinogradov [11], $FMNH^{\cdot}$ produces H_2O_2 through a two-electron reduction, while an iron–sulfur center, probably N-2, produces $O_2^{\cdot-}$. Moreover, they proposed that H_2O_2 would be the predominant species formed by complex I at physiologically relevant concentrations of NADH and/or NAD^+. In all possible mechanisms proposed thus far, formation of $O_2^{\cdot-}/H_2O_2$ by complex I is directed to the matrix.

Complex II

Succinate dehydrogenase (complex II, succinate–ubiquinone oxidoreductase, EC 1.3.5.1) is an enzyme involved both in the tricarboxylic acid cycle and in the mitochondrial electron transport chain that catalyzes the oxidation of succinate to fumarate and the reduction of ubiquinone to ubiquinol:

$$\text{Succinate} + Q \rightarrow \text{fumarate} + QH_2 \tag{1.2}$$

Complex II has four subunits. Two of those subunits, the flavin adenine dinucleotide (FAD)-containing subunit SDHA and the SDHB subunit, which contains three iron–sulfur clusters, are hydrophilic and exposed to the mitochondrial matrix. The transmembrane proteins CybL (SDHC) and CybS (SDHD) are embedded in the inner mitochondrial membrane. They contain a heme *b* and the ubiquinone-binding site [44].

When compared to both complex I and complex III, complex II was shown to make little contribution to $O_2^{\cdot-}/H_2O_2$ formation by mammalian isolated mitochondria and submitochondrial particles under physiological conditions [14,15]. However, mutations in complex II can lead to higher rates of $O_2^{\cdot-}$ formation and are associated with pathological states [45].

Recently, high rates of $O_2^{\cdot-}$ production by complex II isolated mitochondria from rat skeletal muscle [22], under conditions that can occur in vivo in the resting muscle or during hypoxia, have been reported. Furthermore, for rat skeletal muscle isolated mitochondria using glycerol 3-phosphate as a substrate, a substantial portion of $O_2^{\cdot-}/H_2O_2$ formation, commonly attributed to mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH), originates from electron flow into complex II [19,24]. Also, recent data indicate that complex II is partially responsible for the succinate-dependent $O_2^{\cdot-}/H_2O_2$ formation in the presence of rotenone, which had been previously attributed to a reverse electron flow from complex II to complex I [46]. $O_2^{\cdot-}$ is formed at the enzyme flavin site [47].

Complex III

Complex III (ubiquinol–cytochrome c oxidoreductase, EC 1.10.2.2) catalyzes the reduction of cytochrome c by ubiquinol that is coupled to the transmembrane proton translocation and formation of Δp:

$$QH_2 + 2\ \text{ferricytochrome}\,c + 2H^+_{in} \rightarrow Q + 2\ \text{ferrocytochrome}\,c + 4H^+_{out} \qquad (1.3)$$

Complex III exists as a dimer with each monomer consisting of 11 polypeptides. The redox centers of complex I are cytochrome *b*, which contains two hemes, one with low potential (b_L) and another with a high potential (b_H), cytochrome c_1, the Rieske Fe–S protein, and the two ubiquinone centers. The ubiquinol reaction center (called Q_p or Q_o site) is located at the positive side of the membrane (intermembrane space), while the ubiquinone reduction center (Q_n or Q_i site) is located at the negative side of the membrane (matrix). Electron transfers within complex III occur according to the Q cycle proposed by Mitchell [48] starting with the oxidation of ubiquinol at site Q_o, through the donation of one electron to the Rieske Fe–S protein leading to ubisemiquinone formation, followed by the reduction of cytochrome b_L and formation of ubiquinone.

The rate of $O_2^{\cdot-}/H_2O_2$ formation by complex III is about 10% of that mediated by complex I for succinate-mediated respiration, but approximately equal to that of complex I for NADH-dependent respiration in isolated rat skeletal muscle mitochondria [19]. The use of inhibitors has led to the proposal that the site of $O_2^{\cdot-}$ production by complex III is the ubisemiquinone formed at site Q_o [49]. Antimycin A is known to increase the rate of $O_2^{\cdot-}$ formation by complex III [14,50,51]. Antimycin A blocks electron transfer from cytochrome b_L to the ubiquinone at Q_i, which leads to an increased concentration of ubisemiquinone at site Q_o [9], and this antimycin A–induced $O_2^{\cdot-}$ formation is abolished by the Q_o site inhibitors stigmatellin and myxothiazol [52,53].

Unlike complex I that releases $O_2^{\cdot-}$ exclusively to the matrix, studies using isolated mitochondria have shown that complex III releases $O_2^{\cdot-}$ to both sides of the membrane [14,54]. Since the inner membrane is nonpermeable to $O_2^{\cdot-}$ [55], to explain $O_2^{\cdot-}$ release into the matrix, Muller et al. [54] proposed two possible mechanisms. In the first mechanism, a neutral ubisemiquinone would diffuse out of Q_o, along a hydrophobic tunnel, and at the lipid/aqueous phase interface, the ubisemiquinone would deprotonate and react with oxygen to form aqueously solvated $O_2^{\cdot-}$. In the second mechanism, $O_2^{\cdot-}$ formed at Q_o can be protonated to form the hydroperoxyl radical that can diffuse along the membrane to be released in both the matrix and intermembrane space.

Mitochondrial Glycerol 3-Phosphate Dehydrogenase

mGPDH (EC 1.1.99.5) is an integral flavoprotein present in the outer leaflet of the mitochondrial inner membrane [56]. The enzyme is involved in lipid metabolism and in the glycerol phosphate shuttle that connects glycolysis with the mitochondrial respiratory chain. It catalyzes the oxidation of glycerol 3-phosphate to dihydroxyacetone phosphate and the reduction of ubiquinone to ubiquinol:

$$\alpha - \text{glycerophosphate} + Q \rightarrow \text{dihydroxyacetatone phosphate} + QH_2 \qquad (1.4)$$

The mitochondrial content of mGPDH has large variations among different mammalian tissues with the highest content found in brown adipose tissue, while almost negligible levels are present in tissues such as the heart, muscle, or liver [57].

Several studies have established that mGPDH, from both mammalian and insect mitochondria, forms $O_2^{\cdot-}/H_2O_2$ at levels comparable to those formed by complex III in the presence of antimycin A [24,58,59]. mGPDH produces mainly $O_2^{\cdot-}$ that is released approximately equally toward each side of the mitochondrial inner membrane. This suggests the Q-binding pocket of mGPDH as the main site of $O_2^{\cdot-}$ generation [24].

Electron-Transferring Flavoprotein and Electron-Transferring Flavoprotein Ubiquinone Oxidoreductase

During the 1970s, it was shown that the oxidation of palmitoyl carnitine by mitochondria leads to the generation of H_2O_2 [4]. Palmitoyl carnitine is metabolized by the β-oxidation pathway and electrons may enter the respiratory chain at two sites described as follows: complex I, from the NADH formed in the reaction catalyzed by 3-hydroxyacyl-CoA dehydrogenase, and the ubiquinone pool, from the ETF/ETFQOR system that acts as the electron acceptor from nine different mitochondrial FAD-containing acyl-CoA dehydrogenases of fatty acid β-oxidation [60]. Also, oxidation of the end product of β-oxidation, acetyl-CoA, in the tricarboxylic acid cycle leads to further electrons entering the respiratory chain through complex I and complex II [19,61]. Several studies, using isolated mitochondria, identified as sources of $O_2^{\cdot-}/H_2O_2$ during palmitoyl carnitine oxidation, complex I (site I_Q) [14,19,62], complex II [19], complex III [61,62], the ETF/ETFQOR (EC 1.5.5.1) system [14,27,61,62], and acyl-CoA dehydrogenases [27]. Although the ETF/ETF dehydrogenase system has been proposed as being a significant source of $O_2^{\cdot-}/H_2O_2$ into the matrix side of the membrane [14,27,61,62], it is possible that this may only occur either at high [O_2], due the high apparent K_M of ETFQOR for O_2 [62], or in the presence of respiratory chain inhibitors [26]. In fact, recent studies suggest that all $O_2^{\cdot-}/H_2O_2$ formed during oxidation of palmitoyl carnitine by mitochondria can be accounted for by complex I (site I_Q), complex II, and complex III [19,26].

Dihydroorotate Dehydrogenase

DHODH (EC 1.3.5.2) is ubiquitously distributed in mammalian tissues [63]. In upper eukaryotes, class 2 DHODH is an integral protein of the mitochondrial inner membrane with the dihydroorotate-binding site facing the intermembrane space and a hydrophobic tail inserted in the membrane [64]. The enzyme catalyzes the oxidation of dihydroorotate to orotate and the reduction of ubiquinone to ubiquinol during pyrimidine synthesis:

$$\text{(S)} - \text{dihydroorotate} + \text{ubiquinone} \rightleftarrows \text{orotate} + \text{ubiquinol} \qquad (1.5)$$

DHODHs have two redox-active sites: an FMN prosthetic group, which accepts two electrons from dihydroorotate, and a ubiquinone in the quinone-binding site, which accepts the electrons and subsequently joins the ubiquinone pool of the mitochondrial inner

membrane [65]. During the DHODH catalytic cycle, the flavin semiquinone intermediate $FMNH^{\cdot}$ is likely formed [66]. Also, the midpoint potential of the flavin in DHODH is sufficiently negative (–310 mV for the class 2 enzyme purified from *Escherichia coli*) to enable reduction of oxygen to generate $O_2^{\cdot-}$ and/or H_2O_2 by the enzyme [67] even though the activity of DHODH with quinone substrates is 14- to 58-fold higher than with O_2 [66]. In fact, in the absence of its physiological electron acceptor, reduced DHODH can produce H_2O_2 in vitro [63]. Studies made in the 1970s using isolated mitochondria found that DHODH also produced $O_2^{\cdot-}$ [68,69], but later on this $O_2^{\cdot-}$ formation was attributed to complex III [70]. Recently, it was shown that DHODH directly produces $O_2^{\cdot-}$ and/or H_2O_2 at low rates but is also capable of indirect production at higher rates from other sites through its ability to reduce the ubiquinone pool [25]. In mitochondria isolated from rat skeletal muscle and in the presence of inhibitors of complex I, complex III, and complex II, DHODH generates $O_2^{\cdot-}/H_2O_2$ at a rate of about 20–40 pmol H_2O_2 min^{-1} mg $protein^{-1}$, from the ubiquinone-binding site [24,25].

Generation of $O_2^{\cdot-}$ and H_2O_2 by Mitochondrial Outer Membrane Enzymes

There are two enzymes located in the mitochondrial outer membrane that may produce $O_2^{\cdot-}/H_2O_2$, cytochrome b_5 reductase, and monoamine oxidase (MAO).

Cytochrome b_5 Reductase

Cytochrome b_5 reductase (EC 1.6.2.2) is a flavoprotein widely distributed in mammalian tissues. It is an integral membrane protein present in the endoplasmic reticulum, plasma membrane, and also the mitochondrial outer membrane where it catalyzes the reduction of cytochrome b_5 by cytoplasmic NADH [71,72]:

$$\text{NADH} + 2\ \text{ferricytochrome}\ b_5 \rightleftarrows \text{NAD}^+ + \text{H}^+ + 2\ \text{ferrocytochrome}\ b_5 \quad (1.6)$$

However, the enzyme can also act as an NADH oxidase. In fact, rat brain mitochondrial cytochrome b_5 reductase was shown to produce $O_2^{\cdot-}$ with a high rate of ~300 nmol $O_2^{\cdot-}$ min^{-1} mg $protein^{-1}$ [73]. Recently, cytochrome b_5 reductase was purified from pig liver microsomes and shown to catalyze the NADH-dependent production of superoxide anion with a $V_{max} = 3.0 \pm 0.5$ μmol $O_2^{\cdot-}$ min^{-1}mg of purified cytochrome b_5 reductase and a K_M(NADH) = 2.8 ± 0.3 μM NADH [74].

Monoamine Oxidases

MAO-A and MAO-B (EC 1.4.3.4) are flavoproteins ubiquitously expressed in various mammalian tissues that catalyze the oxidative deamination of primary aromatic amines along with long-chain diamines and tertiary cyclic amines. The oxidation of biogenic amines is accompanied by the release of H_2O_2 [75,76]:

$$\text{RCH}_2\text{NHR}' + \text{H}_2\text{O} + \text{O}_2 \rightleftarrows \text{RCHO} + \text{R}'\text{NH}_2 + \text{H}_2\text{O}_2 \quad (1.7)$$

Tyramine oxidation (0.2 mM) by rat brain mitochondria produces H_2O_2 at a rate of 2.71 nmol min^{-1} mg $protein^{-1}$ [75], leading to a steady-state intramitochondrial H_2O_2 concentration of 0.8 μM [76], which is ~50-fold higher than that originating during succinate oxidation in the presence of antimycin A (0.016 μM) [77]. Therefore, in the brain, MAO-dependent H_2O_2 generation may far exceed that of other mitochondrial sources. However, it should be taken into account that dopamine, the physiological substrate, has a concentration of around 1 μM in the cytosol and leads to a 2.7 lower H_2O_2 formation rate by MAO than tyramine [78]. Under these physiological concentrations, MAO would be expected to form less H_2O_2 than the respiratory chain complexes. In other tissues, MAOs may also be a major source of H_2O_2 in the reperfusion following ischemia [79] and in aging [80].

NAD(P)H Oxidase

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4) is a member of the NADPH oxidase family of enzymes NOX1–5 and DUOX1–2. NOX4, which has been reported to be present in the nucleus and endoplasmic reticulum [81], was recently found to have a mitochondrial localization sequence [82] and to be expressed in the mitochondria of cardiomyocytes [83], mesangial cells of the kidney [84], and neurons [85]. The exact location and topology of NOX in the mitochondria is not known and it may be possible that it is located in the inner mitochondrial membrane and not in the outer mitochondrial membrane [86]. NOX4, unlike most NADPH oxidases that catalyze the reduction of O_2 to $O_2^{\cdot-}$, mainly catalyzes the formation of H_2O_2 [87]:

$$NADPH + O_2 + H^+ \rightarrow NADP^+ + H_2O_2 \quad (1.8)$$

Recently, it was shown that a significant increase in both the protein level and activity of mitochondrial complex I–containing supercomplexes I_1III_2 and $I_1III_2IV_{0-1}$ occurred in Nox4-depleted endothelial cells [88]. This may indicate that Nox4-derived H_2O_2 can either damage the supercomplexes or impair complex I assembly into supercomplexes, which have much higher enzymatic activity than individual complexes.

Generation of $O_2^{\cdot-}$ and H_2O_2 by Matrix Enzymes

Isolated mitochondrial 2-oxoglutarate dehydrogenase (OGDH) and pyruvate dehydrogenase (PDH) complexes produce $O_2^{\cdot-}/H_2O_2$ [89]. This formation of $O_2^{\cdot-}/H_2O_2$ has been associated with FAD-linked dihydrolipoamide dehydrogenase (DLDH, EC 1.8.1.4) [39], which catalyzes the reduction of NAD^+ by dihydrolipoamide to form NADH and lipoamide and is present in both these matrix enzyme complexes [89–92] and also in branched-chain 2-oxoacid dehydrogenase (BCOADH) [92]. In fact, DLDH-mediated $O_2^{\cdot-}/H_2O_2$ formation in rat brain mitochondria of heterozygous knockout mice deficient in DLDH ($DLDH^{+/-}$) was shown to be twofold lower than in control mice [89]. This importance of DLDH-mediated $O_2^{\cdot-}/H_2O_2$ formation was reinforced by recent results that showed that in skeletal muscle–isolated mitochondria oxidizing 2-oxoacids, these 2-oxoacid dehydrogenases can produce $O_2^{\cdot-}/H_2O_2$ at higher rates than complex I [92]. The OGDH complex has the greatest capacity followed by the PDH complex and the BCOADH complex.

REMOVAL OF $O_2^{\cdot-}$

The mitochondrial steady-state concentration of $O_2^{\cdot-}$ has been estimated to be in the range 0.08–0.2 nM while that of H_2O_2 is about 5 nM [76]. These low concentrations are the result of very efficient enzymatic antioxidant systems. In fact, most of the $O_2^{\cdot-}/H_2O_2$ produced in the mitochondria is eliminated through these enzymes, with only a minor fraction, estimated to be near 0.1% for $O_2^{\cdot-}$ and 0.001% for H_2O_2, being available to react with other molecules [93]. The superoxide radical main reaction is its dismutation catalyzed by superoxide dismutases (EC 1.15.1.1) present in the matrix (MnSOD) and in the intermembrane space (Cu,Zn-SOD) [94,95]:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \tag{1.8}$$

Other $O_2^{\cdot-}$ reactions may include reaction with iron–sulfur clusters in proteins [96] and with nitric oxide forming peroxynitrite (Equation 1.9) [97]. Also, in its protonated form, hydroperoxyl radical, it may initiate lipid peroxidation (Equation 1.10) through abstraction of an allylic hydrogen atom from an unsaturated fatty acid (RH) [93,98]:

$$O_2^{\bullet-} + {}^{\bullet}NO \rightarrow ONOO^- \tag{1.9}$$

$$HO_2^{\bullet} + RH \rightarrow H_2O_2 + R^{\bullet} \tag{1.10}$$

REMOVAL OF H_2O_2

In the case of H_2O_2, its main removal reaction is the reduction to H_2O catalyzed by glutathione peroxidases (EC 1.11.1.9) and peroxiredoxins (EC 1.11.1.15). Other reactions include the formation through Fenton chemistry of hydroxyl radical (Equation 1.11) [77], a very reactive species, or oxidation of thiols (Equation 1.12), a reaction that mediates a well-known regulatory role of H_2O_2 [99]:

$$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + HO^{\bullet} + H_2O \tag{1.11}$$

$$H_2O_2 + 2R-SH \rightarrow R-S-S-R + 2H_2O \tag{1.12}$$

Next, we describe the antioxidant systems responsible for H_2O_2 removal in the mitochondrial matrix. In liver mitochondria, initially H_2O_2 was assumed to be reduced to water via glutathione peroxidases, as the nonenzymatic reaction of H_2O_2 with thiols is much slower compared with the enzymatic reactions and catalase is absent in this organelle. After the discovery of peroxiredoxins, it became clear that a new player involved in H_2O_2 removal had to be taken into account. In addition to these two enzymatic systems, we will also discuss the role of the nonenzymatic reaction of H_2O_2 with pyruvate, a metabolite that is highly abundant in mitochondria.

Glutathione Peroxidases

Ultimately, both glutathione peroxidase and peroxiredoxin cycles convert H_2O_2 to water at the expense of NADPH, as described by

$$NADPH + H_2O_2 + H^+ \rightarrow 2H_2O + NADP^+ \quad (1.13)$$

The reducing power stored in NADPH, usually used for anabolic pathways, is diverted to detoxify the oxidant H_2O_2 to water. Next, we will describe in detail these two antioxidant systems.

There are two glutathione peroxidase isomers in the mitochondria, Gpx1, a tetramer, and Gpx4, a monomer; both are selenoproteins coded by two different nuclear genes [100]. Their reaction mechanism is similar, with H_2O_2 oxidizing the selenocysteine (–SeH) residue in the active center to a seleninic acid (–SeOH) (Equation 1.14). Then, seleninic acid intermediate is reduced back upon the sequential reaction with two molecules of reduced glutathione (GSH) forming oxidized glutathione (GSSG) (Equations 1.15 and 1.16):

$$H_2O_2 + Gpx - Cys - SeH \rightarrow Gpx - Cys - SeOH + H_2O \quad (1.14)$$

$$Gpx - Cys - SeOH + GSH \rightarrow Gpx - Cys - SeSG + H_2O \quad (1.15)$$

$$Gpx - Cys - SeSG + GSH \rightarrow Gpx - Cys - SeH + GSSG \quad (1.16)$$

GSSG is reduced back to GSH at the expense of NADPH (Equation 1.17), in a reaction catalyzed by glutathione reductase (EC 1.8.1.7), an enzyme present in the mitochondrial matrix [101]:

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \quad (1.17)$$

In rat liver, Gpx1 activity is about 500-fold higher than that of Gpx4 when measured with H_2O_2 as a substrate [102]. The function of Gpx4 is probably the reduction of diacylated phospholipid hydroperoxides, which are not reduced by Gpx1 [103–106], while GPx1 besides removing H_2O_2 may also be responsible for the removal of lysophospholipid hydroperoxides [107,108].

Peroxiredoxins

Two peroxiredoxin isoforms are found in the mitochondria, Prx3 and Prx5; both are coded by two nuclear genes and their catalytic reaction mechanism involves two cysteine residues [109]. In the first step, H_2O_2 oxidizes one cysteine residue (–SH), the so-called peroxidatic cysteine (CysP), to a sulfenic acid (–SOH) (Equation 1.18), and in the second step, this intermediate reacts with a second cysteine residue, the resolving cysteine residue (CysR), to form a disulfide (Equation 1.19). These two cysteine residues are present in a single peroxiredoxin subunit, but while in typical

two-cysteine peroxiredoxins like Prx3, the disulfide is formed between the two identical subunits forming a dimer, in Prx5, an atypical two-cysteine peroxiredoxin, an intramolecular disulfide is formed in the same unit.

$$H_2O_2 + Prx - CysP - SH \rightarrow Prx - CysP - SOH + H_2O \quad (1.18)$$

$$Prx - CysP - SOH + Prx - CysR - SH \rightarrow Prx - CysP - SS - CysR - Prx + H_2O \quad (1.19)$$

Both mitochondrial peroxiredoxins are regenerated following reduction of the disulfide by thioredoxin 2 (Trx2) in a thiol exchange reaction forming a Trx2 intramolecular disulfide (Equation 1.20) [110,111]. The Trx disulfide is reduced back in a reaction catalyzed by thioredoxin 2 reductase (Trx2R, EC 1.8.1.9), a selenoprotein, using NADPH as the cosubstrate (Equation 1.21) [112]. Both Trx2 and its reductase are enzymes present in the mitochondrial matrix [113,114]. In addition, Prx3 is also reduced by the mitochondrial glutaredoxin 2, which may be reduced by glutathione or thioredoxin reductase [115].

$$Prx - CysP - SS - CysR - Prx + Trx2 - (Cys - SH)_2 \rightarrow Prx - CysP - SH + Prx - CysR - SH + Trx2 - (Cys - SS - Cys) \quad (1.20)$$

$$Trx2 - (Cys - SS - Cys) + NADPH + H^+ \rightarrow Trx2 - (Cys - SH)_2 + NADP^+ \quad (1.21)$$

Out of the two peroxiredoxins, Prx3 is mainly responsible for H_2O_2 elimination in the mitochondria because (1) the rate constant for the reaction of Prx3 with H_2O_2, 2×10^7 M^{-1} s^{-1} [116] is two orders of magnitude higher than that of Prx5, 3×10^5 M^{-1} s^{-1} [117] and (2) Prx3 is slightly more abundant in the mitochondria than Prx5 [109].

A distinctive characteristic of peroxiredoxin removal of H_2O_2 is that at large concentrations of H_2O_2, H_2O_2 may react with the sulfenic intermediate (Equation 1.22) before this intermediate is reduced back, inactivating the enzyme. Prx3 is more resistant than the cytosolic Prx2 to this inactivation [118,119] and Prx5 seems resistant to this inactivation [109]. This hyperoxidation facilitates the formation of decamers with chaperone activity. Peroxiredoxins play an important role as chaperones, but this alternative function has not been observed yet with mitochondrial peroxiredoxins [109]. Hyperoxidized Prx3 may be repaired by sulfiredoxin (EC 1.8.98.2) translocated from the cytosol at the expense of one ATP and GSH (Equation 1.23) [120]:

$$Prx - CysP - SOH + H_2O_2 \rightarrow Prx - CysP - SO_2H + H_2O \quad (1.22)$$

$$Prx - CysP - SO_2H + ATP + 2\ GSH \rightarrow Prx - CysP - SOH + ADP + PO_4^{3-} + GSSG \quad (1.23)$$

Relative Contribution of Glutathione Peroxidases and Peroxiredoxins to H_2O_2 Removal

In an excellent review, it was estimated that nearly 90% of mitochondrial H_2O_2 is removed by Prx3, with Gpx1 being responsible for approximately 9%, and the remaining 1% being accounted for other proteins [109]. This estimate of the order of magnitude was based on the reactivity of the reduced enzymes toward H_2O_2, taking also into account their abundance in the mitochondria. So, this estimate is a good approximation if most of the Gpx1 and Prx3 are in the reduced form, that is, if the antioxidant systems that regenerate the reduced enzymes are not rate limiting. Recently, the relative contributions of GPx/GSH and Prx/Trx were addressed experimentally in the heart mitochondria of the mouse, rat, and guinea pig, by measuring the increase in H_2O_2 emission from isolated mitochondria upon addition of auranofin, which inhibits Trx1/2, and dinitrochlorobenzene (DNCB), a GSH-depleting agent [121]. While in the mouse and rat heart mitochondria, addition of DNCB had a larger impact on H_2O_2 emission in either state 3 or state 4 respiration, thus suggesting that GPx1 is more important than Prx3 in eliminating H_2O_2; in guinea pig heart mitochondria, the opposite situation occurred. A mathematical model suggested that the two antioxidant systems act in concert, in which each system can partially replace the other and cooperate to eliminate H_2O_2 [121].

Next, we will estimate the relative contribution of GPx1 and Prx3, taking into account the whole kinetic cycle of both enzymes and focusing our analysis on liver mitochondria. For this, we will use the kinetics of GPx1 and Prx3. Kinetics for GPx1 and Prx3, as judged by *Plasmodium falciparum* peroxiredoxin [122], follows an ordered ping-pong mechanism as described by the following equation:

$$\frac{[E]}{v} = \phi_0 + \frac{\phi_1}{[H_2O_2]} + \frac{\phi_2}{[SH]} \tag{1.24}$$

where

[E] stands for the total enzyme concentration
v stands for the rate of reaction
[SH] is the concentration of the thiol responsible for enzyme regeneration
ϕ_0, ϕ_1, and ϕ_2 are the kinetic parameters characteristic of a ping-pong enzymatic mechanism (Dalziel coefficients)

Equation 1.24 can be rearranged into the more familiar Michaelis–Menten equation:

$$v = \frac{[SH]/(\phi_2 + \phi_0[SH])[E][H_2O_2]}{\phi_1[SH]/(\phi_2 + \phi_0[SH]) + [H_2O_2]} \tag{1.25}$$

For Prx3, $\phi_0 \neq 0$ and, consequently, a finite k_{cat} and K_M (for H_2O_2) are observed for a high thiol concentration (Equation 1.25). On the other hand, for GPx1, $\phi_0 = 0$ [123],

this implies infinite k_{cat} and K_M (for H_2O_2), as can be seen from Equation 1.26, which was obtained from Equation 1.25 by letting $\phi_0 = 0$:

$$v = \frac{\left(\left[SH\right]/\phi_2\right)\left[E\right]\left[H_2O_2\right]}{\phi_1\left(\left[SH\right]/\phi_2\right)+\left[H_2O_2\right]} \tag{1.26}$$

Equations 1.25 and 1.26 are used to estimate the relative contribution of Gpx1 and Prx3. To achieve that, kinetic parameter values as well as enzyme and thiol concentrations are needed, while H_2O_2 will be let as an unknown variable. For rat liver mitochondria Gpx1, ϕ_1 and ϕ_2 have been estimated as 4.7×10^{-8} M s and 2.5×10^{-5} M s, respectively [124]. In rat liver mitochondria, the concentration of Gpx1 has been estimated at 10 μM (monomer concentration) [93] and GSH concentration is 10 mM [125]. This GSH concentration is in excess as a partial GSH depletion of 30%–40% is needed before an increase in mitochondrial H_2O_2 production can be observed [16].

Concerning Prx3, the only kinetic data available for the liver were obtained with saturating H_2O_2 concentrations. Under these conditions, Equation 1.25 simplifies to the following equation:

$$v = \frac{\left(\left[E\right]/\phi_0\right)\left[SH\right]}{\left(\phi_2/\phi_0\right)+\left[SH\right]} \tag{1.27}$$

Thus, ϕ_0 and ϕ_2 may be estimated from K_M (for Trx) and k_{cat} measured under H_2O_2 saturating conditions according to the following equations:

$$\phi_0 = \frac{1}{k_{cat}} \tag{1.28}$$

and

$$\phi_2 = \frac{K_M}{k_{cat}} \tag{1.29}$$

Kinetic parameters for Prx3 obtained by applying these equations under saturating H_2O_2 conditions are shown in Table 1.1. These values are close to those obtained experimentally for *P. falciparum* peroxiredoxin, for which ϕ_0 and ϕ_2 are 1.8 s and 1.85×10^{-5} M s, respectively [122]. Concerning ϕ_1, this parameter is the inverse of the rate constant measured between reduced Prx3 and H_2O_2, which is 2×10^7 M^{-1} s^{-1} [116]. One important information that can be taken from Table 1.1 is the estimation of the apparent K_M for H_2O_2. This value is lower than 10 μM because several investigators have observed that H_2O_2 concentrations of this magnitude saturate the enzyme. However, the value for the apparent K_M for H_2O_2 was never measured, because measuring the low reaction rate at low H_2O_2 concentrations has been challenging

TABLE 1.1
Kinetic Parameters for Purified Prx3 Obtained with Saturating H_2O_2 Concentrations in the Presence of Various Electron Donors

Electron Donor	Mitochondrial Trx (Trx2)		Cytosolic Trx (Trx1)		Glutaredoxin 2 (Grx2)
V_{max} (μmol min^{-1}mg $protein^{-1}$)	1.1	—	2.4	13.3	1.2
k_{cat} (s^{-1})	0.39[a]	0.8–1.2	0.86[a]	4.8[a]	0.43[a]
K_M for Trx/Grx (μM)	11.2	—	4.0	4.3	23.8
ϕ_0 (s)	2.5	0.8–1.2	1.2	0.2	2.3
ϕ_1 (M s)	5.0×10^{-8}	5.0×10^{-8}	5.0×10^{-8}	5.0×10^{-8}	5.0×10^{-8}
ϕ_2 (M s)	2.8×10^{-5}	—	4.6×10^{-6}	9.0×10^{-7}	5.5×10^{-5}
K_M for H_2O_2 (μM)[b]	0.02	0.04–0.06	0.04	0.24	0.02
References	[115]	[126]	[115]	[111]	[115]

[a] Calculated from V_{max} assuming a molecular mass for Prx3 of 21.5 kDa.

[b] Estimated assuming a rate constant for the reaction between reduced Prx3 and H_2O_2 of 2×10^7 M^{-1} s^{-1}.

[109,111,126]. If the enzyme is assumed to be saturated with Trx2, then the K_M for H_2O_2 may be estimated as ϕ_1/ϕ_0 (Equation 1.25). Values obtained are in the range 0.02–0.24 μM (Table 1.1), which is between one and two orders of magnitude lower than the value observed for bacterial peroxiredoxin AhpC (K_M = 1.4 μM) [127].

The last parameters we need to estimate are Prx3 and Trx2 concentrations in liver mitochondria. Prx3 concentration in rat liver mitochondria can be estimated as 160 μM based on a level of 0.7 μg of Prx3/mg of rat liver protein [111]. Trx2 concentration has been estimated to be 10 μM in the mitochondria from bovine adrenal cortex [109,128]. The adrenal gland is extremely rich in Prx3 [111], raising the question whether the levels of its partner Trx2 are also increased compared with liver mitochondria. Taking into account the doubts concerning the concentration of reduced Trx2 in liver mitochondria, calculation with two values, 1 and 10 μM, was performed.

Using these estimations, the relative contribution of GPx1 and PRx3 was calculated (Figure 1.4). For low H_2O_2 concentrations, Prx3 removes most of the H_2O_2, while for larger concentrations, Gpx1 predominates. Table 1.2 summarizes the main data that may be read from Figure 1.4 in the range of endogenous H_2O_2 production. In the lower range of H_2O_2 production (0.2 nmol min^{-1} mg^{-1} of protein), H_2O_2 concentration is expected to be around 1–2 nM with Prx3 being the main contributor for its removal, around 90%, as predicted in [109]. At this condition, both Gpx1 and Prx3 are almost fully reduced, and removal of H_2O_2 may be predicted based simply on the reaction rate constants between the reduced enzymes and H_2O_2. In the high H_2O_2 production range, here assumed to be 2 nmol min^{-1} mg^{-1} of protein [121], the mitochondrial H_2O_2 concentration and the removal pathways of H_2O_2 are strongly dependent on the levels of reduced Trx2. For low Trx2 (1 μM), a high steady-state H_2O_2 is predicted (130 nM), Prx3 saturates, and the contribution of GPx1 becomes fundamental, with 85% of H_2O_2 being removed via Gpx1. This estimate of 130 nM

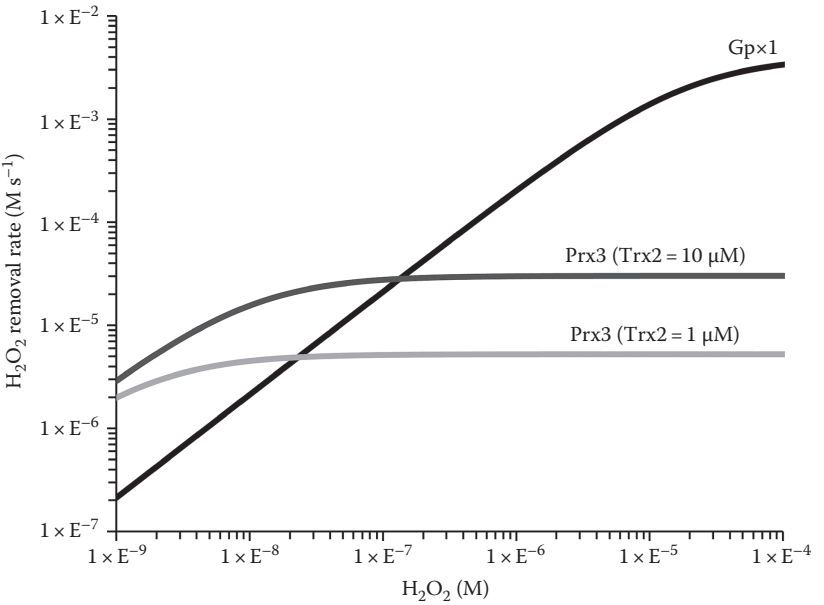

FIGURE 1.4 Removal of mitochondrial H_2O_2 by Prx3 and Gpx1. Equations 1.25 and 1.26 are used, respectively, for Prx3 and Gpx1. Parameters used for Gpx1 were $\phi_1 = 4.7 \times 10^{-8}$ M s, $\phi_2 = 2.5 \times 10^{-5}$ M s, [Gpx1] = 10 μM and [GSH] = 10 mM (black curve). For Prx3, $\phi_0 = 2.5$ s, $\phi_1 = 5.0 \times 10^{-8}$ M s, $\phi_2 = 2.8 \times 10^{-5}$ M s, [Prx3] = 160 μM, [Trx1] = 10 μM (dark gray curve), or [Trx1] = 1 μM (light gray curve).

TABLE 1.2
Estimates for H_2O_2 Concentrations and Relative Contributions for H_2O_2 Removal by Prx3 and Gpx1

H_2O_2 Production (nmol min^{-1} mg^{-1} of protein)	0.2		2	
Reduced Trx (μM)	1	10	1	10
[H_2O_2] (nM)	2	1	130	40
H_2O_2 *removal via* Prx3	87%	93%	15%	74%
Gpx1	13%	7%	85%	26%

Note: Data are taken from Figure 1.4. H_2O_2 production is assumed to be in the range 0.2–2 nmol min^{-1} mg^{-1} of protein, which converts to 3.3–33 μM s^{-1}.

is strongly dependent on the parameters used, with small changes having a significant impact on the predicted H_2O_2 steady state. If reduced Trx2 is assumed to be 10 μM, then H_2O_2 is predicted to be 40 nM, with around 75% of H_2O_2 being removed by Prx3. This dependency on the levels of Trx2 has been observed experimentally, as overexpression of Trx2 improves protection against mitochondrial generation of

H_2O_2 [129]. In other words, the levels of Trx2 are not in the saturating range but near its K_M or lower. This analysis is consistent with experimental observations that indicate that both GPx1 and Prx3 are important for H_2O_2 removal [121,130]. Tissue and species origin of mitochondria are certainly important factors when considering H_2O_2 catabolism.

Pyruvate

In addition to enzymatic antioxidant systems, pyruvate has also been pointed out as having a possible antioxidant role by reacting with H_2O_2. 2-Oxoacids, like pyruvate, undergo a decarboxylation in the presence of H_2O_2, with H_2O_2 being reduced to water, a reaction first described more than a century ago:

$$CH_3COCOO^- + H_2O_2 \rightarrow CH_3COO^- + CO_2 + H_2O \quad (1.30)$$

This reaction is relevant and several observations support a role for pyruvate as an H_2O_2 sink [131–134]. Interestingly, cultured cells export pyruvate to reach levels similar to those observed in human serum (60–150 μM), protecting themselves from added H_2O_2 [133]. Altogether these observations show that pyruvate can have a protective role against H_2O_2. In mitochondria, pyruvate is present in the millimolar range (1.5 mM), and so does pyruvate work as a sink for H_2O_2, competing with enzymatic antioxidant systems. The rate constant between pyruvate and H_2O_2 depends on the pH and ionic force, and a value of 2.2 M^{-1} s^{-1} was determined at pH 7.4 in 0.1 M phosphate buffer containing 0.1 mM DTPA at 37°C [135]. A similar value of 2.4 M^{-1} s^{-1} may be estimated from Figure 1.3 in Desagher et al. [134] at pH 7.4 in Krebs' bicarbonate buffer at 37°C. Thus, a pseudo-order reaction rate constant of 3.6×10^{-3} s^{-1} may be estimated, indicating that pyruvate does not compete with the enzymatic antioxidant systems in the mitochondria. This estimate and the experimental observations showing that pyruvate is effective in decreasing H_2O_2 concentrations when added in cell culture media or perfusion fluids [136], protecting the mitochondria from added H_2O_2 to cell media [137], are not incompatible. When pyruvate is present in the external media, the overall capacity of pyruvate to react with H_2O_2 is large because the ratio between the external media and the cellular volume is very large, and so the amount of pyruvate able to react with H_2O_2 is large, even if the rate constant with H_2O_2 is slow. For example, Desagher et al. [134] observed that only half of the 200 μM H_2O_2 initially present in the cellular growth medium remained after 2 min in the presence of 2 mM pyruvate. However, pyruvate cannot compete with antioxidant systems when present in the same compartment such as the mitochondria.

CONCLUSION

Nearly half a century has passed since the initial discovery of H_2O_2 production in mitochondria. However, a number of uncertainties still remain concerning both the mechanisms of the formation and removal of $O_2^{\cdot-}$/H_2O_2 in the mitochondria.

Some open questions are as follows: (1) To what extent is $O_2^{\cdot-}/H_2O_2$ formation an unavoidable consequence of O_2 reduction to H_2O in the respiratory chain or, alternatively, is this formation under the regulation with the formation rates of $O_2^{\cdot-}/H_2O_2$ having a relevant physiological role in cellular processes? (2) To what extent is the knowledge obtained from isolated mitochondria, respiratory complexes, or other isolated H_2O_2-forming enzymes a good picture of the in vivo situation, where the mitochondria are part of a dynamic network, at both functional and morphological levels, interacting with other cellular components? (3) To what extent do peroxiredoxins and glutathione peroxidases cooperate in the removal of H_2O_2?

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