Nitric Oxide, Cell Signaling, and Gene Expression

edited by

Santiago Lamas
Enrique Cadenas

Nitric Oxide, Cell Signaling, and Gene Expression

OXIDATIVE STRESS AND DISEASE

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Series Introduction

Oxygen is a dangerous friend. Through evolution, oxygen—itself a free radical—was chosen as the terminal electron acceptor for respiration. The two unpaired electrons of oxygen spin in the same direction; thus, oxygen is a biradical. Other oxygen-derived free radicals, such as superoxide anion or hydroxyl radicals, formed during metabolism or by ionizing radiation are stronger oxidants (i.e., endowed with a higher chemical reactivity). Oxygen-derived free radicals are generated during oxidative metabolism and energy production in the body, and are involved in regulation of signal transduction and gene expression; activation of receptors and nuclear transcription factors; oxidative damage to cell components; the anti-microbial and cytotoxic action of immune system cells, neutrophils, and macrophages; and in aging and age-related degenerative diseases. Overwhelming evidence indicates that oxidative stress can lead to cell and tissue injury; however, the same free radicals that are generated during oxidative stress are produced during normal metabolism and, as a corollary, are involved in both human health and disease.

In addition to reactive oxygen species, research on reactive nitrogen species has been gathering momentum to develop an area of enormous importance in biology and medicine. Nitric oxide or nitrogen monoxide (NO) is a free radical generated by nitric oxide synthase (NOS). This enzyme modulates physiological responses in the circulation, such as vasodilation (eNOS) or signaling in the brain (nNOS). During inflammation, however, a third isoenzyme is induced—iNOS—resulting in the overproduction of NO and causing damage to targeted infectious organisms and to healthy tissues in the vicinity. More worrisome, however, is the fact that NO can react with superoxide anion to yield a strong oxidant—peroxynitrite. Oxidation of lipids, proteins, and DNA by peroxynitrite increases the likelihood of tissue injury.

Both reactive oxygen and nitrogen species are involved in the redox regulation of cell functions. Oxidative stress is increasingly viewed as a major upstream component in the signaling cascade involved in inflammatory responses and stimulation of adhesion molecule and chemoattractant production. Hydrogen peroxide decomposes in the presence of transition metals to the highly reactive hydroxyl radical, which by two major reactions—hydrogen abstraction and addition—accounts for most of the oxidative damage to proteins, lipids, sugars, and nucleic acids. Hydrogen peroxide is also an important signaling molecule that, among others, can activate NF-κB, an important transcription factor involved in inflammatory responses. At low concentrations, hydrogen peroxide regulates cell signaling and stimulates cell proliferation; at higher concentrations, it triggers apoptosis and, at even higher levels, necrosis.

Virtually all diseases thus far examined involve free radicals. In most cases, free radicals are secondary to the disease process, but in some instances, free radicals are causal. Thus, a delicate balance exists between oxidants and antioxidants in health and disease. Their proper balance is essential for ensuring healthy aging.

The term oxidative stress indicates that the antioxidant status of cells and tissues is altered by exposure to oxidants. The redox status is thus dependent on the degree

to which cells' components are in the oxidized state. In general, the reducing environment inside cells helps to prevent oxidative damage. In this reducing environment, disulfide bonds (S–S) do not spontaneously form because sulfhydryl groups are maintained in the reduced state (SH), thus preventing protein misfolding or aggregation. This reducing environment is maintained by oxidative metabolism and by the action of antioxidant enzymes and substances, such as glutathione, thioredoxin, vitamins E and C, and enzymes such as superoxide dismutases, catalase, and the selenium-dependent glutathione reductase, as well as glutathione and thioredoxin hydroperoxidases, which serve to remove reactive oxygen species (hydroperoxides).

Changes in the redox status and depletion of antioxidants occur during oxidative stress. The thiol redox status is a useful index of oxidative stress mainly because metabolism and NADPH-dependent enzymes maintain cell glutathione (GSH) almost completely in its reduced state. Oxidized glutathione (glutathione disulfide [GSSG]) accumulates under conditions of oxidant exposure and this changes the ratio GSSG/GSH; an increased ratio is usually taken as indicating oxidative stress. Other oxidative stress indicators are ratios of redox couples such as NADPH/NADP, NADH/NAD, $\text{thioredoxin}_{\text{reduced}}/\text{thioredoxin}_{\text{oxidized}}$, dihydrolipoic acid/α-lipoic acid, and lactate/pyruvate. Changes in these ratios affect the energy status of the cell, largely determined by the ratio ATP/ADP + AMP. Many tissues contain large amounts of glutathione, 2–4 mM in erythrocytes or neural tissues, and up to 8 mM in hepatic tissues. Reactive oxygen and nitrogen species can oxidize glutathione, thus lowering the levels of the most abundant non-protein thiol, sometimes designated as the cell's primary preventative antioxidant.

Current hypotheses favor the idea that lowering oxidative stress can have a health benefit. Free radicals can be overproduced or the natural antioxidant system defenses weakened, first resulting in oxidative stress, and then leading to oxidative injury and disease. Examples of this process include heart disease, cancer, and neurodegenerative disorders. Oxidation of human low-density lipoproteins is considered an early step in the progression and eventual development of atherosclerosis, thus leading to cardiovascular disease. Oxidative DNA damage may initiate carcinogenesis. Environmental sources of reactive oxygen species are also important in relation to oxidative stress and disease. A few examples include: UV radiation, ozone, cigarette smoke, and others are significant sources of oxidative stress.

Compelling support for the involvement of free radicals in disease development originates from epidemiological studies demonstrating that an enhanced antioxidant status is associated with reduced risk of several diseases. Vitamins C and E, in the prevention of cardiovascular disease, are a notable example. Elevated antioxidant status is also associated with decreased incidence of cataracts, cancer, and neurodegenerative disorders. Some recent reports have suggested an inverse correlation between antioxidant status and the occurrence of rheumatoid arthritis and diabetes mellitus. Indeed, the indications in which antioxidants may be useful in the prevention or the treatment of disease are increasing in number.

Oxidative stress, instead of being the primary cause of disease, is more often a secondary complication in many disorders. Oxidative stress diseases include inflammatory bowel diseases, retinal ischemia, cardiovascular disease and restenosis, AIDS, adult respiratory distress syndrome, and neurodegenerative diseases such as

stroke, Parkinson's disease, and Alzheimer's disease. Such indications may prove amenable to antioxidant treatment (in combination with conventional therapies) because a clear involvement of oxidative injury exists in these disorders.

In this series of books, the importance of oxidative stress and disease associated with organ systems of the body is highlighted by exploring the scientific evidence and the medical applications of this knowledge. The series also highlights the major natural antioxidant enzymes and antioxidant substances such as vitamins E, A, and C, flavonoids, polyphenols, carotenoids, lipoic acid, coenzyme Q_{10}, carnitine, and other micronutrients present in food and beverages. Oxidative stress is an underlying factor in health and disease. More evidence indicates that a proper balance between oxidants and antioxidants is involved in maintaining health and longevity, and that altering this balance in favor of oxidants may result in patho-physiological responses that cause functional disorders and disease. This series is intended for researchers in the basic biomedical sciences and clinicians. The potential of such knowledge for healthy aging and disease prevention warrants further knowledge about how oxidants and antioxidants modulate cell and tissue function.

Lester Packer
Enrique Cadenas
Series Editors

Preface

The role of nitric oxide (NO) as a physiological mediator was established with the discovery in the early 1980s of its capacity to regulate the vascular tone through cyclic GMP. Over the past 10 years, newer roles for NO have emerged, related to the ability of NO to interact with and modify a wide variety of other molecules, such as the free radical superoxide anion, key redox regulators such as glutathione, and macromolecules such as DNA and proteins. This forms the basis for the possibility of NO to influence crucial processes within the cell, such as the response to redox perturbations, protein function, and gene expression through non-enzymatic modifications. Among the mechanisms that underlie these effects, S-nitrosylation of proteins has attracted increasing interest in recent years, and it has been postulated as a possible new paradigm of signal transduction. The capacity of NO to interact with crucial mitochondrial enzymes, such as cytochrome oxidase, and the discovery of mitochondrial NO add even more relevance to the wide array of cellular functions on which NO may have an influence.

Nitric Oxide, Cell Signaling, and Gene Expression is a collection of chapters written by experts on various aspects of NO functions: regulation of mitochondrial respiration by NO; mitochondrial NO signaling in redox modulation of cell behavior, synaptic plasticity, and cell death; and deleterious effects of NO on mitochondria, partly caused by peroxynitrite. The importance of NO in hypoxia is exemplified and analyzed in a chapter where the relationship between NO and the hypoxia-sensor HIF-1 is described in detail. Other chapters address modulation of cell metabolism by NO, regulation of cell signaling by cGMP, protein nitrosylation/denitrosylation, the Ras superfamily GTPases, and ceramide, as well as the involvement of NO in apoptosis through activation of caspases. A subset of chapters is devoted to the role of NO in gene expression and the post-transcriptional control of gene expression as well as a role for NO in tumor biology.

The editors are grateful to the contributors for having shared their expertise in the completion of this work.

Santiago Lamas
Enrique Cadenas

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Enrique Cadenas is professor and chairman of molecular pharmacology and toxicology at the University of Southern California School of Pharmacy in Los Angeles. He earned his M.D. and Ph.D. in biochemistry from the University of Buenos Aires, Argentina. His research programs focus on mitochondrial oxidative/nitrosative stress, with implications for neurodegeneration and aging. Dr. Cadenas has authored 200 publications, edited numerous books, and serves on the editorial boards of several prestigious journals.

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CONTENTS

Over the past 10 years, evidence has been collected that suggests a role for nitric oxide (NO) in cell bioenergetics, and many reviews have highlighted the relevance and the multiple aspects of the issue [1–12]. This chapter summarizes some of the experimental information available, particularly focusing on the structural basis and the mechanisms of the reactions between NO and mitochondrial complex IV, and their functional relevance.

1.1 THE MITOCHONDRIAL PRODUCTION OF NO

Nitric oxide (i.e., the nitrogen monoxide NO) has been recognized as ubiquitous, its presence inducing a variety of intra- and intercellular physiological actions [13–17], all virtually prone to become of pathological relevance due to the high reactivity of NO. In the cell as well as *in vivo*, NO is enzymatically produced by the NO-synthases (NOSs), converting L-arginine to L-citrulline in the presence of NADPH, O_2 and other co-factors [18–20]. Three NOS isoforms have been isolated; these are almost ubiquitously expressed, although more typically by different cell lines or under inducible metabolic or experimental conditions (see Table 1.1). The so-called neuronal NOS (nNOS) and the endothelial NOS (eNOS) are the constitutive Ca^{++}-dependent NOSs, whereas the expression of the inducible Ca^{++}-independent NOS (iNOS) can be enhanced in immunocompetent cells such as macrophages [21, and references therein]. The iNOS activity is up-regulated during the inflammatory response, and can be stimulated by effectors, such as the lipopolysaccharide and the cytokines interferon γ, and/or the tumor necrosis factor α (TNF-α). The three types of NOS have been sequenced and characterized [22], whereas the existence of a fourth isoform, initially proposed as a specific mitochondrial NOS (mtNOS), has been ruled out [21]. Kanai et al. [23] proposed that the mtNOS is actually an nNOS, based on the observation that mitochondria of cardiomyocytes from mice knockout for nNOS do not produce NO, contrary to wild-type mice. Later, Elfering et al. [24] reported that the mtNOS most likely is the isoform alfa of the nNOS, excluding the existence of an additional splicing product of the nNOS alternative to the three identified. According to these authors [21, 24], the mtNOS is a constitutive nNOS-alfa bearing two post-translational modifications, namely an acylation and a phosphorylation, accounting for the interplay of this NOS with the mitochondrial inner membrane [21, and references therein]. Thus, the mtNOS is a membrane-bound NOS, localized in the mitochondrion as proposed originally [25–30]. Accordingly, NO has been demonstrated to be produced by mitochondria isolated to a high degree of purity from brain, heart [31], and other organs [23, 31–34]. Purified mitochondria in the presence of L-arginine produced NO and nitrite; thereby, respiration is inhibited, and inhibition is released by the NOS inhibitor L-nitrosoarginine, or similar compounds [35]. It is worth noting that NO like O_2 can diffuse very rapidly through biological membranes [36, 37], making the existence of a local mtNOS perhaps less relevant to physiology.

Regardless of where NO is produced, more than 10 years of investigation has proven the rapid inhibition of the aerobic mitochondrial respiration by submicromolar NO [11, 38–40]. As the release of mitochondrial inhibition is also rapid, the interaction between NO and the respiratory chain displays the characteristics of a functional control reaction. For this reason, the effect of NO on cell respiration is of particular interest and, depending on circumstances, might be of physiological or pathological relevance.

TABLE 1.1
A Synopsis of the NO Synthases

Isoform	Nickname	Tissue/cell specificy[a]	MW (kDa)	Ca++-dependence
NOS-1	nNOS[b]	Neurons	157	+
NOS-2	iNOS	Macrophages	135	-
NOS-3	eNOS	Endothelium	140	+
NOS-1	mtNOS[c]	Mitochondria	130–147	+

[a] All NOSs isoforms are widely distributed through most cells and tissues; tissue specificity is herein intended as to where NOSs typically have been predominantly found and historically purified.

[b] Four splice variants of the nNOS-1 (nNOS-α) have been described, namely the nNOS-β, nNOS-γ, nNOS-μ, and nNOS-2.

[c] The most accredited hypothesis is that the mtNOS is an nNOS-α that is post-translationally modified [21].

1.2 THE FUNCTIONAL RELEVANCE OF THE REACTIONS BETWEEN NO AND MITOCHONDRIA

A simple way to directly unveil in cultured cells the inhibition of cell respiration by NO is to perform fluorescence-microscopy experiments aimed at functionally visualizing mitochondria. In such experiments, the cells respiring on physiological substrates and glucose electrophoretically accumulate in the mitochondria a cationic fluorescent dye, typically rhodamine or JC-1. The import is driven by the membrane potential component, $\Delta\psi$, of the proton electrochemical potential gradient, $\Delta\mu_{H+}$ [41, 42]. Under these conditions, mitochondria rapidly accumulate the probe and light up (Figure 1.1a). The same experiment performed in the presence of NO-donors, or after stimulation of the endogenous NO-synthase, demonstrated a marked decrease of mitochondrial fluorescence, indicative of the respiratory chain inhibition (Figure 1.1b). Mitochondrial inhibition appears rapidly and is reversible upon interruption of the NO flux, or washing the cells, or by specifically inhibiting NOS with 7-nitroindazole [42].

In 1994, clear-cut experiments [43, 44] demonstrated that the time course of NO inhibition of respiration sustained by mitochondria of neuronal synaptosomes is fully compatible with the interaction of NO with the terminal acceptor of the

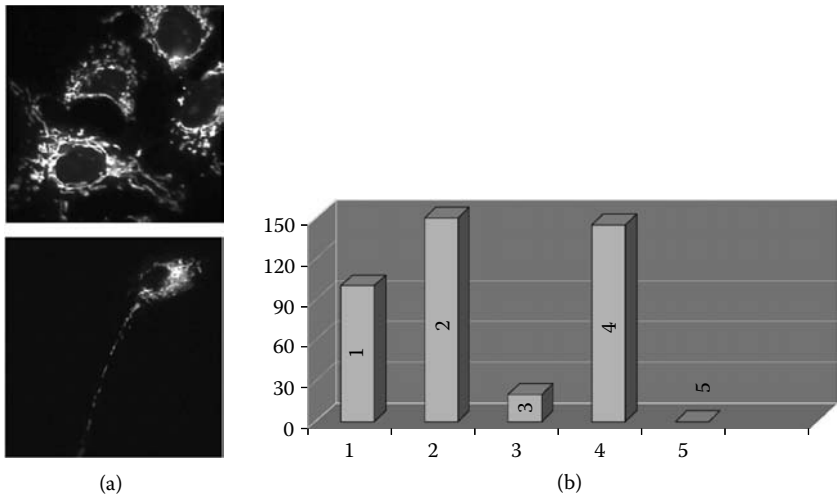

FIGURE 1.1 Mitochondrial fluorescence microscopy of cells importing rhodamines. Typical images of the mitochondrial network in astrocytes and neurons (left panel). Computer-aided image analysis allows quantitation of fluorescence as observed: (1) in the presence of nigericin alone, to fully convert $\Delta\mu_{H+}$ into $\Delta\Psi$; (2) by inhibiting the NOS (7-nitroindazole, 7-N); (3) by stimulating the NOS (N-methyl-D-aspartate, NMDA); (4) as 3, but in the presence of 7-N, or (5) after collapsing $\Delta\Psi$ with valinomycin.

respiratory chain, the mitochondrial complex IV (i.e., cytochrome c oxidase [CcOX]) [43]. Since then, a wealth of evidence has been produced pointing to CcOX as the primary mitochondrial target for NO, and the fast (seconds) phenomenology triggered by the production/supplementation of NO to respiring cells appears to be due to the reaction of NO with CcOX. On a much longer time scale (tens of minutes to hours) and higher NO concentrations (>> μM), other mitochondrial complexes also react and are inhibited [45, 46].

This chapter describes in detail the cell respiratory changes observed in the presence of NO, particularly focusing on the mechanism(s) by which NO reacts with CcOX.

1.3 THE FAST-RESPONDING MITOCHONDRIAL TARGET OF NO IS CYTOCHROME C OXIDASE

Cytochrome c oxidase belongs to the heme-copper oxidase superfamily. Ubiquitous in the aerobic organisms, the heme-copper oxidases transfer electrons from reduced cytochrome c or quinols (in some bacteria) to O_2 [49]. This redox reaction is coupled to a vectorial proton translocation (pump) across the inner mitochondrial (eukaryotes) or the periplasmic (bacteria) membrane. The free energy release contributes to formation and maintenance of the proton electrochemical gradient $\Delta\mu_{H+}$ used to synthesize ATP [47]:

$$4\ \text{cyt.c}^{2+} + O_2 + 8\ H^+_{in} \rightarrow 4\ \text{cyt.c}^{3+} + 2\ H_2O + 4\ H^+_{out}$$

In 1995, the structure of the *aa₃*-type *Paracoccus denitrificans* and that of the enzyme purified from beef heart were simultaneously published. The latter is a dimer of 200 kDa monomers, each comprising 13 different polypeptides/subunits [49]. In the monomer (Figure 1.2), three Cu and two Fe ions are organized into four redox-active metal centers, namely the Cu_A (bimetallic), the low-spin heme *a,* and the Cu_B and the high-spin heme a_3 (the so-called binuclear center). A Zn and a Mg atom (Mn in bacteria) were found to be present in the structure [50]. The Mg/Mn site, located close to the heme a_3-Cu_B site, was suggested to be involved in the exit pathway for protons/water molecules [51]. The role of these additional metals is still obscure; they are redox-silent and, as far as we know, do not participate to the NO or other ligand/substrate binding chemistry. They likely contribute to stabilizing a trans-membrane structure, suitable for electron and proton transfer via pathways and channels of the protein moiety. X-ray structures of the bacterial aa_3-type CcOX from *Rhodobacter sphaeroides* and of the ba_3-type CcOX from the thermophilic bacterium *Thermus thermophilus* have been also reported, together with the structure of a ubiquinol oxidase, the bo_3 from *Escherichia coli* [52].

The bimetallic Cu_A site of CcOX is the electron-entry door of the enzyme (Figure 1.2) [53]; CuA accepts electrons from reduced cytochrome c, located in the intermembrane space of the mitochondrion (the periplasmic space of bacteria) or from other reducing substrates [47]. Cu_A is in rapid equilibrium with heme *a*, and electrons are thereby rapidly transferred intramolecularly to the active binuclear site where O_2, NO, and other ligands can bind. Interestingly, O_2 and CO only bind to the fully (two-electrons) reduced binuclear site, whereas NO can also bind to the half (one-electron) reduced or even to the oxidized site, as further discussed next [3, and references therein]. The possibility of NO to react with several CcOX intermediates/species is important to understand its peculiar efficacy as inhibitor of respiration.

Thus, the reaction with NO occurs at the level of the active site (i.e., in the same site where O_2 binds and reacts) [3]. In all heme-copper oxidases, this conserved bimetallic site (Figure 1.3) is constituted by a high-spin heme (a_3, b_3, o_3 depending on the organism) and a copper ion (called Cu_B). In the beef-heart enzyme, heme a_3 is coordinated by H_{376}, whereas Cu_B is coordinated by H_{240}, H_{290}, and H_{291} [49, 50]. A tyrosine residue, Y_{244}, is covalently bound to H_{240} and is highly conserved, being presumably absent only in the cbb_3-type oxidases, the most divergent members of the heme-copper oxidases superfamily [54, 55]. Tyrosine 244 has been proposed to become a radical during catalysis [56]; it is, therefore, a putative additional target for NO, although this is, at present, a speculation. By reacting with NO, activated tyrosines and thiols yield, respectively, the nitro- and the nitroso-derivatives. In addition to Tyr_{244}, beef heart CcOX contains the bulk-exposed cys_{115} in subunit III, a potential additional reaction site for NO [57, 58].

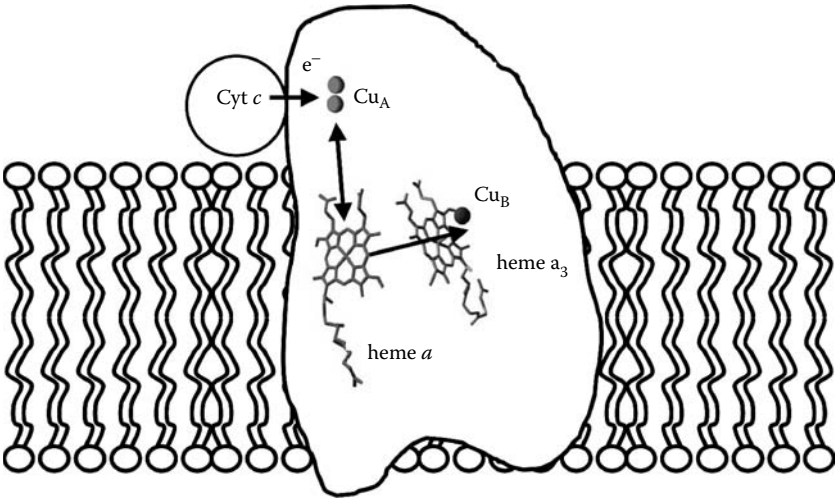

FIGURE 1.2 Cytochrome c oxidase. Purified from ox heart as a dimer, each 200 kDa monomer comprises 13 different polypeptides/subunits. In the monomer, three Cu and two Fe ions are organized into four redox-active metal centers; namely a bimetallic Cu_A center accepting the electrons donated by cytochrome c, heme *a* in rapid equilibrium with Cu_A, wherefrom electrons are intramolecularly transferred to heme a_3 and Cu_B, the binuclear active site of the enzyme.

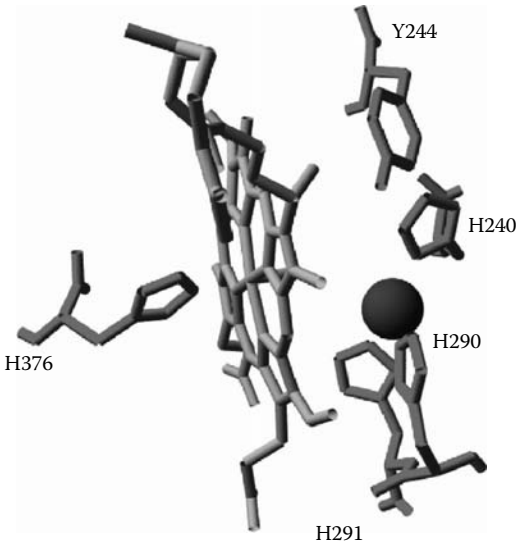

FIGURE 1.3 The active site of cytochrome c oxidase. Notice the tyrosine residue, Y_{244}, covalently bound to H_{240}; this highly conserved residue may possibly react with NO (reaction not reported, so far). (From the Protein Data Bank coordinates deposited by Tsukihara et al., *Science* (1995) 269: 1069–74.)

Despite the existence of several potential reaction sites, at physiological NO concentrations (micromolar or less), NO appears to react with CcOX, only at the level of the redox metals heme a_3 and Cu_B [3]. Peroxynitrite ($ONOO^-$), exogenously added and only in large excess over CcOX (≥ 100-fold), induces protein nitration [59]. Thus, when dealing with the reaction of NO with CcOX, the attention should be focused on the reactions involving the metals in the active site (Fe and Cu). As discussed next, these reactions lead to accumulation of products with an impact on mitochondria metabolism that might be substantially different. The reaction between NO and CcOX has to take into account the fact that, during catalysis, the metals in the active site undergo rapid (micro/milliseconds) redox and ligation changes, forming oxidized, partially reduced, and O_2-bound species (Figure 1.4). Particularly the so-called half-reduced binuclear site (i.e., a species where only one electron resides on the site) has been recently demonstrated to rapidly react with NO, providing an additional rationale to the advantage that NO appears to have over O_2 when reacting with CcOX [60].

When attempting to draw an overall picture of the NO to CcOX interactions, one should keep in mind that:

1. All redox species and intermediates of CcOX react with NO
2. The complete, though schematic, catalytic cycle includes two slow (milliseconds) reductive steps, followed by the diffusion limited ($k \geq 10^8\ M^{-1}s^{-1}$) O_2 binding and three fast (microseconds) oxidative steps
3. Moreover, at non-limiting O_2 concentration (i.e., in a first approximation above $K_{M,O2}$ (≥ 1– 10 μM)) the fraction of intermediates populated during turnover depends on the concentration of reduced cytochrome c, whereas the balance between the oxidative and the reductive equivalents at the CcOX site becomes relevant when O_2 becomes limiting—below $K_{M,O2}$ [61].

A schematic view of the catalytic cycle is reported in Figure 1.4 to allow a clearer understanding of the reactions of NO with CcOX during turnover. In this respect, it is worth knowing that most of the spectroscopic information on the chemistry of the binuclear site has been gathered by flashing the fully reduced CO-bound derivative of CcOX in the presence of oxygen [62] or, alternatively generating oxygen *in situ* on a nanosecond (or faster) time scale, by photolysing a synthetic caged O_2-carrier [63].

In summary, a catalytic cycle includes a reductive and an oxidative limb [64, 65]; in Figure 1.4, the species populated are labeled with capital letters. Reduction of the oxidized active site O proceeds via two sequential electron donations from Cu_A via heme *a*. The first electron leads to formation of the half-reduced intermediate **E** (**E_1** and **E_2**, indicating respectively the two half-reduced species with the electron residing on heme a_3, or on Cu_B [66]); the second electron leads to the fully reduced **R**. After the relatively slow (ms) reduction [56, 67], the much faster (μs) oxidation restores the initial species **O** through the transient formation of the so-called intermediates **P** and **F**; these are both oxo-ferryl adducts [68],

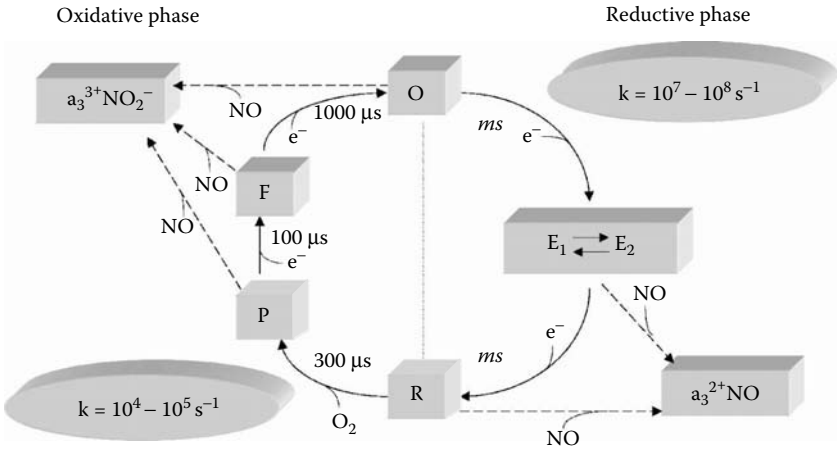

FIGURE 1.4 The catalytic cycle and the reaction of NO with the intermediates of cytochrome c oxidase. This is an oversimplified scheme of the catalytic redox cycle of the active site. **O**, **R**, **P**, and **F** stand for oxidized, reduced, peroxy, and ferryl state. **E** identifies the half-reduced active site (see text). The rate constant values are within *ms* for the slow reductive phase (**O → R**) and hundreds of *µs* for the fast oxidative phase (**R → O**). NO reacts very rapidly ($k = 10^7 - 10^8$ $M^{-1}s^{-1}$) with the reduced species R more slowly ($k = 10^4 - 10^5$ $M^{-1}s^{-1}$) with intermediates **O**, **P** and **F**. Differences in rate constant can be compensated by a higher occupancy in turnover of the **O**, **P**, and **F** species. (Modified from Sarti et al., *Free Radic. Biol. Med.* (2003) 34:509–20.)

although their detailed chemical identity is still controversial. Regardless of the number of CcOX species that can react with NO, only two adducts have been observed and identified, namely a nitrosyl- [$Cu_B^+a_3^{2+}$ NO] and a nitrite- [$Cu_B^{++}a_3^{3+}NO_2^-$] derivative. It is worth mentioning that, in the nitrite-derivative, although the oxidation state of the heme-Fe can be observed and is thus defined, the redox state of Cu_B is only assumed.

1.4 TWO MECHANISMS OF THE INHIBITION OF CcOX BY NO

Although the first report of a reaction between NO and CcOX dates to 1955 [69], the physiological relevance of this reaction was ignored until the late 1980s. Meanwhile, the reaction of NO with the reduced CcOX [70] was investigated, leading to the conclusion that NO is a very efficient reactant for the reduced heme-iron, and thus is a tool alternative to CO to stabilize the heme a_3 Fe^{2+} [71]; however, NO, differently from CO and O_2, proved to also react with the oxidized active site of the enzyme [72]. In 1994, however, NO proved to affect mitochondrial respiration via a fully reversible, transient inhibition of CcOX, displaying a competition with O_2 [73].

With purified mitochondrial CcOX, ligand binding to the fully reduced enzyme in the absence of O_2 is not associated to a redox reaction. Combination occurs at the reduced heme a_3, following bimolecular kinetics [70, 74, 75]. Under anaerobic conditions, NO reacts very quickly ($k \approx 10^8\ M^{-1}\ s^{-1}$) and with high affinity ($K_{aff} \approx 10^{11}\ M^{-1}$, at 20°C), yielding a typical Fe^{2+}-NO nitrosyl-adduct [74, 75]:

$$Cu_B^{+}\ a_3^{2+} + NO \quad \rightleftarrows \quad Cu_B^{+}a_3^{2+}\text{-}NO$$

On the contrary, the reaction of NO with oxidized Cu_B (see next paragraph) leads to the oxidative degradation of NO to nitrite, presumably via the transient formation of a nitrosonium ion (NO^+); the newly formed nitrite binds momentarily to the active site of the enzyme leading to inhibition [76]:

$$Cu_B^{2+}a_3^{3+} + NO \rightarrow Cu_B^{+}\ a_3^{3+}\text{-}NO^{+} + OH^{-} \rightarrow Cu_B^{2+}\ a_3^{3+}\text{-}NO_2^{-} + H^{+} + e^{-}$$

At a given NO concentration and during turnover, both the reactions just described can occur, although to a different extent depending on the relative occupancy of the intermediates bearing reduced heme a_3 or oxidized Cu_B. A third type of reaction has been proposed [77–79], and according to Pearce et al. [77], may yield the transient formation of peroxynitrite bound to the site and its subsequent reduction to nitrite.

For the sake of clarity, we will dissect the catalytic cycle and treat separately the reactions with NO of all CcOX species and intermediates, having either oxidized Cu_B as in species **O**, **P**, **F**, or reduced heme a_3 as in species **R** and $\mathbf{E_1}$. Interestingly, $\mathbf{E_1}$ bears at the same time reduced heme a_3 and oxidized Cu_B; whether this peculiar redox state plays a special role in NO binding/degradation is still obscure.

1.5 THE REACTION OF NO WITH OXIDIZED Cu_B YIELDS THE NITRITE-INHIBITED CcOX, RAPIDLY RECOVERING FUNCTION

The ability of the CcOX intermediates to react with NO has been studied in detail by mixing, in a stopped flow, NO with CcOX oxidized, **O**, or in the **R**, **P**, and **F** state [61, 78]. All these species can be independently generated and react with NO. The reaction of the oxidized CcOX with NO yields the inhibited nitrite-bound form of the enzyme, but removal of chloride is necessary to observe this reaction [79]. It is worth mentioning, in fact, that chloride is commonly bound to the active site of the oxidized enzyme *as prepared* and affects its reaction with ligands [80]. Although direct X-ray crystallographic evidence for the presence of Cl^- in the site is missing, a wealth of indirect measurements (EXAFS, EPR) suggest that chloride is bound, probably to Cu_B^{2+}. Thus, to observe the reaction of the oxidized enzyme with NO, CcOX has to be preliminarily stripped from

chloride, usually by a reduction/reoxidation cycle in a chloride free medium. In the reaction with oxidized Cu_B, NO is oxidized to nitrite, which then binds to the binuclear site perturbing the spectrum of the heme a_3^{3+} [61, 78]; the nitrite ion, by occupying the site, inhibits the enzyme [81].

The fully oxidized species **O** is (obviously) the most stable CcOX species, in air equilibrated buffer and at room temperature. Intermediates **P** and **F** can also be prepared in a sufficiently pure and stable state to be characterized. Similar to **O**, intermediates **P** and **F** bear oxidized Cu_B in the active site, and on this basis, Torres et al. [78] proposed a mechanism common to the three intermediates, involving the oxidation of NO to nitrite via formation of the nitrosonium ion NO^+ at the level of Cu_B. Interestingly, the reaction of NO with these species rapidly generates in the absence of reductants a stable spectral perturbation of heme a_3 identical to that observed when nitrite is added to the oxidized chloride-free enzyme (Figure 1.5). Taken together, both the optical spectroscopy [61, 78] and the NO amperometry [61] measurements suggest that intermediates O, P, and F react with NO with the same stoichiometry (1:1) and kinetics ($k_O = 2 \times 10^5\ M^{-1}s^{-1}$ to be compared with $k_{P,F} \approx 10^4 \div 10^5\ M^{-1}\ s^{-1}$, at 20°C). The interesting finding is that the adduct, which accumulates in all cases, is the inhibited nitrite-bound oxidized heme a_3, regardless of whether the experiment has been performed with **O**, **P**, or **F** (Figure 1.5). Owing to the lower affinity of the reduced active site for nitrite [61, 81], and relevant to the mitochondrial respiratory chain function, the nitrite CcOX-derivative promptly recovers activity upon reduction by cytochrome c or other artificial electron donors [61]. To better focus on the functional role of the reaction between NO and the oxidized Cu_B, it is worth noticing that the reaction is two to three orders of magnitude slower than that with **R** ($k \approx 1 \times 10^8\ M^{-1}s^{-1}$). Nevertheless the reaction is still relevant when the overall occupancy of the intermediates **O**, **P**, and **F** is predominant, as it happens when turnover is sustained by a slow electron supply to CcOX [61].

1.6 THE REACTION OF NO WITH THE FULLY REDUCED (R) OR THE HALF REDUCED (E) SPECIES YIELDS THE NITROSYL-INHIBITED CcOX, SLOWLY RECOVERING FUNCTION

The reaction of the partially or the fully reduced CcOX with NO yields the inhibited nitrosyl form of the enzyme, bearing NO bound to reduced heme a_3 (a_3^{2+}NO). Until the kinetics of the reaction of NO with the oxidized Cu_B was reexamined and the reaction with the partially reduced active site discovered, the formation of a complex with the fully reduced CcOX was the only pathway considered responsible for CcOX inhibition. As outlined previously, the reaction of NO with R is a fast bimolecular process ($k = 0.4 - 1.0 \times 10^8\ M^{-1}\ s^{-1}$ at 20°C, [70, 82]), yielding a tight heme a_3^{2+}-NO adduct [74, 75]. Inhibition of CcOX is reversible, and a competition between NO and O_2 was clearly demonstrated [73], proving that the fully reduced active site is one of the targets of NO. Removal

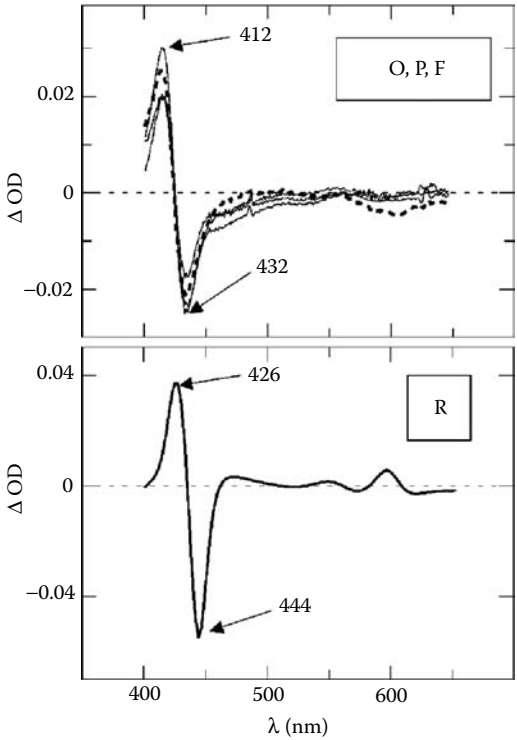

FIGURE 1.5 Spectroscopic features of the nitrosyl- and the nitrite-derivative of cytochrome c oxidase. Difference spectra recorded by mixing intermediates **O**, **P**, **F** (top panel), or **R** (bottom panel), ~2 μM CcOX functional unit (i.e., containing 2 hemes), with NO; notice the different position of the peaks. In the top panel, the difference spectra are reported after subtraction of cytochrome *a* contribution; they display high similarity and closely match the spectrum obtained by mixing oxidized CcOX with excess NO_2^-. (Modified from Giuffrè et al., *Biochemistry* (2000) 39: 15446–53.)

of free NO is associated to recovery of activity, by slow dissociation NO from the Fe^{2+} of heme a_3 ($k' = 0.01\ s^{-1}$ at 37°C [83]). This dissociation process, which is relatively fast for a heme protein [84 and references therein], is compatible with a fairly rapid respiration recovery and preservation of mitochondrial function, but it is still quite slow if compared with dissociation of nitrite from the active site under reducing conditions. Interestingly, from the experimental point of view, the NO dissociation rate is light sensitive (see below) [83, 85]. In the dark at 20°C, the functional recovery of respiration occurs at $k' = 4 \times 10^{-3}\ s^{-1}$, a value that can be dramatically accelerated by white light (e.g., up to 20- to 30-fold, by a 150 W xenon lamp, heat filtered, see Figure 1.6). As indicated by amperometric measurements performed anaerobically, in the reaction with the fully reduced enzyme, a single NO molecule binds to heme a_3 with no redox changes [86]. No evidence exists for binding of a second NO molecule to Cu_B^+,

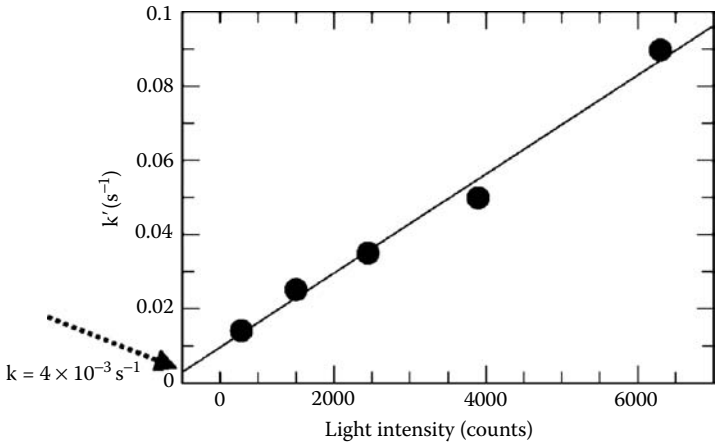

FIGURE 1.6 Light-induced dissociation of NO from fully reduced nitrosyl-cytochrome c oxidase. Fully reduced CcOX nitrosylated with stoichiometric NO is mixed with O_2 in a photodiode array stopped flow, and the intensity of the incident white light beam is varied. In the dark (back extrapolation), the observed dissociation proceeds at $k = 4 \times 10^{-3} s^{-1}$ (T = 20°C).

up to ~20 μM NO (the upper limit for amperometric measurements of the type reported in Reference 86). This finding suggests that NO has a low affinity for reduced Cu_B, and makes the hypothesis of a NO reductase-like activity of CcOX less likely, consistently with the report of Stubauer et al. [86].

The two single-electron reduced species, $\mathbf{E_1}$ and $\mathbf{E_2}$, have never been produced separately in a stable form so to allow differentiation of their reactivity, thus both have been proposed to react with NO. We tend to believe that $\mathbf{E_1}$ (i.e., the species with the reduced heme a_3) instead of $\mathbf{E_2}$ reacts with NO based on the just mentioned higher affinity of heme a_3 for the ligand in the **R** state of CcOX. Consistently, the reaction of NO with the single-electron reduced intermediate **E** also apparently yields the nitrosyl-derivative of CcOX [60]. This conclusion has been obtained by studying the reactivity toward NO of a K-channel mutant of *Paracoccus denitrificans* oxidase (K354M), in which electron transfer to the active site is severely slowed down [60]. In the presence of excess reductants, the **E** species of the K354M mutant does not display a significant reactivity toward O_2, this reaction demanding two electrons in the heme a_3-Cu_B site, whereas it promptly reacts with NO yielding the nitrosyl-derivative; see Figure 1.7.

1.7 THE REACTION BETWEEN NO AND CcOX IN TURNOVER

Both the nitrosylated- and the nitrite-bound (native) enzyme recover function by dissociation of the inhibitor (NO or NO_2^-) from the active site. In the presence of O_2, the reduced nitrosylated enzyme recovers activity at the rate of dissociation

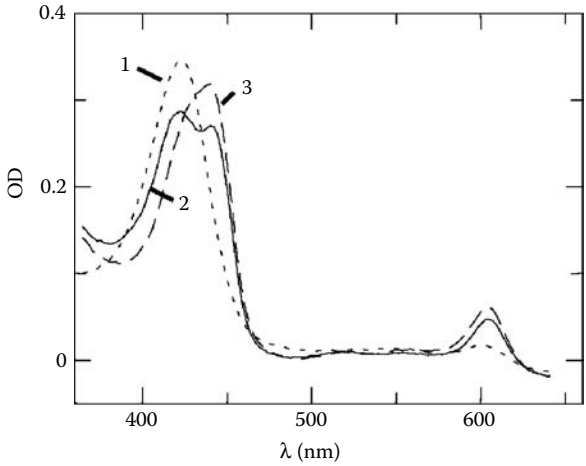

FIGURE 1.7 Absolute spectra of the K354M mutant of the *Paracoccus denitrificans* cytochrome oxidase. Absolute spectra of cytochrome c oxidase oxidized (1), heme *a*-reduced (2), and NO bound, fully reduced nitrosylated (3). (Modified from Giuffrè et al., *J. Biol. Chem.* (2002) 277: 22402–6.)

of NO from Fe^{2+} of heme a_3. Still unclear, though possibly relevant to physiology, appears to be the fate of dissociated NO. According to recent reports [77, 87], NO would be released in the bulk not as such, as originally assumed [3 and references therein] but oxidized to nitrite instead. Pearce et al. [77] claim that in the nitrosylated enzyme Cu_B^+ would react with O_2 leading to formation of superoxide, which would react within the active site with NO producing peroxynitrite, thereafter reduced to nitrite before dissociation from the enzyme. If this hypothesis holds, activity recovery of the fully reduced nitrosylated enzyme in the presence of O_2 would also be associated with the CcOX-mediated oxidative degradation of NO to nitrite.

It is agreed that the NO inhibition occurs in competition with O_2. The dependence of inhibition on O_2 concentration, however, is a complex issue; both ligands target the same site, but NO appears to react with **R** and $\mathbf{E_1}$, whereas O_2 reacts only with **R**. Moreover, NO reacts also with **O**, as well as with **P** and **F**, these two intermediates being significantly populated when turnover is sustained by low levels of reductants [61]. Finally, when dealing with the NO inhibition under turnover conditions, some attention should be paid also to the bulk reaction of NO and O_2 occurring according to the equation:

$$V = k\,[NO]^2\,[O_2];\ k_{(aq)} = 2 \times 10^6\ M^{-2}\ s^{-1},\ \text{at } 25°C\ [88]$$

a process that is faster in hydrophobic environments given the increase in the concentration of both gases [5]. Despite complexity, an apparent $K_I = 60$ nM at $[O_2] = 30$ μM has been measured using respiring synaptosomes [44]. Thus, as

outlined by Brown [89], one interesting conclusion is that in the presence of nM concentrations of NO (i.e., a condition that is fully compatible with *in vivo* physiological NO fluxes), there might be a finite amount of inhibited CcOX. The results of fluorescence microscopy experiments aimed at measuring the mitochondrial membrane potential of cultured neuroblastoma cells support this contention. In the presence of NOSs inhibitors, such as the 7-nitroindazole or the L-nitroso-arginine, the import of rhodamine is significantly higher than in their absence (Figure 1.1), demonstrating that, at least in cultured cells, a measurable NOS-mediated inhibitory effect occurs on cell respiration. Together, these observations account for the fact that the apparent K_m of CcOX for O_2 measured in tissues (≥ 1 μM) is higher than that determined *in vitro* (0.1 μM), with the purified enzyme. By using the oversimplified reaction mechanism including CcOX in turnover with reduced cytochrome c and O_2, the apparent $K_{M,O2}$ rises in the range measured *in vivo* [90]. Another interesting aspect of this issue, discussed by Moncada and Erusalimski [9], is the possible cross talk between CcOX and guanylyl-cyclase (GC). Namely, at 10 μM O_2, one may predict that 20 nM NO would inhibit 50% of CcOX; interestingly, half activation of GC by NO is also achieved at 20 nM [91]. Thus, both enzymes sense NO with comparable affinities but opposite effects (i.e., activation for GC and inhibition for CcOx); the cross talk between the two pathways appears likely, but is still obscure and needs to be further investigated.

Which one of the two NO inhibition mechanisms may predominate? By probing purified CcOX in turnover at different concentrations of reductants [83], it was found that at high reductants the nitrosyl-derivative accumulates, whereas at low reductants the nitrite-derivative is preferentially formed (Figure 1.8) [83, 92]. Amperometric measurements performed using mammalian CcOX demonstrated that in all cases, and regardless of the redox state of the enzyme, NO binds to the active site in a 1:1 stoichiometry (Figure 1.9) [61], apparently making less likely the possibility of forming N_2O.

Studies of the chemical modifications induced by NO in tissues/organs as well as *in vivo* have also been performed. In these cases, the direct assignment to a given chemical change induced by NO into any of the respiratory chain complexes might be difficult, unless a stable type of adduct is formed, such as S-nitrosothiols and nitro-tyrosines, the formation of which has been reported for only complex I so far [93].

Simultaneous measurements of cell respiration and spectral perturbation of the respiratory complexes are most informative [83, 94]. The redox changes of the mitochondrial cytochromes can be detected by multi-wavelength visible spectroscopy and correlated at given concentrations of O_2 and exogenous NO (by NO-releasers) to simultaneously measured changes in O_2 consumption. This methodological approach appears promising to elucidate the mechanism(s) by which endogenous NO produced by transiently activated constitutive NOS (Ca^{++} transient) controls cell respiration. Fluorescence microscopy [41, 42] and O_2 polarography (respirometry) [83, 92] experiments have been successfully performed to study the functional effects of the reaction between NO and CcOX.

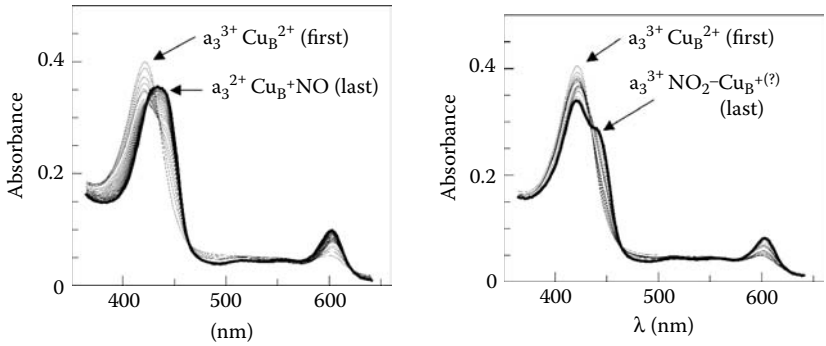

FIGURE 1.8 Spectral changes induced by NO on cytochrome c oxidase in turnover at high (left panel) and low (right panel) reductant concentration. The experiment was performed by using a sequential mixing stopped-flow, thus avoiding incubation of NO with reducing agents; the sequential mode allows the rapid (~ 1 ms) premixing of NO with chosen concentrations of reductants, followed (≥ 10 ms) by mixing with oxidized CcOX. At higher reductant concentration, the fully reduced NO-bound enzyme is formed, whereas at low reductant concentration, the nitrite-inhibited enzyme is populated. The question mark on Cu_B outlines that the redox state of the metal when nitrite is in the site is still unknown. (Modified from Sarti et al., *Biochem. Biophys. Res. Comm.* (2000) 274:183–7.)

The former approach provides information on the membrane potential set across the mitochondrial membrane; its use is particularly helpful in cytology as it allows measurements on living cells. Respirometry also may provide information on intact cells, but most importantly yields insight into the NO inhibition mechanism prevailing under a given experimental condition; it is simple, powerful and will therefore be illustrated more in detail.

In a typical polarographic experiment, purified CcOX, mitochondria, or cells are allowed to respire on reducing substrates in a reaction chamber monitoring amperometrically both O_2 and NO. As schematically depicted in Figure 1.10, when low (μM) NO is added to the system, respiration is abolished until NO is either consumed or scavenged (typically by HbO_2). In the absence of free NO, two different respiration recovery patterns can be observed. In the presence of reductants and O_2 (i.e., as in the cell or *in vivo*), the nitrite-derivative recovers immediately, whereas the nitrosyl-derivative recovers more slowly at the NO off-rate from the active site ($k \approx 0.004\ s^{-1}$ at 20°C). Thus, if nitrite-CcOX is formed, upon addition of the NO scavenger respiration reactivates immediately; on the contrary, if the nitrosyl-CcOX predominates, recovery is slow and appears autocatalytic [83, 92]. The light sensitivity of the nitrosyl-derivative of CcOX may add additional information, since illumination by increasing the rate of dissociation of NO from the nitrosylated site induces the prompt recovery of respiration (Figure 1.10). This very simple protocol allows us to distinguish between the two pathways and has been used with detergent-solubilized CcOX [83], mitochondria, and cells [92]. Interestingly, cultured neuroblastoma (SY-5Y) cells revealed a prompt recovery of respiration after addition of HbO_2, the NO scavenger, indicative of nitrite

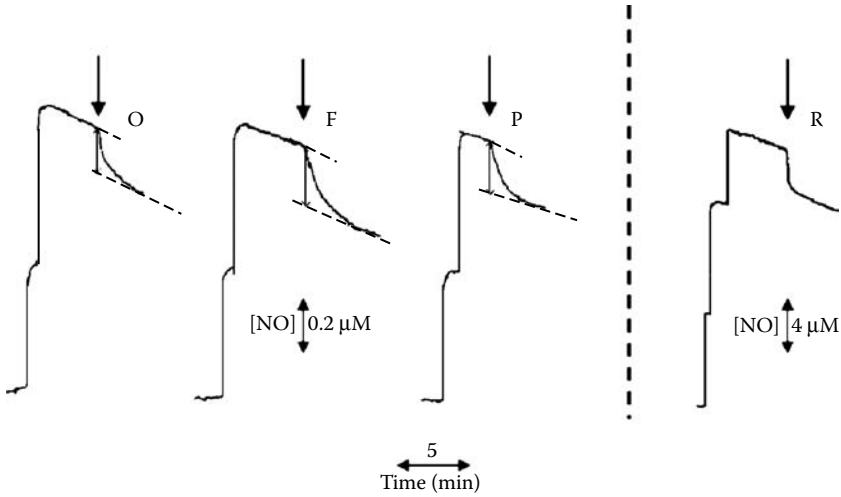

FIGURE 1.9 Amperometric determination of the NO to cytochrome c oxidase binding stoichiometry. Experiments have been performed anaerobically, using the ISO-NO World Precision Instrument apparatus equipped with a 2-mm NO-sensitive electrode. The protein at suitable concentration (0.2 and 4 μM for **O**, **P**, **F**, and **R**, respectively) is added (arrows). Notice that, even in the presence of large excess reductants, the binding of approximately 1 NO/CcOX functional unit is followed by a drift with a slope that is identical to that recorded before CcOX addition, ruling out enzymatic NO degradation. (Modified from Giuffrè et al., *Biochemistry* (2000) 39: 15446–53; Giuffrè et al., *Proc. Natl. Acad. Sci. USA* (1999) 96: 14718–23.)

formation. On the other hand, in the presence of artificial (externally added) reductants able to increase the electron transfer flux through the respiratory chain, formation of the nitrosyl-derivative was strongly enhanced, with the appearance of the slow and light-sensitive recovery from inhibition (Figure 1.11). This finding has been interpreted as indicative of a particular attitude of these cells to safely dispose of NO at the mitochondrial level, by degrading NO to nitrite. Coupled rat liver mitochondria (RCR = 4 – 6) consuming O_2 in the presence of standard concentrations of reductants (mM), such as malate and succinate, also appear to degrade NO to nitrite, particularly if under state 4 conditions. Whether mitochondria are more susceptible to nitrosylation, and thus more severely inhibited by NO, in state 3 than in state 4 still needs to be clarified [92, 95].

In synthesis, two principal reaction pathways can be observed, and the prevalence of one over the other can make the difference in terms of persistence of mitochondrial inhibition by NO. This conclusion is based on the consideration that the scavenging of free NO from the bulk phase leaves CcOX in two different conditions, depending on the prevailing mechanism. The nitrite derivative recovers activity rapidly by dissociating (innocuous) nitrite. In contrast, the nitrosylated CcOX can only recover function at the rate of NO dissociation from Fe^{2+} of heme a_3 (k ~0.01 s^{-1}, at 37°C [83]), although in this case, it is

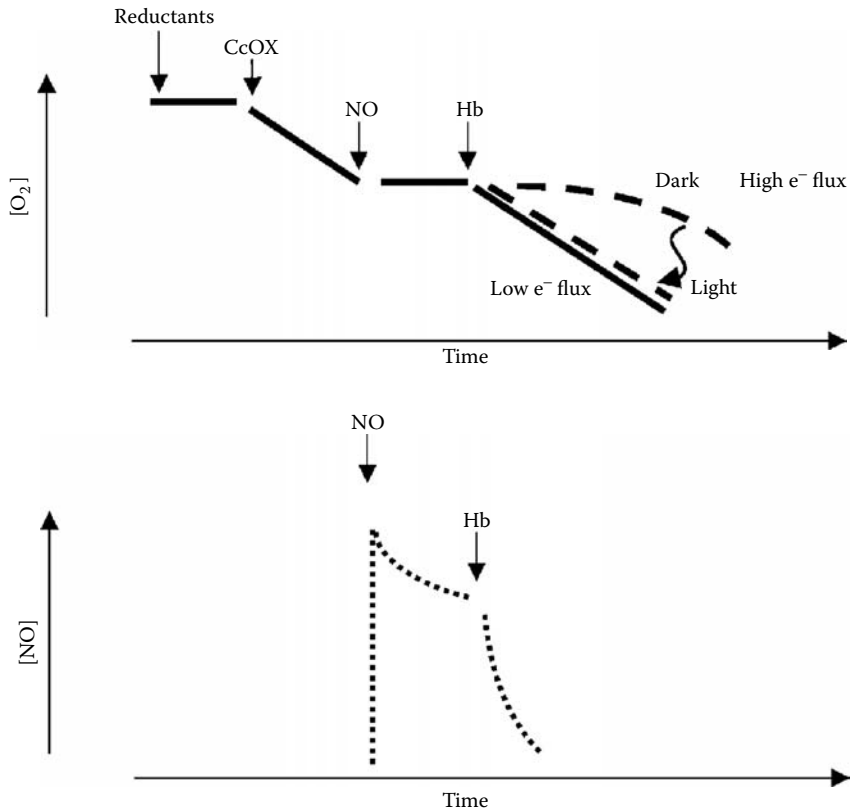

FIGURE 1.10 Investigating the NO inhibition mechanism by polarography. Typical oxygraphic (top) and nitroxygraphic (bottom) profiles observed upon inhibiting purified CcOX (or any other respiring system) by μM NO, and releasing inhibition with oxy-hemoglobin used as NO scavenger. After NO scavenging, the kinetics of recovery is either slow and lags behind NO dissociation (high electron flux through the respiratory chain) or is fast (low electron flux); further supporting this hypothesis, the light facilitates removal of inhibition by accelerating NO dissociation.

debated whether dissociated NO is released into the medium as such or as nitrite [77, 87].

1.8 EXPERIMENTAL DESIGNS

The study of the chemical reactions between NO and CcOX has been made possible thanks to the development of protocols allowing the characterization of:

1. The NO/CcOX adduct formed during turnover
2. The final state of NO after reaction, whether NO radical, oxidized to NO_2^- or reduced to N_2O

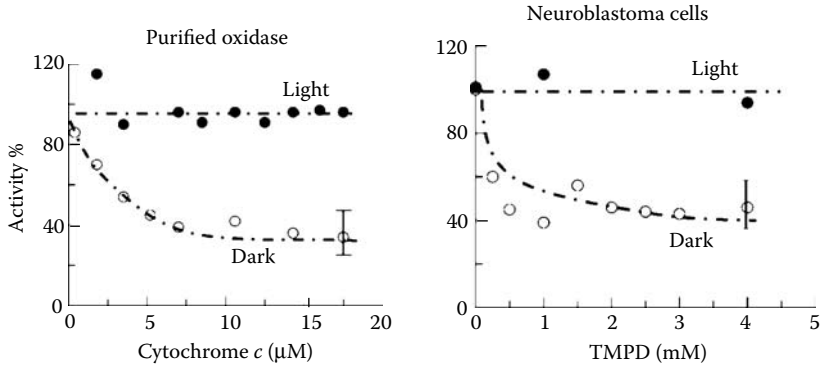

FIGURE 1.11 Effect of the reductant concentration on the NO inhibition mechanism. The increase of reduced cytochrome *c*, or TMPD (in the presence of ascorbate) favors the accumulation of the nitrosyl light-sensitive CcOX-adduct, in assays employing either purified CcOX (left panel) or cells (right panel).

The oxygraphic assay just described allows for the collection of indirect but meaningful evidence on the accumulation of either the nitrosyl or the nitrite derivative of CcOX, turning over in the presence of NO. As already pointed out this protocol is particularly useful when dealing with cells and mitochondria, which are very difficult to analyze by spectrophotometry.

Multi-wavelength stopped-flow spectroscopy proved very useful in identifying the intermediate state (adduct formed) in the reaction between detergent-solubilized CcOX and NO [83]. This approach allowed us to assign transient optical signals to either the nitrosyl- or the nitrite-derivative, by comparing experiments performed under high or low reductive pressure, mostly using ruthenium hexamine, as efficient as cytochrome c in reducing CcOX, but spectroscopically silent. In a typical experiment, oxidized N_2-equilibrated CcOX is mixed in a diode-array stopped-flow with a solution containing large excess ascorbate and variable ruthenium hexamine. Figure 1.8 illustrates the spectral changes and the time courses observed at two extreme concentrations (high and low) of ruthenium hexamine. In the absence of NO, the enzyme becomes half-reduced (with heme *a* reduced) at steady state and eventually becomes fully reduced upon O_2 exhaustion [83]. In the presence of NO, in small excess over CcOX, two different behaviors can be detected: at high reductant concentration, the enzyme becomes fully reduced nitrosylated (Figure 1.8, left panel), while at low reductant concentration (Figure 1.8, right panel) after approaching steady state, the enzyme transiently displays the features of the nitrite-bound derivative.

The reduction of NO to N_2O catalyzed by some bacterial heme-copper oxidases has been clearly detected [96–98]. Contrary to the mammalian (beef heart) enzyme that has no NO reductase activity [86], both the ba_3 and caa_3 oxidases from *Thermus thermophilus* and the cbb_3 oxidase from *Pseudomonas stutzeri* proved able to reduce NO to N_2O, as also demonstrated by head-space gas chromatography analysis [96]. The NO reductive degradation activity by terminal

oxidases can be observed under strictly anaerobic conditions both spectroscopically (Figure 1.12) and amperometrically (Figure 1.13). Measurements have to be performed (virtually) in the absence of O_2 (i.e., by degassing and N_2 equilibrating the sample) and in the presence of an O_2 scavenging system, such as glucose-glucose oxidase, in the presence of catalase to avoid accumulation of H_2O_2. The spectroscopic determination consists in measuring at different time intervals the residual concentration of NO, during anaerobic incubation with oxidase and excess reductants (Figure 1.12). In more detail, one driving syringe of the stopped flow apparatus is filled with such a solution of NO and oxidase, and mixed at the appropriate times, with deoxy-hemoglobin (or myoglobin) in excess over NO. In the presence of an enzymatic NO degradation activity, a time-dependent decrease of free NO is observed, detected as a decrease in the yield of nitrosyl-hemoglobin [96] (Figure 1.12, right panel). Similar results can be obtained amperometrically by directly monitoring NO in solution using a selective electrode [96–98]; such an experiment is depicted in Figure 1.13, where the NO reductase activity of purified *cbb*$_3$ oxidase from *P. stutzeri* is compared with the activity of bona fide NO-reductase purified from *P. denitrificans*.

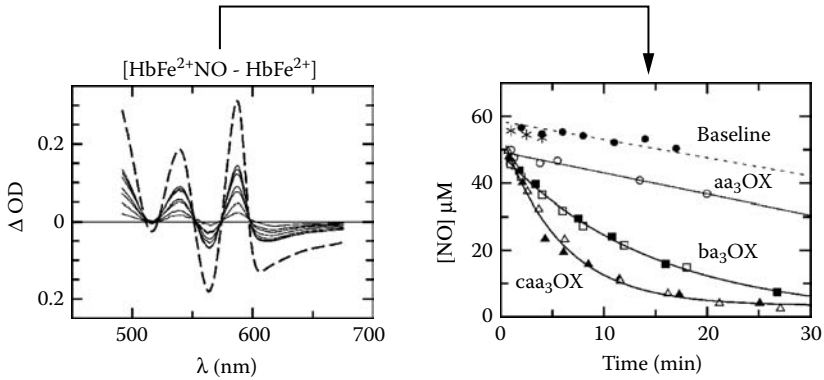

FIGURE 1.12 Reductive degradation of NO by heme-copper oxidases: spectroscopic assay. Typically, one driving syringe of the photodiode-array stopped flow apparatus is filled with an oxidase solution (mammalian or bacterial) and incubated anaerobically with NO for a suitable period of time (minutes). At appropriate times, the enzyme-NO solution is mixed with deoxy hemoglobin in excess over NO; the reaction between NO and Hb is very rapid and is complete within 100 ms, yielding the difference spectra depicted in the left panel. Notice that, at longer incubation times, the extent of the difference spectra decreases due to NO disappearance from solution. Right panel: The very slow NO degradation observed in the presence of mammalian *aa*$_3$ oxidase (5 μM) is identical to baseline, and is likely due to chemical reaction with reductants. In contrast, *ba*$_3$ (1.5 μM) and *caa*$_3$ (0.3 μM) oxidases from *T. thermophilus* bacteria degrade NO at a significant rate. (Modified from Giuffrè et al., *Proc. Natl. Acad. Sci. USA* (1999) 96: 14718–23.)

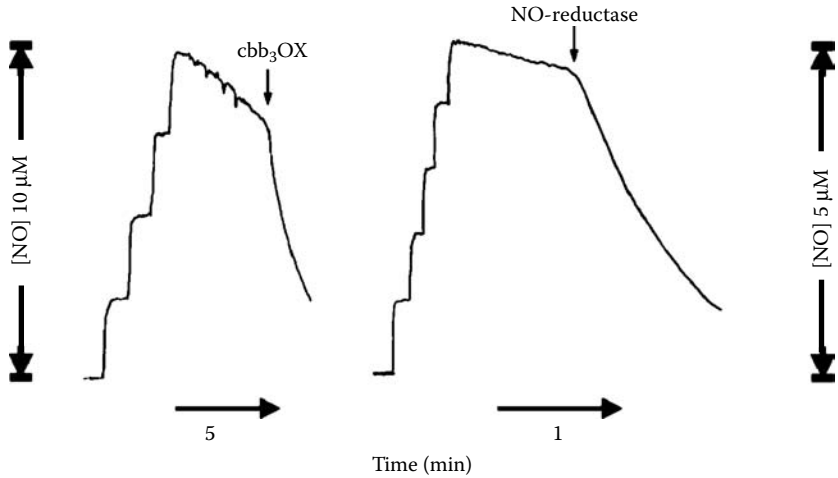

FIGURE 1.13 Reductive degradation of NO by heme-copper oxidases: amperometric assay. The NO consumption by the *cbb$_3$* oxidase (0.1 μM) from *P. stutzeri* (left panel) is measured anaerobically in the presence of excess reductants, ascorbate and tetra-methyl-p-phenylenediamine (TMPD), and is compared with the activity of bona fide NO-reductase (3 nM) purified from *P. denitrificans*. (Modified from Forte et al., *Eur. J. Biochem.* (2001) 268: 6486–91.)

1.9 PERSISTENCE OF NO IN THE MITOCHONDRION

The almost incredible number of physiological actions that NO appears to have suggests that, if out of control, its interaction with biological targets may become pathological. NO in cells and tissues is freely permeable through the membranes [36, 37]. It is worth recalling that both NO and O_2 are more soluble in the phospholipid membranes than in water [5, 99, and references therein]. Thus, the reaction between NO and O_2 is facilitated in the membrane (hydrophobic) compartments where a decreased lifetime of NO is expected. As pointed out by Shiva et al. [5] and relevant to pathophysiology, in the presence of O_2 the lipid reach environment of atherosclerotic plaques, for instance, can be responsible for a lower NO bioavailability to the mitochondrial targets (CcOX) and the close-by endothelial sites. In addition, the enhanced intramembrane reaction between NO and O_2 would yield an overproduction of N_2O_3, an efficient NO^+ donor, leading to an increase of S-nitrosated derivatives [5].

According to the two-inhibition mechanisms presented earlier, when dealing with NO and CcOX, one would expect the production of nitrite at low electron flow levels through the respiratory chain, whereas at higher levels the release of NO is expected [3]. The production of nitrite by isolated mitochondria, attributed to a mitochondrial NOS, is well documented [21, and references therein]. It is still unclear whether inhibited CcOX releases NO in the environment as such, or

as peroxynitrite or oxidized to NO_2^- [77, 87]. This is not a trivial point because all these species, including the nitroxyl anion (NO^-) as recently put forward by Shiva et al. [100], can participate in reactions relevant to mitochondrial metabolism and more, in general, to cell/tissue physiology or pathology [12].

Some apparently futile reactions, such as NO binding to and release from reduced CcOX, or the back production of NO from oxidized products, may lead to a stationary persistence of NO in the cell/mitochondrion environment. In addition, the reaction of NO with ubiquinol (QH_2) recycles NO [5, 12, 101]. This reaction generates ubisemiquinone ($Q^{\cdot}$) and a nitroxyl anion (NO^-), which is cytotoxic and elicits biological responses similar to those of NO and peroxynitrite [100]. Interestingly, the nitroxyl anion can also readily be converted back to NO by metalloproteins [12, 101, 102]. If released in excess and persistent in the mitochondrial environment, NO upon increasing of superoxide ions (O_2^-) would react forming $ONOO^-$, a powerful oxidizing agent detrimental not only to mitochondrial complexes [103, 104], but also for membranes, many other proteins, and nucleic acids. The reactions of $ONOO^-$ with all putative targets and the role played by mitochondria is a matter of intensive investigation [102, 105, 106, and references therein], but it is outside the scope of this review. In addition, peroxynitrite can be back reduced to NO by ascorbate; whether the conversion of $ONOO^-$ to NO demands or not the presence of CcOX still needs to be clarified [107]. The presence of NO is, indeed, of physiological relevance in the proximity of the endothelium, where a *higher* NO tension is required to maintain the correct vessel tone [15]. Particularly under chronic hypoxic conditions such as those characteristic of neurodegeneration, however, a detrimental cycle could be triggered by NOSs activation because the NO released, by further inhibiting respiration, might worsen the hypoxic effects.

As outlined by Moncada and Erusalimski [9], the persistent blockage of CxOX by NO may induce different responses of patho-physiological relevance; depending on the type of cell facing the NO pulse, whether able or unable to promptly activate glycolysis (e.g., astrocytes or neurons, respectively), a two-stage time response has been proposed [9, 108, 109]. The initial event is the efficient inhibition of the respiratory chain by NO, and the accumulation of reducing equivalents leading to an overproduction of superoxide ions. In the first stage, the mitochondrial superoxide dismutase would generate hydrogen peroxide at a concentration level compatible with cell signaling and physiology; at this stage, a correct balance between the radical species NO and O_2^- is set, and production of highly reactive (toxic) peroxynitrite is minimal. In contrast, in the second stage, the persistence of CcOX inhibition should lead to the accumulation of peroxynitrite, thereby triggering a more stable modification (inactivation) of the respiratory chain components, including the S-nitrosation of complex I, and activating cell responses such as apoptosis and necrosis. From the bioenergetic point of view, one key event seems to be the prompt activation of glycolysis, with production of ATP used to maintain the mitochondrial membrane potential and prevent cell death [109]. Consequently, parallel experiments were performed exposing nervous cells to pulses of exogenous NO (DETA-NO), causing no

significant toxicity in astrocytes but irreversible damage of neurons [110]. Among the numerous issues still to be elucidated, the important question regarding the NO concentration level that needs to be maintained in the cell remains to be addressed, including the length of time required to shift from a physiological to a pathological type of cell response.

1.10 ACKNOWLEDGMENTS

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