CMLS, Cell. Mol. Life Sci. 55 (1999) 1003–1014
1420-682X/99/091003-12 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Interactions of nitric oxide with hemoproteins: roles of nitric oxide in mitochondria

Y. Henry* and A. Guissani

INSERM U 350, Institut Curie (Section de Recherche), Bât. 112, Centre universitaire, F-91405 Orsay (France), Fax + 33 1 69075327, e-mail: yann.henry@curie.u-psud.fr

Abstract. Nitric oxide (NO) binds to metalloproteins, and particularly to hemoproteins in both ferrous and ferric states, with association and dissociation rate constants which cover many orders of magnitude. These chemical properties often provide clear explanations of enzymatic specificity. A basic and straightforward description of the versatility of NO chemistry and of the biological relevance of NO effects, as understood by biochemists as opposed to physiologists, is presented. NO effects on hemoglobin and soluble guanylate cyclase, two proteins directly involved in arterio-venous oxygen transport at quite different biological levels, are compared. NO and other *N*-oxides also play primary roles in several mitochondrial functions. Specific interactions with cytochrome *c* oxidase and cytochrome *c* are reviewed, and the effects of NO and other *N*-oxides on other iron-cluster-containing components of mitochondrial respiration are discussed.

Key words. Nitric oxide; peroxynitrite; hemoproteins; guanylate cyclase; cytochrome *c* oxidase; mitochondria.

Introduction

Versatility of nitric oxide chemistry and biochemistry, and the biological relevance of nitric oxide effects

After more than 10 years of research dealing explicitly with nitric oxide (nitrogen monoxide, NO), the most striking aspect is the versatility of the biological effects of NO in cells, organs, and whole bodies, from bacteria to plants and human beings. Many research groups have tried to suggest correlations of measurable biological effects with the prodigiously varied chemical reactivity of NO itself; it is associated with its molecular orbital electronic configuration and its population in a paramagnetic excited state level at room temperature [1–3]. Its versatility in aerobic medium also arises from the numerous NO-derived *N*-oxides of various oxidation number, from + 1 (nitroxyl anion or oxonitrate and its acid NO^-/HNO; and nitrous oxide gas N_2O) to + 5 (peroxynitrite or oxoperoxonitrate and its *cis* and *trans* acidic forms $ONOO^-$/ONOOH, its CO_2-adduct, nitrate ion, and the nitrosonium or nitryl cation NO_2^+), and even + 6 for the ONOO· and NO_3· radicals [1–3]. The main difficulties in establishing any kind of correlations between raw chemical or biochemical data obtained in vitro and biological data arise from the well-meant attempts by biologists to assign 'biological relevance' and by biochemists to 'give sense' to any demonstrated reaction, reversible or not, of NO or any of the *N*-oxides.

The best example of this apparent misunderstanding is the fact that many physiologists, performing in vivo or ex vivo experiments, continue to think and write that NO is short-lived and highly reactive, while chemists and biochemists know very well that this is not true in vitro. NO is neither a strong oxidant nor a strong reductant and does not usually react very fast, with several notable exceptions such as the reaction with superoxide anion, another free radical. It diffuses as rapidly and is as soluble as O_2 in water. It is more polar than O_2 and thus diffuses more rapidly through proteins and is much more soluble in organic phases. Some *N*-oxides of higher oxidation number are truly unstable in neutral water solutions and are highly reactive with nearly any neighboring compound; their action is thus often unspecific. Are these facts transposable from solutions to cells in culture and to organs?

* Corresponding author.

Very often, consideration of dissociation constants measured at equilibrium, and of on and off rate constants, are the only basis of such reasoning. Rapid, short-term and reversible chemical effects are considered as 'most important and therefore...' biologically relevant, being good candidates for regulation of physiological systems; naively NO is then termed as 'good.' Long-term and irreversible effects can be involved in pathophysiological processes, with the label 'bad' attached to NO.

At this point, in order to attempt a balanced discussion, a few very trivial considerations need to be recalled. First, irreversible, usually oxidative chemical effects can be rapidly and effectively repaired in a living cell, through highly active pools, such as that of thiols. Second, a chemical effect leading to cell killing could, depending upon the cell type involved, serve either good or bad purposes: for instance, apoptosis, a regulated and programmed cell-death process, which appears necessary for organ differentiation, appears to be inhibited in cancer development. Depending on many known and unknown factors, NO can be pro-apoptotic or anti-apoptotic [4, 5], or play a role in tumor angiogenesis [6]. There are numerous other instances in which NO has been suggested to be protective or destructive, such as cerebral ischemic injury [7]. NO possibly plays very intricate roles in cells, particularly in macrophage infection and parasitology [8]. Third, some irreversible and unspecific chemical effects, apparently suicidal and too costly in terms of energy to be repaired by certain cell types, could turn out to be truly pathological, as in chronic inflammatory processes and human autoimmune diseases [9–13]. Obviously, multiple shades exist between these extreme black and white cases; for example, all inflammatory processes, whatever the primary tissue source, are under multiple controls [14].

In vitro reactions of NO (nitrosylation) with the iron (ferrous or ferric) atom of hemoproteins are usually reversible, the dissociation time range varying from hours to fractions of seconds, resulting in either the formation of detectable nitrosyl-metal complexes or redox chemistry leading to NO^- or NO^+ characters. NO reacts through the electron pair on its nitrogen atom forming a σ-bond and through the anti-bonding $2p\pi^*$ unpaired electron forming a π-bond with transition metal d-electrons [3, 15]. Thus NO can act as a three-electron donor. NO binding modifies other iron-ligand bonds, particularly that in the *trans* position of heme. The reactions of higher-oxidation-number *N*-oxides are usually irreversible; some, however, can be reversed in vivo by the action of cellular reductive pools. The same is true for [FeS], [ZnS] clusters, and copper-containing proteins. *S*-nitrosylations of free thiols and proteins often appear reversible and can therefore be part of intra- and intercellular signalling (see the review by M. C. Broillet in this issue). In contrast, tyrosine residue nitration seems irreversible in vitro, leads to enzyme inactivation, and has been linked to many pathologies. Some degree of reversibility of protein nitration could occur in vivo.

In fact, concentrations of reactants and their targets are somewhat difficult to estimate in cells and organelles. Interesting attempts have been made to estimate the action range or 'target areas' of given compounds and to draw some conclusions about which chemical or biological effects might be expected to occur in one cell type rather than another, and thus establish their biological relevance [2, 16]. The target area around the source of a compound is defined by the product of its initial or steady-state molar concentration and the second-order rate constant for its reaction with a second compound (although expressed in s^{-1}, Crow and Beckman chose this expression of area as a conceptual image) [16]. Comparison of target areas can hint at the relative importance of two competitive reactions [16]. Given research work and time, many of these crucial considerations will probably be revealed as pertinent to cellular compartmentation and to specific cell types.

Oxygen, superoxide, and NO play blind man's bluff around hemoproteins

The reactions of NO with O_2, in gas phase and in water solution, are now well established, following rather slow third-order kinetic laws, leading to ONOONO, $NO_2^{\bullet}$ and N_2O_3 as oxidation intermediates, although the actual mechanisms are still under discussion [17–20]. The reaction appears to be much more rapid (300-fold) within biological membranes [21].

The reaction of NO with the superoxide anion or its acid form also appears now to be clear-cut with general agreement on a diffusion-limited rate constant ($6.7 \times 10^9\ M^{-1}\ s^{-1}$) (see the review by C. Ducrocq et al. in the present issue). The oxidizing and nitrating properties of the $ONOO^-$/ONOOH produced seem to be highly dependent on the presence of redox-active iron. An important conclusion was reached by Miles et al. [22], showing that 'in the absence of iron, equimolar fluxes of NO and $O_2^{-\bullet}$ interact to yield potent oxidants such as $ONOO^-$/ONOOH.' Furthermore 'excess production of either radical remarkably inhibits these oxidative reactions' [22]. These effects could have extremely variable consequences, depending on optimal conditions and on the cells producing NO and $O_2^{-\bullet}$ and on any nearby target cell. The half-life of peroxynitrite is of the order of 1 s under physiological conditions, which is considered short-lived by some and relatively long-lived by others! Thus it seems obvious that in vitro experiments making use of peroxynitrite given as a bolus at high concentrations cannot be transposed in a straightforward manner to in vivo conditions. Another reaction

producing peroxynitrite, that of molecular oxygen O_2 with the nitroxyl anion NO^-, is not often considered because its rate is estimated to be a 100-fold slower ($5.7 \times 10^7\ M^{-1}\ s^{-1}$) [23]. But knowing that O_2 concentrations could be many orders of magnitude higher than that of $O_2^{-\bullet}$, and that O_2 can diffuse freely, the formation of NO^- in vivo could also be important for cellular $ONOO^-$ production. The reaction of nitrite anion with hydrogen peroxide could also produce peroxynitrite.

The fate and the kinetic rates of diffusion of the $ONOO^-$/ONOOH system across phospholipid membranes as compared to water solutions [24, 25] and its decay are still under active discussion, particularly concerning the involvement of highly reactive radical intermediates [26–31]. The existence of the CO_2-adduct of peroxynitrite could well modify our views of peroxynitrite oxidation and nitration reactions [32–34]. All this is further complicated by the involvement of buffer composition and metal-catalyzed reactions [35, 36] (see the review by C. Ducrocq in this issue).

This basic versatility of NO and *N*-oxide chemistry has important consequences for many enzymatic systems. A fascinating example is that of NO synthases (NOSs) which have O_2 as one obligatory substrate, NO as one main product and as an inhibitor/regulator, and NO^- and $O_2^{-\bullet}$ as by-products under various conditions. All these 'mildly reactive' compounds, with low redox potentials, co-exist within rather short distances, perhaps of the order of the size of the enzyme dimer, but yield highly oxidative, nitrosating, and nitrating agents. Such complexity is frightening and discouraging. However, there is no doubt, given the current outpouring of results, that a clear picture of the intricate regulations of NOS systems will appear soon [20, 37–41] (see the review by J.-L. Boucher et al. in this issue).

Analogous problems have to be recognized for other heme-containing systems, also depending on O_2, NO and $O_2^{-\bullet}$, dioxygenases and monooxygenases, such as L-tryptophane 2,3-dioxygenase and indoleamine 2,3-dioxygenase, prostaglandin H synthase (cyclooxygenase), and P-450 monooxygenases. The same is true of mononuclear iron-containing dioxygenases, such as lipoxygenases or phenolytic dioxygenases [42, 43]. Binuclear iron-containing enzymes have also been widely studied, particularly ribonucleotide reductase, a rate-limiting enzyme in DNA synthesis, whose reversible inhibition induces cell cytostasis [44] (see review by O. Guittet et al. in this issue). A final example, which we will review in this paper, is cytochrome *c* oxidase (CcO), particularly the most studied, mitochondrial cytochrome aa_3.

Keeping in mind this introductory discussion, we shall now review the most recent data involving two hemoproteins, hemoglobin (Hb) and soluble guanylate cyclase (sGC), both directly involved in oxygen transport at quite different levels. We shall also try to explain some of the observed effects of NO and related *N*-oxides on the mitochondrial respiratory chain, mostly through the interaction with CcO.

Hemoproteins as targets of NO

NO reacts with hemoproteins in the ferrous, ferric, and ferryl ($Fe^{4+}=O$) forms, with widely different kinetics. While the association (on) rate constants of NO with ferrous hemoproteins vary little between 10^7 and 10^8 $M^{-1}\ s^{-1}$, those with the ferric forms are usually slower by many orders of magnitude (10^2–$10^7\ M^{-1}\ s^{-1}$). Differences of several orders of magnitude also exist in the dissociation (off) rate constants, dissociation being faster from ferric nitrosyl-hemoproteins. These differences often reflect whether the heme is penta- or hexacoordinated. NO is sometimes able to reduce ferric hemes, such as that of metHb, but this is not a general property, and probably depends on the redox potential of the hemoprotein considered. As we shall see, exceptions to the general rules just mentioned, reflect enzyme specificity; the best example appears in the comparison between Hb and sGC.

NO reactions with Hb

Hb has long been considered a prototype of hemoproteins and NO has been a useful paramagnetic analogue of O_2, leading to compounds observable by electron paramagnetic resonance (EPR) spectroscopy. This point has been reviewed several times [45–50]. NO binds to the heme of deoxyHb in vitro very rapidly (1×10^8–$2 \times 10^7\ M^{-1}\ s^{-1}$), depending on the R ('relaxed,' oxy, 'high-affinity') and T-('tense,' deoxy, 'low-affinity') states, respectively, and dissociates slowly (10^{-5} and $10^{-3}\ s^{-1}$, respectively), corresponding to rather long 'half-residence times' of NO on heme iron, ranging from 20 h to 12 min, respectively. As a consequence, hybrid Hb tetramers such as $\alpha(\text{Fe-NO})_2\beta(\text{Fe-deoxy})_2$ (T state) and $\alpha(\text{Fe-NO})_2\beta(\text{Fe-O}_2)_2$ (R state) have been observed both in vitro [45, 46, 51] and in vivo in animal models of e.g., pathological states, septic shock, autoimmune diseases, organ transplantations, and vascular diseases [45, 52–54]. The proportion of accumulated HbNO detected in red blood cells (RBCs) is certainly minor compared to their Hb content; its detection merely indicates NO synthesis in generator cells (e.g., macrophages, hepatocytes) and transfer to RBCs, in a given pathology.

The preferential binding of NO to α heme within the Hb tetramer and the consequences of this for allosteric properties [51, 55] may have only negligible direct ef-

fects on in vivo O_2 affinity because with NO in the 0.1 to 10 μM range, the hybrid nitrosylated Hb concentration accumulated in RBCs would probably be small (less than 100 μM compared to a 20 mM total heme concentration). A similar conclusion can be suggested for the *S*-nitrosylation of Cysβ93, which has been evoked as demonstrating a role for NO in O_2 transport, regulation of blood flow and delivery of O_2 to tissues [56–58]. The amount of accumulated *S*-nitrosylated Hb would have to be large to act effectively on O_2 transport in vivo. Note that models implying the release of NO from an α-subunit heme iron and its binding on Cysβ93 imply both redox and NO transport processes.

Finally, bearing in mind that through the arterio-venous cycle, a mean of three to four of the Hb heme sites are oxygenated, the very rapid NO-induced oxidation of HbO_2 to metHb and nitrate, with a proposed peroxynitrite intermediate ($3–5 \times 10^7$ M^{-1} s^{-1}), allows for the main catabolic pathway of NO in blood [59]. MetHb is rapidly turned over in RBCs by the metHb-reductase (cytochrome b_5 reductase) system [60]. The reaction of peroxynitrite with HbO_2 is more than three orders of magnitude slower (1×10^4 M^{-1} s^{-1}); its contribution to metHb formation should thus be negligible [25].

These rate constants measured in vitro could, however, give a false picture of what happens in whole blood. From the above-mentioned rate of HbO_2 reaction with NO, and given the Hb concentration in RBCs (30–36%, that is 20 mM heme), the NO half-life within a RBC would be of the order of 2 μs, quite outside the range of action of free NO as endothelium-derived relaxing factor (EDRF). In fact, Liu et al. [61] have shown that the apparent rate of the above reaction is considerably slowed by the rate of NO diffusion into the cell, the estimated half-life in whole blood being increased a 1000-fold to less than 2 ms, still more than a 1000-fold shorter than the half-life implied in an EDRF function. An attempt at modelling the NO concentration in blood vessels, taking into account all the above-mentioned reactions with Hb and the limit of NO dissociation from sGC (0.25 μM) (see below), led to the following conclusions: the effective diffusion distance of NO is heterogeneous in the arterio-venous cycle depending on the vessel diameter, the apparent rates of reactions of NO with Hb within RBCs are much slower than in vitro and, finally, 'the microcirculation is the optimal site for NO to exert its regulatory function' [62]. The two recent papers by Liu et al. [61] and Vaughn et al. [62] are very fine attempts at reconciling in vitro and in vivo data.

NO binding to sGC

Following the discovery of the activation of sGC by e.g., nitroglycerin, sodium nitrite and nitroprusside by Murad's group (1975), the effect of NO gas itself [63] was actively studied by many groups, particularly those of Murad (1977) and Ignarro (1979), prior to the discovery of EDRF by Furchgott and Zawadski (1980). The elaboration of new purification procedures for sGC by Marletta and coworkers, and a few other groups, raising its NO activation up to 400-fold [64, 65], and the early proposed models for this activation process [66–68], have led to recent progress in understanding this mechanism. sGC is an $\alpha\beta$ heterodimer containing *b*-type heme, with a stoichiometry of 1.5 hemes per heterodimer, suggesting that the native enzyme contains one heme *b* per monomer [69]. In contrast to its binding to Hb subunits, heme *b* is very labile in sGC and its site on the β subunit seems to be quite unique and totally different from that of known hemoproteins. In the ferrous state of unactivated sGC, the heme is high-spin pentacoordinate with a likely His residue (Hisβ105) as a proximal ligand. This reduced heme *b* does not bind O_2 and is not autoxidizable. It readily binds NO to form a pentacoordinate heme-NO complex, thus breaking the probable imidazole-iron bond *trans* to NO [64]. This was confirmed by Raman resonance (RR) and EPR spectroscopy of the ferrous-[^{14}N/^{15}N]nitrosyl complexes of sGC [70–72]. A minor hexacoordinated heme-NO component is, however, observable by EPR which could well involve another His residue, supposing a bis-imidazole enzyme intermediate [70]. Ferric sGC has an unusual high-spin heme complex with a rhombic symmetry, with weak affinities for ligands [65]. It retains a low enzymatic activity. This state probably has nothing to do with sGC activation by NO.

As indicated by the ultraviolet (UV)-visible spectra, the sGC nitrosyl-heme complex is formed during in vitro activation in the presence of GTP at low concentrations of NO (~ 8 μM NO in solution in the presence of 0.4 μM sGC) [71]. Stone and Marletta [73] have reported the rates of NO binding to the reduced heme of sGC measured by stopped-flow spectrophotometry. They proposed in a model that the enzyme is found in two populations (of 28% and 72%), with NO first binding very rapidly (7×10^8 M^{-1} s^{-1}) and reversibly (800 s^{-1}) to heme to form a hexacoordinate complex, which converts by two different slower (20 s^{-1} or 0.1–1 s^{-1} depending on the enzyme state) reversible processes into a pentacoordinate nitrosylated complex. This model also suggests extra binding of one molecule of NO to a non-heme site within the enzyme. An upper limit for the equilibrium dissociation constant of NO for the activation of the enzyme was estimated to be 0.25 μM, four orders of magnitude lower than the mean dissociation of NO from Hb in vitro [73]. In fact, the dissociation rate of NO from sGC is increased in the presence of substrate GTP, Mg^{2+}, and product cGMP, by two orders of magnitude, from $k_{obs} = 6 \times 10^{-4}$ s^{-1} to $4 \times$

10^{-2} s^{-1}, the fastest NO dissociation rates from a ferrous hemoprotein [74, 75]. This would correspond to a half-life for NO dissociation, extrapolated from 20 °C measurements to 37 °C, of 2 min and 5 s, respectively. The influence of GTP and cGMP can be explained by the proposal, based on RR spectroscopy, that either of them could bind to the distal side of the heme close to bound NO [76]. This finding was fully confirmed by experiments on the truncated heme-bound β1 N-terminal recombinant fragment of sGC (β1-385), able to bind NO both under hexa- and pentacoordinated forms [77, 78]. UV-visible and EPR spectroscopic results by Zhao et al. [78] confirm that NO binding to sGC not only leads to the cleavage of the *trans* Fe-His bond but induces a conformational change which opens the heme proximal pocket significantly. Comparisons with nitrosylated-heme model systems and other hemoproteins do not readily explain the unique activation process of sGC by NO [66–68, 74, 75].

NO as a modulator of mitochondrial functions

Among the first roles recognized for L-arginine-derived NO were its effects on some mitochondrial functions, mitochondrial aconitase, and complexes I, II and IV of the electron transport chain, in various cell types, such as macrophages, tumor cells, and hepatocytes [79–86]. Other results obtained since 1994 have demonstrated a reversible inhibition, at low levels of NO, of the last component of the respiratory chain, CcO. These findings led to reappraisals of previous data, of the exact nature of molecular targets and mechanisms involved, and to controversial points of view with respect to their biological relevance (see above). Although quantitative data obtained under physiological conditions are scarce, it is generally admitted that mitochondria produce superoxide in vivo. Competition between NO and peroxinitrite effects on the various targets are under discussion, the question centering on the diverse compartments in which the reactants NO and $O_2^{-\bullet}$ are produced. Other mitochondrial targets involving e.g., cellular Ca^{2+} homeostasis, creatine kinase and cGMP-dependent protein kinase have been suggested [87, 88]. All these alterations of mitochondrial functions have been proposed as being implicated in some neurodegenerative diseases and ischemic pathologies [89–92].

Biosynthesis of NO in mitochondria

Evidence, making use of monoclonal antibodies against endothelial NOS, has been presented for the localization of a mitochondrial NOS (mtNOS) in rat liver, heart, skeletal muscle, and kidney mitochondria [93, 94]. The existence of such a mtNOS activity has recently been confirmed in rat liver mitochondria and is associated with the inner mitochondrial membrane; it is Ca^{2+} dependent [95]. The actual production of NO was demonstrated by spin-trapping and EPR spectroscopy, in percoll-purified rat liver mitochondria, in mitochondrial homogenates, and in a crude preparation of NOS [96, 97]. Purification of mtNOS and cross-reactivity with monoclonal antibodies against macrophage NOS suggested some similarities with inducible NOS [97]. The presence of a functional mtNOS may have important implications in energy metabolism and mitochondrial respiration.

Interactions of NO with cytochrome *c* oxidase

Since the early findings, based mostly on spectroscopic methods, that NO interacts with CcO, kinetic studies have helped to discriminate various mechanisms. These in turn have allowed interpretation of the reversible functional inhibition of CcO observed more recently both in vitro and in vivo.

The target, CcO (cytochrome *aa*$_3$). CcO is a member of the heme-copper cytochrome oxidase superfamily of redox-driven proton pumps, that uses cytochrome *c* as substrate and is found in mitochondria (the prototype being bovine heart) and many bacteria.

CcO is a complex integral membrane protein made of two or three subunits in bacteria and of 13 dissimilar subunits in mammals. They all contain two inequivalent hemes *a* and two inequivalent copper centers. The electrons enter CcO, imbedded in the 40-Å-thick mitochondrial inner membrane, from the cytosol side and the protons consumed are taken up from the matrix side. All four redox-active metal centers participate in the catalytic activity, with different specific functions. Cytochrome *a* which contains a hexacoordinate low-spin heme *a* and Cu_A participates in the electron flow from reduced cytochrome *c* to the O_2-binding site, formed by the binuclear cluster, cytochrome a_3, a high-spin heme in both the oxidized and reduced forms of CcO, and Cu_B.

In the resting ferric state, the heme a_3 is strongly antiferromagnetically coupled to Cu_B yielding a net $S = 2$ paramagnetic state, which does not exhibit any EPR signal with conventional instrumentation. In the reduced state, heme a_3 is high-spin ferrous and also $S = 2$. On the open side of heme a_3 lies Cu_B with three His residues as ligands. While heme a_3 and Cu_B interact magnetically and behave as a unit, heme *a* exhibits an anti-cooperative interaction with heme a_3 and Cu_B, while Cu_A interacts allosterically with heme a_3. NO binding to these various metal sites has been characterized both structurally, by EPR, UV-visible, infrared (IR), and RR spectroscopic methods, and kinetically, by stopped-flow and double-mixing experiments [for a review see ref. 42].

NO binding to reduced CcO. When hydroxylamine NH_2OH is added aerobically to oxidized CcO, heme a_3 is reduced and a heme a_3^{2+}-NO complex is formed. A characteristic EPR spectrum of the heme a_3^{2+}-NO moiety is obtained when CcO is reduced by dithionite with authentic NO or by ascorbate in the presence of nitrite, or when mitochondria are reduced by succinate in the presence of NO.

NO binding to heme a_3^{2+} in fully reduced CcO is, as that of O_2, very fast ($0.4–1 \times 10^8$ M^{-1} s^{-1}) [98]. In fact, the cytochrome a_3^{2+}-Cu_B^+ binuclear center seems to be involved [99], and NO dissociates relatively fast from this center (0.13 s^{-1}), then reactivating the enzyme [100]. Reversal of enzyme NO-induced inhibition by O_2 does not seem to involve a reaction with the a_3^{2+}-NO complex but simply NO dissociation. These rate constants provided a clue to the mechanism of CcO inhibition by NO, at nanomolar levels (see below), and its reversal by O_2 under steady-state conditions [100]. Reaction with a partially reduced cytochrome a_3-Cu_B binuclear center may also be involved, implicating oxidized Cu_B. Inhibition by NO occurs during turnover by binding to ferrous cytochrome a_3, and is dependent on the oxygen concentration (see below) [101]. It appears that NO and O_2 compete for the same site, ferrocytochrome a_3. This means that NO at low physiological nanomolar concentrations can act as an effective regulator of the mitochondrial respiratory chain, under physiological conditions where the O_2 concentration is low [101–103].

NO binding to oxidized CcO. NO also binds to ferric CcO as measured by the appearance of a rhombic high-spin EPR signal assigned to uncoupled heme a_3^{3+}. The binding is totally reversible as the high-spin signal disappears upon NO removal. The half-binding NO pressure is 65 mmHg, i.e., $\sim$ 170 μM. It suggests that NO coordinates to Cu_B^{2+} and breaks the anti-ferromagnetic couple by forming a heme a_3^{3+}-Cu_B^{2+}-NO complex. The formation of a bridged complex a_3^{2+}-NO-Cu_B^{2+} would be irreversible, in contrast to that of the a_3^{3+}-Cu_B^{2+}-NO complex [104–106]. These reactions have probably little impact on the reversible inhibition of CcO in mitochondria (see below).

Reaction cycles catalyzed by CcO. The original observation that N_2O evolved when a mixture of N_3^- plus NO was added to oxidized CcO was confirmed by use of ^{15}N nuclear magnetic resonance, mass spectroscopy and IR spectroscopy, with different mixtures combining ^{14}NO, ^{15}NO, $^{14}N_3^-$, and $^{15}N^{14}N_2^-$ [104, 105, 107]. Depending on the redox state of the enzyme when NO or a mixture of N_3^- plus NO were added, three different catalytic cycles were hypothesized, yielding either N_2O, nitrite or possibly NO_2 as an intermediate:

$$a_3^{2+}Cu_B^+ + 2NO + 2H^+ \rightarrow a_3^{3+}Cu_B^{2+} + N_2O + H_2O$$

$$a_3^{3+} + N_3^- + NO \rightarrow a_3^{2+} + N_2O + N_2$$

$$Cu_B^{2+} + NO + H_2O \leftrightarrow Cu_B^+ + NO_2^- + 2H^+$$

and $$a_3^{3+}Cu_B^{2+} + NO + H_2O \rightarrow a_3^{2+}Cu_B^+ + NO_2 + 2H^+$$

All these reactions were observed under strictly anaerobic incubation conditions. These reactions seem too slow, some requiring hours, although CcO catalyzed, to represent a true NO-reductase activity [108]. Similar reactions occur with other copper-containing oxidases [42, 43, 103].

The whole notion of respiration inhibition by NO should be reconsidered in kinetic terms—some of which have still to be measured or remeasured quantitatively—to determine whether NO regulates the electron flows, or merely competes with O_2 as electron acceptor, or really inhibits aerobic respiration (see below). The controversial production of anesthetic N_2O could be of great importance, because it is known to occupy sites in the interior of CcO and to modify its turnover rate.

Inhibition or regulation of CcO. Complex IV was early found to be significantly inhibited by L-arginine-derived NO in murine activated macrophages and in tumor target cells [80]. Results obtained since 1994 have shown that in mitochondrial preparations from various cell types, respiration is inhibited reversibly at the distal end of the respiratory chain, CcO [101]. Incubation of rat skeletal muscle mitochondria with *S*-nitrosoglutathione and dithiothreitol reversibly inhibits CcO, determined polarographically on intact mitochondria, but not complexes I, II, and III, determined spectrophotometrically on freeze-thawed mitochondria [89]. Cleeter et al. [89] suggested that the inhibitions of complexes I and II described by previous authors are dependent on differences in methodology, particularly in mitochondrial preparations, and could be long-term effects due to NO exposure rather than the reversible inhibition of CcO. Similar conclusions were drawn by Cooper and Brown [109], who suggested that CcO inhibition is the primary event of brain cell respiration, in contrast to the inhibition of [FeS]-containing proteins of complexes I and II. Another effect of NO on rat brain and liver mitochondria is the reversible de-energization at low oxygen tension which is paralleled by release and reuptake of mitochondrial Ca^{2+} [110].

Similar experiments were carried out on rat brain synaptosomes, by following simultaneously NO and O_2 concentrations with two independent Clark-type electrodes, one NO-electrode and one O_2-electrode in the same stirred vessel, for CcO activity measurements. Brown and Cooper [101] estimated that inhibition by NO in brain synaptosomes is competitive with O_2, with half-inhibition by 270 nM NO at an O_2 concentration around the 145 μM (as in arterial blood) and by 60 nM NO at around 30 μM O_2 (around the tissue O_2 concentration). The source was NO, either gas or released from sodium nitroprusside under light, both in synapto-

somes or isolated CcO, with similar results. These levels of NO are within plausible physiological and pathological ranges, suggesting that NO inhibition of CcO and the competition with O_2 may occur in vivo [111]. NO could regulate mitochondrial respiration across the O_2 physiological range [111].

NO produced by the inducible NOS II pathway caused a similar reversible inhibition of cellular respiration in activated cultured Wistar rat astrocytes [112]. The two-electrode method showed that the activated NOS II produced a steady-state level of up to 1 μM NO which caused respiration inhibition within the same time scale. The respiration inhibition was rapidly reversed by addition of a NOS inhibitor or HbO_2. A similar inhibition was observed when astrocytes, not activated by lipopolysaccharide or interferon-γ, were incubated with pure exogenous NO. These effects on cellular respiration could perhaps partly explain neuropathological disorders, such as encephalitis, ischemia or multiple sclerosis [112].

The above studies were confirmed with Wistar rat heart or liver mitochondria exposed to different states ($\pm$ ADP) and functioning with various substrates, e.g., succinate, pyruvate plus malate, under the action of NO-saturated water solutions [113, 114]. Similar results were obtained on intact purified rat liver mitochondria in which NO synthezised from mtNOS was monitored by the reaction with HbO_2, CcO activity, and O_2 consumption measured by a Clark-electrode [115]. The dependence of CcO respiration on mitochondrial NADPH, ADP and substrates, on L-arginine and on an mtNOS inhibitor (N^{ω}-monomethyl-L-arginine) confirmed a role for NO as a physiological regulator in intact mitochondria [115].

A very important article reported the kinetics of the inhibition of isolated brown fat mitochondria by NO, and provided for the first time a quantitative mechanism of respiration inhibition [116]. The K_i of NO for respiration was $\sim$27 nM, with the IC_{50} of NO increasing in proportion to the square of an increase of O_2 tension. The K_m of O_2 for respiration was $\sim$16 μM and in the presence of NO, the dependence of respiration on O_2 tension had a Hill coefficient close to 2. CcO seems therefore to be able to use two NO or only one O_2 as electron acceptor. The presence of NO induces a 'pseudocooperative interaction' between O_2 and the respiratory system, explaining the extreme sensitivity of respiration to decreasing O_2 tension in the presence of physiological levels of NO [116].

Other targets of NO within mitochondria

We have already mentioned the effects of L-arginine-derived NO on other mitochondrial functions, particularly in macrophages and tumor target cells [79–86].

Effects on mitochondrial respiration components. Most of the five components of the mitochondrial respiratory chain in mammal cells contain [FeS] clusters. We have mentioned complex I (NADH-ubiquinone oxidoreductase) containing five [2Fe-2S], three [4Fe-4S] clusters and one flavin mononucleotide site within a complex of 25 unlike proteins, and complex II (succinate-ubiquinone oxidoreductase) containing within four different polypeptides, two [2Fe-2S] and one [4Fe-4S] clusters, a flavin adenine dinucleotide site and a cytochrome b_{560}. Complex III (ubiquinol-cytochrome *c* oxidoreductase) contains within nine to ten unlike polypeptides, one [2Fe-2S] Rieske cluster, two cytochromes *b* and one cytochrome *c*. Finally complex IV is CcO and complex V is ATPase. Thus the respiratory chain offers a host of 40 different potential metal targets for NO, altogether eight [2Fe-2S] clusters, four [4Fe-4S] clusters with different stoichiometry and in different proteic environments, two hemes *a*, three hemes *b*, one heme *c* and two copper sites. In contrast to the interaction of NO with complex IV (see above), very little is known of the molecular processes involved in the interaction of NO with complexes I and II.

The study of cytotoxic activated macrophages as a component of the immune response contributed to the discovery of the L-arginine-NO pathway. The earliest and best characterized cytostatic effect of activated macrophages was the inhibition of [FeS]-containing enzymes, particularly those involved in mitochondrial respiration. Citrate-dependent respiration (aconitase) is inhibited first ($\tau_{1/2}$ $\sim$4 h), followed by the inhibition of mitochondria complex I ($\tau_{1/2}$ $\sim$8 h) and complex II ($\tau_{1/2}$ $\sim$14 h), while complex III with a [2Fe-2S] Rieske cluster remains unaffected (see the review by Hibbs et al. [84]).

These results were extended using EPR spectroscopy, which demonstrated that L-arginine-derived NO could bind to [FeS]-cluster-containing proteins, forming an EPR signal now assigned to $Fe^{I}(NO)_2(SR)_2$ complexes. This offered an explanation of the observed inhibition of mitochondrial aconitase, a [4Fe-4S]-containing enzyme of the Krebs cycle, and of complexes I and II [117, 118]. It also demonstrated that NO synthesized in macrophages could reach cocultured tumor cells and inhibit these same enzymes in the target cells [86]. Later comparisons between the respective roles of NO and peroxynitrite have led to controversial points of view, and we will discuss, successively, [FeS]-cluster-containing respiratory components, and cytochrome *c*.

The respective roles of NO and peroxynitrite. $Fe^{I}(NO)_2(SR)_2$ complexes can be stable and reversible, and, at least in the case of aconitase and other dehydratases, the iron-sulfur cluster may also be disrupted by the loss of a labile iron (Feα). This effect of NO, and/or NO-derived oxides, is similar to those of e.g., O_2, $O_2^{-\cdot}$ on

this cluster, forming a [3Fe-4S] cluster, which leads to enzyme inactivation. In vitro experiments on purified and iron-reactivated aconitases showed that, in fact, NO formed a reversible iron-nitrosyl complex and caused only a transient inhibition and no cluster disruption [15, 119, 120]. On the other hand, according to the same authors, peroxynitrite reacts with aconitase (1.4×10^5 M^{-1} s^{-1}), and causes both a cluster disruption and irreversible aconitase inhibition. Similar effects of peroxynitrite were observed on *Escherichia coli* dehydratases [121].

In cellular systems, the iron-sulfur clusters can be reversibly repaired. New results, obtained in vitro by measurements of enzymatic activity and EPR spectroscopy on cytosolic and mitochondrial purified aconitases, confirmed the earlier results obtained with cell cultures, that NO itself under anaerobic conditions inhibits both aconitase forms [122]. Similar results were obtained on purified electron transport complexes, succinate-cytochrome *c* reductase, succinate-ubiquinone reductase, and ubiquinol-cytochrome *c* reductase, supporting the early results of Drapier and Hibbs, and others [123]. Enzyme purity, an effect of the enzyme iron-reactivation, sensitivity to pH, and other unknown factors could explain these discrepancies between the results of different groups [122, 124]. Other cellular factors, such as the effects of glutathione and other thiol pools, or glucose, could also intervene in the distinct effects of NO and peroxynitrite [125–127]. In particular, NO effect on mitochondrial complex I could be accounted for by its *S*-nitrosylation, which is in no way incompatible with $Fe^{I}(NO)_2(SR)_2$ complex formation [127].

Although the various results on the effects of NO and *N*-oxides on mitochondrial functions are often presented as being exclusive and incompatible, it is highly probable that a better understanding of experimental conditions and of the time scale of the observed effects will help in describing a full picture of what is occurring in organelles and cells. While NO may regulate cell respiration physiologically by its rapid and reversible action on complex IV, long-term (hours) exposure to NO leads to persistent inhibition of other mitochondrial functions, e.g., aconitase, complexes I and II, which may be reversed or otherwise lead to cell pathology. NO and peroxynitrite have differential roles to play in these respects.

Cytochrome *c*. Cytochrome *c* was, with Hb, one of the first nitrosylated-hemoproteins characterized by EPR spectroscopy [128]. One axial ligand Met80 is replaced by NO both in the ferric and ferrous forms, while the other His18 remains heme bound [129]. The diamagnetic nitrosylated-ferricytochrome *c* complex does not seem to be auto-reducible by NO as is that of metHb [130]. The affinity of NO for native ferrocytochrome *c* is 8.2×10^{-6} M in anaerobic conditions at pH 7 (10 °C). A recent paper by Sharpe and Cooper [131] clarifies some of these reactions. Under aerobic conditions, NO, followed by a NO-electrode, reacts slowly (200 M^{-1} s^{-1}) with ferrocytochrome *c* to yield irreversibly ferricytochrome *c* and the nitroxyl anion NO^-, which reacts further with dissolved O_2 to form peroxynitrite, as assayed by dihydrorhodamine-123 oxidation. The formation of NO^- is in agreement with the redox potential difference: $E_7^1 = +390$ mV for the NO/NO^- couple, versus $+250$ mV for the ferri/ferro couple of cytochrome *c*. NO binds slightly more rapidly to ferricytochrome *c* (1.3×10^3 M^{-1} s^{-1}) and also dissociates slowly (0.087 s^{-1}). The dissociation constant K_d, measured by direct optical observation, of 22 μM is a third of the ratio of the off over on rate constants (66 μM). These constants are within the physiological range of concentration for cytochrome *c*, the substrate of CcO. These reactions could have an impact both on the inhibition of CcO by NO, which occurs at very low NO concentrations, and on the binding of NO^- by CcO [131]. They could also be relevant to the observation of cytochrome *c* release into the cytoplasm during apoptosis.

Conclusion

Hemoproteins are primary targets of NO, which can thus exert many different biological functions, the reversibility of its effects allowing fine biological regulation. Other *N*-oxides, particularly peroxynitrite, also play important roles, often leading to irreversible and, thus, drastic biological effects. Comparison of the interactions of NO and other *N*-oxides with Hb, sGC, NOS, CcO and cytochrome *c* offers a nearly complete survey of their chemical and biological versatility.

Acknowledgements. We wish to thank our colleagues C. Ducrocq, J.-C. Drapier, M. Lepoivre, J.-P. Tenu and J.-L. Boucher for collaboration and helpful discussions over many years. Our work is supported by Centre National de la Recherche Scientifique (France), and by grants to INSERM Unité 350 from Institut National de la Santé et de la Recherche Médicale (France) and from Institut Curie, section de Recherche (France).

1 Koppenol W. H., Moreno J. J., Pryor W. A., Ischiropoulos H. and Beckman J. S. (1992) Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. Chem. Res. Toxicol. **5:** 834–842

2 Beckman J. S. (1996) The physiological and pathological chemistry of nitric oxide. In: Nitric Oxide: Principles and Actions, pp. 1–82, Lancaster J. Jr. (ed.), Academic Press, San Diego

3 Henry Y. A., Ducastel B. and Guissani A. (eds.) (1997) Basic chemistry of nitric oxide and related nitrogen oxides. In: Nitric Oxide Research from Chemistry to Biology: EPR Spectroscopy of Nitrosylated Compounds, pp. 15–46, Springer, Heidelberg

4 Albina J. E. and Reichner J. S. (1998) Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. Cancer Metastasis Rev. **17:** 39–53
5 Brüne B., Von Knethen A. and Sandau K. B. (1998) Nitric oxide and its role in apopotosis. Eur. J. Pharmacol. **351:** 261–272
6 Garcia-Cardeña G. and Folkman J. (1998) Is there a role for nitric oxide in tumor angiogenesis? J. Natl. Cancer Inst. **90:** 560–561
7 Iadecola C. (1997) Bright and dark sides of nitric oxide in ischemic brain injury. Trends Neurosci. **20:** 132–139
8 MacMicking J., Xie Q. W. and Nathan C. (1997) Nitric oxide and macrophage function. Annu. Rev. Immunol. **15:** 323–350
9 Matthys K. E. and Bult H. (1997) Nitric oxide function in atherosclerosis. Mediators Inflamm. **6:** 3–21
10 Abe K., Pan L. H., Watanabe M., Konno H., Kato T. and Itoyama Y. (1997) Upregulation of protein-tyrosine nitration in the anterior horn cells of amyotrophic lateral sclerosis. Neurol. Res. **19:** 124–128
11 Belmont H. M., Levartovsky D., Goel A., Amin A., Giorno R., Rediske J. et al. (1997) Increased nitric oxide production accompanied by the up-regulation of inducible nitric oxide synthase in vascular endothelium from patients with systemic lupus erythematosus. Arthritis Rheum. **40:** 1810–1816
12 Kimura H., Hokari R., Miura S., Shigematsu T., Hirokawa M., Akiba Y. et al. (1998) Increased expression of an inducible isoform of nitric oxide synthase and the formation of peroxynitrite in colonic mucosa of patients with active ulcerative colitis. Gut **42:** 180–187
13 Ormerod A. D., Weller R., Copeland P., Benjamin N., Ralston S. H., Grabowksi P. et al. (1998) Detection of nitric oxide and nitric oxide synthases in psoriasis. Arch. Dermatol. Res. **290:** 3–8
14 Cirino G. (1998) Multiple controls in inflammation: extracellular and intracellular phospholipase A(2), inducible and constitutive cyclooxygenase, and inducible nitric oxide synthase. Biochem. Pharmacol. **55:** 105–111
15 Radi (1996) Reactions of nitric oxide with metalloproteins, Chem. Res. Toxicol. 9: 828–835
16 Crow J. P. and Beckman J. S. (1995) Reactions between nitric oxide, superoxide, and peroxynitrite: footprints of peroxynitrite in vivo. In: Nitric Oxide: Biochemistry, Molecular Biology, and Therapeutic Implications, pp. 17–43, Ignarro L. and Murad F. (eds.), Academic Press, San Diego
17 Czapski G. and Goldstein S. (1995) The role of the reactions of NO with superoxide and oxygen in biological systems: a kinetic approach. Free Rad. Biol. Med. **19:** 785–794
18 Goldstein S. and Czapski G. (1995) Kinetics of nitric oxide autoxidation in aqueous solution in the absence and presence of various reductants: the nature of oxidizing intermediates. J. Am. Chem. Soc. **117:** 12078–12084
19 Lewis R. S., Tannenbaum S. R. and Deen W. M. (1995) Kinetics of N-nitrosation in oxygenated nitric oxide solutions at physiological pH: role of nitrous anhydride and effects of phosphate and chloride. J. Am. Chem. Soc. **117:** 3933–3939
20 Mayer B., Klatt P., Werner E. R. and Schmidt K. (1995) Kinetics and mechanism of tetrahydrobiopterin-induced oxidation of nitric oxide. J. Biol. Chem. **270:** 655–659
21 Liu X. P., Miller M. J. S., Joshi M. S., Thomas D. D. and Lancaster J. R. (1998) Accelerated reaction of nitric oxide with O_2 within the hydrophobic interior of biological membranes. Proc. Natl. Acad. Sci. USA **95:** 2175–2179
22 Miles A. M., Bohle D. S., Glassbrenner P. A., Hansert B., Wink D. A. and Grisham M. B. (1996) Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. J. Biol. Chem. **271:** 40–47
23 Wink D. A. and Feelisch M. (1996) Formation and detection of nitroxyl and nitrous oxide. In: Methods in Nitric Oxide Research, pp. 403–412, Feelisch M. and Stamler J. S. (eds.), Wiley, Chichester
24 Marla S. S., Lee J. and Groves J. T. (1997) Peroxynitrite rapidly permeates phospholipid membranes. Proc. Natl. Acad. Sci. USA **94:** 14243–14248
25 Denicola A., Souza J. M. and Radi R. (1998) Diffusion of peroxynitrite across erythrocyte membranes. Proc. Natl. Acad. Sci. USA **95:** 3566–3571
26 Goldstein S., Squadrito G. L., Pryor W. A. and Czapski G. (1996) Direct and indirect oxidations by peroxynitrite, neither involving the hydroxyl radical. Free Rad. Biol. Med. **21:** 965–974
27 Pryor W. A., Jin X. and Squadrito G. L. (1996) Insensitivity of the rate of decomposition of peroxynitrite to changes in viscosity; evidence against free radical formation. J. Am. Chem. Soc. **118:** 3125–3128
28 Pfeiffer S., Gorren A. C. F., Schmidt K., Werner E. R., Hansert B., Bohle D. S. et al. (1997) Metabolic fate of peroxynitrite in aqueous solutions: reaction with nitric oxide and pH-dependent decomposition to nitrite and oxygen in a 2:1 stoichiometry. J. Biol. Chem. **272:** 3465–3470
29 Kissner R., Nauser T., Bugnon P., Lye P. G. and Koppenol W. H. (1997) Formation and properties of peroxynitrite as studied by laser flash photolysis, high-pressure stopped-flow technique, and pulse radiolysis. Chem. Res. Toxicol. **10:** 1285–1292
30 Jourd'heuil D., Mai C. T., Laroux F. S., Wink D. A. and Grisham M. B. (1998) The reaction of S-nitrosoglutathione with superoxide. Biochem. Biophys. Res. Commun. **244:** 525–530
31 Merényi G. and Lind J. (1998) Free radical formation in the peroxynitrous acid (ONOOH)/peroxynitrite ($ONOO^-$) system. Chem. Res. Toxicol. **11:** 243–246
32 Uppu R. M., Squadrito G. L. and Pryor W. A. (1996) Acceleration of peroxynitrite oxidations by carbon dioxide. Arch. Biochem. Biophys. **327:** 335–343
33 Gow A., Duran D., Thom S. R. and Ischiropoulos H. (1996) Carbon dioxide enhancement of peroxynitrite-mediated protein tyrosine nitration. Arch. Biochem. Biophys. **333:** 42–48
34 Lymar S. V. and Hurst J. K. (1996) Carbon dioxide: physiological catalyst for peroxynitrite-mediated cellular damage or cellular protectant? Chem. Res. Toxicol. **9:** 845–850
35 Kirsch M., Lomonosova E. E., Korth H.-G., Sustmann R. and de Groot H. (1998) Hydrogen peroxide formation by reaction of peroxynitrite with HEPES and related tertiary amines: implications for a general mechanism. J. Biol. Chem. **273:** 12716–12724
36 Schmidt K., Pfeiffer S. and Mayer B. (1998) Reaction of peroxynitrite with HEPES or MOPS results in the formation of nitric oxide donors. Free Rad. Biol. Med. **24:** 859–962
37 Xia Y., Dawson V. L., Dawson T. M., Snyder S. H. and Zweier J. L. (1996) Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. Proc. Natl. Acad. Sci. USA **93:** 6770–3774
38 Xia Y. and Zweier J. L. (1997) Direct measurement of nitric oxide generation from nitric oxide synthase. Proc. Natl. Acad. Sci. USA **94:** 12705–12710
39 Xia Y., Roman L. J., Masters B. S. S. and Zweier J. L. (1998) Inducible nitric-oxide synthase generates superoxide from the reductase domain. J. Biol. Chem. **273:** 22635–22639
40 Vásquez-Vivar J., Kalyanaraman B., Martásek P., Hogg N., Masters B. S. S., Karoui H. et al. (1998) Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proc. Natl. Acad. Sci. USA **95:** 9220–9225
41 Dweik R. A., Laskowski D., Abu-Soud H. M., Kaneko F. T., Hutte R., Stuehr D. J. et al. (1998) Nitric oxide synthesis in the lung: regulation by oxygen through a kinetic mechanism. J. Clin. Invest. **101:** 660–666
42 Henry Y. A., Guissani A. and Ducastel B. (eds.) (1997) Utilisation of nitric oxide as a paramagnetic probe of the molecular oxygen binding site of metalloenzymes. In: Nitric Oxide Research from Chemistry to Biology: EPR Spec-

troscopy of Nitrosylated Compounds, pp. 99–143, Springer, Heidelberg
43 Henry Y. A., Guissani A. and Ducastel B. (eds.) (1997) General discussion: crossregulations of metalloenzymes triggered by nitric oxide. In: Nitric Oxide Research from Chemistry to Biology: EPR Spectroscopy of Nitrosylated Compounds, pp. 307–328, Springer, Heidelberg
44 Guittet O., Ducastel B., Salem J. S., Henry Y., Rubin H., Lemaire G. et al. (1998) Differential sensitivity of the tyrosyl radical of mouse ribonucleotide reductase to nitric oxide and peroxynitrite. J. Biol. Chem. **273:** 22136–22144
45 Henry Y., Ducrocq C., Drapier J.-C., Servent D., Pellat C. and Guissani A. (1991) Nitric oxide, a biological effector: electron paramagnetic resonance detection of nitrosyl-iron-protein complexes in whole cells. Eur. Biophys. J. **20:** 1–15
46 Henry Y., Lepoivre M., Drapier J.-C., Ducrocq C., Boucher J.-L. and Guissani A. (1993) EPR characterisation of molecular targets for NO in mammalian cells and organelles. FASEB J. **7:** 1124–1134
47 Singel D. J. and Lancaster J. R. (1996) Electron paramagnetic resonance spectroscopy and nitric oxide biology. In: Methods in Nitric Oxide Research, pp. 341–356, Feelisch M. and Stamler J. S. (eds.), Wiley, Chichester
48 Henry Y. and Singel D. J. (1996) Metal-nitrosyl interactions in nitric oxide biology probed by electron paramagnetic resonance spectroscopy. In: Methods in Nitric Oxide Research, pp. 357–372, Feelisch M. and Stamler J. S. (eds.), Wiley, Chichester
49 Kosaka H. and Shiga T. (1996) Detection of nitric oxide by electron spin resonance using hemoglobin. In: Methods in Nitric Oxide Research, pp. 373–381, Feelisch M. and Stamler J. S. (eds.),Wiley, Chichester
50 Henry Y. A., Guissani A. and Ducastel B. (eds.) (1997) EPR characterization of nitric oxide binding to hemoglobin. In: Nitric Oxide Research from Chemistry to Biology: EPR Spectroscopy of Nitrosylated Compounds, pp. 61–86, Springer, Heidelberg
51 Yonetani T., Tsuneshige A., Zhou Y. X. and Chen X. S. (1998) Electron paramagnetic resonance and oxygen binding studies of α-nitrosyl hemoglobin: a novel oxygen carrier having NO-assisted allosteric functions. J. Biol. Chem. **273:** 20323–20333
52 Henry Y. A., Guissani A. and Ducastel B. (eds.) (1997) Overproduction of nitric oxide in physiology and pathophysiology: EPR detection. In: Nitric Oxide Research from Chemistry to Biology: EPR Spectroscopy of Nitrosylated Compounds, pp. 235–270, Springer, Heidelberg
53 Weinberg J. B., Gilkeson G. S., Mason R. P. and Chamulitrat W. (1998) Nitrosylation of blood hemoglobin and renal nonheme proteins in autoimmune MRL-lpr/lpr mice. Free Rad. Biol. Med. **24:** 191–196
54 Takahashi Y., Kobayashi H., Tanaka N., Sato T., Takizawa N. and Tomita T. (1998) Nitrosyl hemoglobin in blood of normoxic and hypoxic sheep during nitric oxide inhalation. Am. J. Physiol. 274 (Heart Circul. Physiol.) **43:** H349–H357
55 Kosaka H. and Seiyama A. (1996) Physiological role of nitric oxide as an enhancer of oxygen transfer from erythrocytes to tissues. Biochem. Biophys. Res. Commun. **218:** 749–752
56 Jia L., Bonaventura C., Bonaventura J. and Stamler J. S. (1996) *S*-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. Nature **380:** 221–226
57 Stamler J. S., Jia L., Eu J. P., McMahon T. J., Demchenko I. T., Bonaventura J. et al. (1997) Blood flow regulation by *S*-nitrosohemoglobin in the physiological oxygen gradient. Science **276:** 2034–2036
58 Gow A. J. and Stamler J. (1998) Reactions between nitric oxide and haemoglobin under physiological conditions. Nature **391:** 169–173
59 Eich R. F., Li T. S., Lemon D. D., Doherty D. H., Curry S. R., Aitken J. F. et al. (1996) Mechanism of NO-induced oxidation of myoglobin and hemoglobin. Biochemistry **35:** 6976–6983
60 Henry Y. A., Guissani A. and Ducastel B. (eds.) (1997) Effects of nitric oxide on red blood cells. In: Nitric Oxide Research from Chemistry to Biology: EPR Spectroscopy of Nitrosylated Compounds, pp. 87–98, Springer, Heidelberg
61 Liu X. P., Miller M. J. S., Joshi M. S., Sadowska-Krowicka H., Clark D. A. and Lancaster J. R. (1998) Diffusion-limited reaction of free nitric oxide with erythrocytes. J. Biol. Chem. **273:** 18709–18713
62 Vaughn M. W., Kuo L. and Liao J. C. (1998) Effective diffusion distance of nitric oxide in the microcirculation. Am. J. Physiol. 274 (Heart Circul. Physiol.) **43:** H1705–H1714
63 Arnold W. P., Mittal C. K., Katsuki S. and Murad F. (1977) Nitric oxide activates guanylate cyclase and increases guanosine 3′:5′-cyclic monophosphate levels in various tissue preparations. Proc. Natl. Acad. Sci. USA **74:** 3203–3207
64 Stone J. R. and Marletta M. A. (1994) Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. Biochemistry **33:** 5636–5640
65 Stone J. R., Sands R. H., Dunham W. R. and Marletta M. A. (1996) Spectral and ligand-binding properties of an unusual hemoprotein, the ferric form of soluble guanylate cyclase. Biochemistry **35:** 3258–3262
66 Traylor T. G. and Sharma V. S. (1992) Why NO? Biochemistry **31:** 2847–2849
67 Traylor T. G., Duprat A. F. and Sharma V. S. (1993) Nitric oxide-triggered heme-mediated hydrolysis: a possible model for biological reactions of NO. J. Am. Chem. Soc. **115:** 810–811
68 Tsai A.-l. (1994) How does NO activate hemeproteins? FEBS Lett. **341:** 141–145
69 Stone J. R. and Marletta M. A. (1995) Heme stoichiometry of heterodimeric soluble guanylate cyclase. Biochemistry **34:** 14668–14674
70 Yu A. E., Hu S., Spiro T. G. and Burstyn J. N. (1994) Resonance Raman spectroscopy of soluble guanylate cyclase reveals displacement of distal and proximal heme ligands by NO. J. Am. Chem. Soc. **116:** 4117–4118
71 Stone J. R., Sands R. H., Dunham W. R. and Marletta M. A. (1995) Electron paramagnetic resonance spectral evidence for the formation of a pentacoordinated nitrosyl-heme complex on soluble guanylate cyclase. Biochem. Biophys. Res. Commun. **207:** 572–577
72 Deinum G., Stone J. R., Babcock G. T. and Marletta M. A. (1996) Binding of nitric oxide and carbon monoxide to soluble guanylate cyclase as observed with resonance Raman spectroscopy. Biochemistry **35:** 1540–1547
73 Stone J. R. and Marletta M. A. (1996) Spectral and kinetic studies on the activation of soluble guanylate cyclase by nitric oxide. Biochemistry **35:** 1093–1099
74 Kharitonov V. G., Sharma V. S., Magde D. and Koesling D. (1997) Kinetics of nitric oxide dissociation from five- and six-coordinate nitrosyl hemes and heme proteins including soluble guanylate cyclase. Biochemistry **36:** 6814–6818
75 Kharitonov V. G., Russwurm M., Magde D., Sharma V. S. and Koesling D. (1997) Dissociation of nitric oxide dissociation from soluble guanylate cyclase. Biochem. Biophys. Res. Commun. **239:** 284–287
76 Tomita T., Ogura T., Tsuyama S., Imai Y. and Kitagawa T. (1997) Effects of GTP on bound soluble guanylate cyclase probed by resonance Raman spectroscopy. Biochemistry **36:** 10155–10160
77 Zhao Y. and Marletta M. A. (1997) Localization of the heme binding region in soluble guanylate cyclase. Biochemistry **36:** 15959–15964
78 Zhao Y., Hoganson C., Babcock G. T. and Marletta M. A. (1998) Structural changes in the heme proximal pocket induced by nitric oxide binding to soluble guanylate cyclase. Biochemistry **37:** 12458–12464
79 Drapier J.-C. and Hibbs J. B. Jr. (1986) Murine cytotoxic activated macrophages inhibit aconitase in tumor cells: inhibition involves the iron-sulfur prosthetic group and is reversible. J. Clin. Invest **78**: 790–797

80 Drapier J.-C. and Hibbs J. B. Jr. (1988) Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. J. Immunol. **140**: 2829–2838

81 Hibbs J. B., Jr., Vavrin Z. and Taintor R. R. (1987) L-Arginine is required for expression of the activated macrophage effector mechanism causing selective metabolism inhibition in target cells. J. Immunol. **138**: 550–565

82 Lancaster J. R., Jr., Laster S. M. and Gooding L. R. (1989) Inhibition of target cell mitochondrial electron transfer by tumor necrosis factor. FEBS Lett. **248**: 169–174

83 Stuehr D. J. and Nathan C. F. (1989) Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J. Exp. Med. **169:** 1543–1555

84 Hibbs J. B., Jr., Taintor R. R., Vavrin Z., Granger D. L., Drapier J.-C., Amber I. J. et al. (1990) Synthesis of nitric oxide from a terminal guanidino nitrogen atom of L-arginine: a molecular mechanism regulating cellular proliferatiuon that targets intracellular iron. In: Nitric Oxide from L-Arginine: A Bioregulatory Mechanism, pp. 189–223, Moncada S. and Higgs E. A. (eds.), Elsevier, Amsterdam

85 Stadler J., Billiar T. R., Curran R. C., Stuehr D. J., Ochoa J. B. and Simmons R. L. (1991) Effects of exogenous and endogenous nitric oxide on mitochondrial respiration of rat hepatocytes. Am. J. Physiol. **260:** C910–C916

86 Drapier J.-C., Pellat C. and Henry Y. (1991) Generation of EPR-detectable nitrosyl-iron complexes in tumor target cells cocultured with activated macrophages. J. Biol. Chem. **266:** 10162–10167

87 Stachowiak O., Dolder M., Wallimann T. and Richter C. (1998) Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. J. Biol. Chem. **273:** 16694–16699

88 Young M. E. and Leighton B. (1998) Fuel oxidation in skeletal muscle is increased by nitric oxide/cGMP: evidence for involvement of cGMP-dependent protein kinase. FEBS Lett. **424:** 79–83

89 Cleeter M. W. J., Cooper J. M., Darley-Usmar V. M., Moncada S. and Schapira A. H. V. (1994) Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide: implications for neurodegenerative diseases. FEBS Lett. **345:** 50–54

90 Shergill J. K., Cammack R., Cooper C. E., Cooper J. M., Mann V. M. and Schapira A. H. V. (1996) Detection of nitrosyl complexes in human substantia nigra, in relation to Parkinson's disease. Biochem. Biophys. Res. Commun. **228:** 298–305

91 Bolaños J. P., Almeida A., Stewart V., Peuchen S., Land J. M., Clark J. B. et al. (1997) Nitric oxide-mediated mitochondrial damage in the brain: mechanisms and implications for neurodegenerative diseases. J. Neurochem. **68:** 2227–2240

92 Brown G. C. (1997) Nitric oxide inhibition of cytochrome oxidase and mitochondrial respiration: implications for inflammatory, neurodegenerative and ischaemic pathologies. Mol. Cell. Biochem. **174:** 189–192

93 Kobzik L., Stringer B., Balligand J.-L., Reid M. B. and Stamler J. S. (1995) Endothelial type nitric oxide synthase in skeletal muscle fibers: mitochondrial relationships. Biochem. Biophys. Res. Commun. **211:** 375–381

94 Bates T. E., Loesch A., Burnstock G. and Clark J. B. (1996) Mitochondrial nitric oxide synthase: a ubiquitous regulator of oxidative phosphorylation? Biochem. Biophys. Res. Commun. **218:** 40–44

95 Ghafourifar P. and Richter C. (1997) Nitric oxide synthase activity in mitochondria. FEBS Lett. **418:** 291–296

96 Giulivi C., Poderoso J. J. and Boveris A. (1998) Production of nitric oxide by mitochondria. J. Biol. Chem. **273:** 11038–11043

97 Tatoyan A. and Giulivi C. (1998) Purification and characterization of a nitric-oxide synthase from rat liver mitochondria. J. Biol. Chem. **273:** 11044–11048

98 Blackmore R. S., Greenwood C. and Gibson Q. H. (1991) Studies of the primary intermediate in the reaction of fully reduced cytochrome oxidase. J. Biol. Chem. **266:** 19245–19249

99 Zhao X. J., Sampath V. and Caughey W. S. (1994) Infrared characterization of nitric oxide bonding to bovine heart cytochrome *c* oxidase and myoglobin. Biochem. Biophys. Res. Commun. **204:** 537–543

100 Giuffrè A., Sarti A., D'Itri E., Buse G., Soulimane T. and Brunori M. (1996) On the mechanism of inhibition of cytochrome c oxidase by nitric oxide. J. Biol. Chem. **271:** 33404–33408

101 Brown G. C. and Cooper C. E. (1994) Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. FEBS Lett. **356:** 295–298

102 Torres J., Darley-Usmar V. and Wilson M. T. (1995) Inhibition of cytochrome c oxidase in turnover by nitric oxide: mechanism and implications for control of respiration. Biochem. J. **312:** 169–173

103 Cooper C. E., Torres J., Sharpe M. A. and Wilson M. T. (1997) Nitric oxide ejects electrons from the binuclear centre of cytochrome *c* oxidase by reacting with oxidised copper: a general mechanism for the interaction of copper proteins with nitric oxide? FEBS Lett. **414:** 281–284

104 Stevens T. H., Brudvig G. W., Bocian D. P. and Chan S. I. (1979) Structure of cytochrome a_3-Cu_{a3} couple in cytochrome *c* oxidase as revealed by nitric oxide binding studies. Proc. Natl. Acad. Sci. USA **76:** 3320–3324

105 Brudvig G. W., Stevens T. H. and Chan S. I. (1980) Reactions of nitric oxide with cytochrome *c* oxidase. Biochemistry **19:** 5275–5285

106 Rousseau D. L., Sing S., Ching Y.-C. and Sassaroli M. (1988) Nitrosyl cytochrome *c* oxidase: formation and properties of mixed valence enzyme. J. Biol. Chem. **263:** 5681–5685

107 Zhao X. J., Sampath V. and Caughey W. S. (1995) Cytochrome *c* oxidase catalysis of the reduction of nitric oxide to nitrous oxide. Biochem. Biophys. Res. Commun. **212:** 1054–1060

108 Stubauer G., Giuffrè A., Brunori M. and Sarti P. (1998) Cytochrome *c* oxidase does not catalyze the anaerobic reduction of NO. Biochem. Biophys. Res. Commun. **245:** 459–465

109 Cooper C. E. and Brown G. C. (1995) The interactions between nitric oxide and brain nerve terminals as studied by electron paramagnetic resonance. Biochem. Biophys. Res. Commun. **212:** 404–412

110 Schweizer M. and Richter C. (1994) Nitric oxide potently and reversibly deenergizes mitochondria at low oxygen tension. Biochem. Biophys. Res. Commun. **204:** 169–175

111 Brown G. C. (1995) Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. FEBS Lett. **369:** 136–139

112 Brown G. C., Bolaños J. P., Heales S. J. R. and Clark J. B. (1995) Nitric oxide produced by activated astrocytes rapidly and reversibly inhibits cellular respiration. Neurosci. Lett. **193:** 201–204

113 Borutaité V. and Brown G. C. (1996) Rapid reduction of nitric oxide by mitochondria, and reversible inhibition of mitochondrial respiration by nitric oxide. Biochem. J. **315:** 295–299

114 Okada S., Takehara Y., Yabuki M., Yoshioka T., Yasuda T., Inoue M. et al. (1996) Nitric oxide, a physiological modulator of mitochondrial function. Physiol. Chem. Phys. Med. NMR **28:** 69–82

115 Giulivi C. (1998) Functional implications of nitric oxide produced by mitochondria in mitochondrial metabolism. Biochem. J. **332:** 673–679

116 Koivisto A., Matthias A., Bronnikov G. and Nedergaard J. (1997) Kinetics of the inhibition of mitochondrial respiration by NO. FEBS Lett. **417:** 75–80

117 Pellat C., Henry Y. and Drapier J.-C. (1990) IFN-γ-activated macrophages: detection by electron paramagnetic resonance of complexes between L-arginine-derived nitric oxide

and non-heme iron proteins. Biochem. Biophys. Res. Commun. **166:** 119–125
118 Lancaster J. R., Jr. and Hibbs J. B., Jr. (1990) EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. Proc. Natl. Acad. Sci. USA 87: 1223–1227
119 Hausladen A. and Fridovich I. (1994) Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not. J. Biol. Chem. **269:** 29405–29408
120 Castro L., Rodriguez M. and Radi R. (1994) Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. J. Biol. Chem. **269:** 29409–29415
121 Keyer K. and Imlay J. A. (1997) Inactivation of dehydratase [4Fe-4S] clusters and disruption of iron homeostasis upon cell exposure to peroxynitrite. J. Biol. Chem. **272:** 27652–27659
122 Kennedy M. C., Antholine W. E. and Beinert H. (1997) An EPR investigation of the products of the reaction of cytosolic and mitochondrial aconitases with nitric oxide. J. Biol. Chem. **272:** 20340–20347
123 Welter R., Yu L. and Yu C.-A. (1996) The effects of nitiric oxide on electron transport complexes. Arch. Biochem. Biophys. **331:** 9–14
124 Gardner P. R., Costantino G., Szabó C. and Salzman A. L. (1997) Nitric oxide sensitivity of the aconitases. J. Biol. Chem. **272:** 25071–25076
125 Lizasoain I., Moro M. A., Knowles R. G., Darley-Usmar V. and Moncada S. (1996) Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose. Biochem. J. **314:** 877–880
126 Cheung P.-Y., Danial H., Jong J. and Schulz R. (1998) Thiols protect the inhibition of myocardial aconitase by peroxynitrite. Arch. Biochem. Biophys. **350:** 104–108
127 Clementi E., Brown G. C., Feelisch M. and Moncada S. (1998) Persistent inhibition of cell respiration by nitric oxide: crucial role of *S*-nitrosylation of mitochondrial complex I and protective action of glutathione. Proc. Natl. Acad. Sci. USA **95:** 7631–7636
128 Kon H. (1969) Electron paramagnetic resonance of nitric oxide cytochrome *c*. Biochem. Biophys. Res. Commun. **35:** 423–427
129 Ascenzi P., Coletta M., Santucci R., Polizio F. and Desideri A. (1994) Nitric oxide binding to ferrous native horse heart cytochrome *c* and to its carboxymethylated derivative: a spectroscopic and thermodynamic study. J. Inorg. Biochem. **53:** 273–280
130 Ehrenberg A. and Szczepkowski T. W. (1960) Properties and structure of the compounds formed between cytochrome *c* and nitric oxide. Acta Chem. Scand. **14:** 1684–1692
131 Sharpe M. A. and Cooper C. E. (1998) Reactions of nitric oxide with mitochondrial cytochrome *c*: a novel mechanism for the formation of nitroxyl anion and peroxynitrite. Biochem. J. **332:** 9–19