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Nitric oxide: a radical molecule in quest of free radicals in proteins

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Abstract. A number of enzymes use an amino acid free radical cofactor. Tyrosyl and tryptophanyl radicals react with nitric oxide (NO) with an almost diffusion-limited rate. The catalytically competent tyrosyl radical in ribonucleotide reductase (RR) and prostaglandin H synthase (PGHS) recombines with NO in a radical-radical reaction. The unstable adduct formed can dissociate to regenerate the tyrosyl radical. However, upon prolonged incubation with NO, the diiron center of mouse RR leaks out, while the adduct is sucessively oxidized into an iminoxyl radical and a nitrotyrosine in PGHS. These data provide a plausible mechanism for the physiological inactivation of RR observed in various models, and may help in understanding the inhibition of PGHS reported in some cases. Reversible combination with NO is an intrinsic property of tyrosyl radicals, which also occurs with $Y_D^{\bullet}$ and $Y_Z^{\bullet}$ in photosystem II, where NO has been useful in the analysis of the oxygen-evolving complex.

Key words. Nitric oxide; tyrosyl radical; radicals; nitrosotyrosine; nitrotyrosine; ribonucleotide reductase; prostaglandin H synthase; photosystem II.

Introduction

Nitric oxide (NO) is a free radical molecule and is therefore susceptible to react with other free radicals in addition reactions. However, until the last decade, the free radical chemistry of NO was relatively ignored. Recently, the identification of NO as a biological mediator of fundamental importance in mammalian physiology and pathology has led to reexamination of this important facet of its reactivity. Several radicals of biological relevance have been found to react with NO. Most importantly, the biosynthesis of peroxynitrite results from a radical combination reaction involving NO and the superoxide anion $O_2^{\bullet -}$. Peroxynitrite is now known to be a potent oxidant and also a superoxide trap suspected to support some of the biological functions previously attributed to NO itself [reviewed in ref. 1]. A consequent protective role for NO in atherogenesis has been inferred from radical-radical termination reactions between NO and the alkoxyl $RO^{\bullet}$ and peroxyl $ROO^{\bullet}$ radical intermediates catalyzing lipid peroxidation [2, 3]. Most of these reactions, as well as others involving inorganic radicals like $NO_2^{\bullet}$ and $OH^{\bullet}$ [3], proceed at an almost diffusion-limited rate ($k \geq 1 \times 10^9$ M^{-1} s^{-1}), several orders of magnitude higher than that for the reaction of NO with oxygen ($k = 7 \times 10^6$ M^{-2} s^{-1}) or oxyhemoglobin ($k = 3 \times 10^7$ M^{-1} s^{-1}) [3]. This holds also for the very rapid radical-radical coupling reactions of NO with tyrosyl ($Tyr^{\bullet}$) and tryptophanyl ($Trp^{\bullet}$) radicals in free amino acids or polypeptides, as shown by pulse-radiolysis experiments (8×10^8 M^{-1} $s^{-1} \leq k \leq 2 \times 10^9$ M^{-1} s^{-1}) [4]. Such amino acid free radicals either appear in oxidized, altered proteins or, conversely, fulfill a tightly controlled, essential function in enzyme systems [5]. The increasingly recognized role of free radicals in protein function, associated with their extremely fast reaction rate with NO, has led to the idea that protein radicals are likely an important biological target for nitrogen oxides (NO). This review will summarize the current knowledge in this research area, which has provided important information regarding the role of protein-centered free radicals in enzyme chemistry. As extremely reactive species, free radicals usually exhibit a very brief half-life. Most essential amino acid free radicals are thus generated transiently

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during the catalytic cycle, a characteristic that can limit investigations. It is not surprising that the earliest reports considered ribonucleotide reductase (RR) and photosystem II (PSII), two favorable models in which the free radical exhibits an unusual stability [6, 7]. Recently, investigations have been extended to the more transient tyrosyl free radical in prostaglandin H synthase (PGHS) [8].

Ribonucleotide reductase

A docked radical in RR

The first piece of evidence for the existence of an amino acid free radical in a protein, generated as an intrinsic part of its polypeptide structure and involved in electron transfer, came from Swedish studies on RR in the late 1970s. The radical was attributed to a tyrosyl residue in the *Escherichia coli* enzyme and subsequently, in the reductase from mouse and phage T4 [9; and refs in 10]. Since these pioneering studies, many other reductases have been characterized. RR activity is essential to all living organisms, because the reduction of ribonucleotides into deoxyribonucleotides provides the building blocks for DNA synthesis and repair. Despite considerable structural and functional differences, all RRs catalyze a radical-driven reduction of the substrates and all employ an amino acid free radical to initiate this radical chemistry [recently reviewed in refs 10, 11]. Three classes of reductases have been delineated, based on the identity of the radical cofactor as well as on structural and regulatory properties. A catalytically competent tyrosyl radical is present in class Ia and Ib enzymes. Those in class II are adenosylcobalamin-dependent reductases found in eubacteria and archaebacteria. Class III enzymes are anaerobic reductases containing a [FeS] center and a glycyl free radical.

So far, only the two prototypes of subclass Ia (i.e., the mouse and *E. coli* enzymes) have been investigated for NO reactivity [7, 12, 13]. Reductases from this group are $\alpha_2\beta_2$ complexes [see refs 10, 11, 14 for reviews]. They are expressed in all types of eukaryotes and in some prokaryotes including DNA viruses (for instance vaccinia and herpes viruses), and a few bacteria. The large α_2 dimer, called R1, binds the four diphosphate substrates ADP, CDP, UDP, and GDP. It also contains two additional sites per protomer that bind allosteric effectors regulating the specificity and the catalytic rate of the reductase. This finely tuned regulation controls the formation of non-identical desoxyribonucleotide pools required for the high-fidelity replication of DNA [15]. The R1 protein also provides redox-active cysteines involved in substrate reduction. The small β_2 subunit harbors the tyrosyl free radical, which exhibits unusual stability. For example, the radical first identified in an *E. coli* R2 protein was detected after a 2-week purification procedure [9]. With a half-life of 30 min at 37 °C, the free radical of the mouse R2 protein is also very stable [16]. Several factors contribute to this surprising stability. First, tyrosyl free radicals are usually delocalized over the aromatic ring, with highest electronic densities not only on the phenolic oxygen but also on the C1 and C3/C5 carbon atoms [5, 17]. This delocalization renders Tyr$^\bullet$ less reactive. Second, elucidation of three-dimensional X-ray structures of the *E. coli* and mouse R2 dimers has revealed a tyrosine residue buried deep inside the protein core where it is protected from solvent reactivity, although it appears to be more accessible in the mammalian protein [18, 19]. No oxidizable amino acid side chains are in van der Waals' contact with the free radical in the protein. In contrast, the environment of the radical-carrying tyrosine comprises hydrophobic residues, including three invariant ones, which form a hydrophobic pocket that greatly influences the stability of the radical. Mutational studies of the invariant residues have shown that minor changes (e.g., F212Y) resulted in a dramatic decrease in radical half-life [20]. It has been proposed that the apolar residues constitute an insulator that shields the radical from the solvent. Finally, the tyrosyl free radical of all class Ia enzymes is magnetically coupled with a pair of (μ-oxo)-bridged non-heme iron atoms. They are close to the radical and involved in its production, stabilization, and catalytic activity [21].

The existence of Tyr$^\bullet$ is linked to a functional iron center. Removing the metal from the R2 protein with iron chelators produces a radical-free inactive enzyme [16, 22], since Tyr$^\bullet$ in protein R2 participates in ribonucleotide reduction on protein R1. The crystallographic structures of R1 [23] and R2 dimers [18, 19], allowing a plausible structural model of a tetrameric R1R2 enzyme [23], have shown that Tyr$^\bullet$ was much too far from the active site to directly interact with the substrate. Instead, these studies have suggested a hydrogen-bonded network running through the holoenzyme, implicating three residues in R1, five in protein R2 plus one iron atom, and connecting the buried free radical to the substrate binding site (fig. 1) [18, 23]. Mutation of these amino acids leads to catalytically inert proteins, substantiating the hypothesis of a long-range radical transfer pathway in class Ia reductases. According to this scheme, substrate binding on R1 would be immediately followed by a transfer of the free radical from the tyrosine residue in R2 onto a residue located near the ribose ring of the substrate in the active site on R1. The mechanism of ribonucleotide reduction by the *E. coli* reductase has been a matter of intense research over the past few years. As initially proposed by Mao et al. [24] and by analogy with class II reductases, there is now good evidence that the reaction starts with the forma-

tion of a thiyl radical on Cys439, formed at the expense of Tyr• (fig. 2) [25]. X-ray crystallography of protein R1 binding the GDP substrate has shown that Cys439 is in van der Waals' contact with the 3′-hydrogen of the substrate ribose [26], in a convenient position to generate a transient radical on the substrate by removing a hydrogen atom at the 3′-position. This event is proposed to trigger a radical chemistry between the 3′- and 2′-carbon atoms of the ribose ring, also implicating the two vicinal cysteines Cys225 and Cys462 of protein R1, which are capable of forming a disulfide bond and provide the reducing equivalents necessary for substrate reduction. Cys439 then retrocedes the abstracted 3′-hydrogen to the newly formed deoxyribose; afterwards, the free radical is believed to be transferred back from Cys439 to the tyrosine residue in protein R2. It has to be noted that the radical intermediates at the active site are extremely transient species, which have not yet been detected in a functional enzyme preparation. This can be important when considering the likelihood of an exogenous molecule like NO interacting with one of the endogenous radicals present in a catalytically cycling reductase.

Protein R2 can exist in several states: the active form (two ferric atoms and the radical Tyr•), a partially oxidized form metR2 (a diferric iron center and a normal Tyr residue), a fully reduced form redR2 (two ferrous iron atoms and a normal Tyr residue), and the radical-free, iron-free form apoR2. The stable free radical is produced during the reaction of dioxygen with the diferrous iron center of redR2 [11]. The X-ray structure of redR2 has been determined [27]. It shows a more accessible iron center with a lower coordination number, compared to the diferric center, allowing oxygen to bind to a free coordination site. Upon O_2 binding, a high-valent iron complex is transiently produced that generates the tyrosyl free radical.

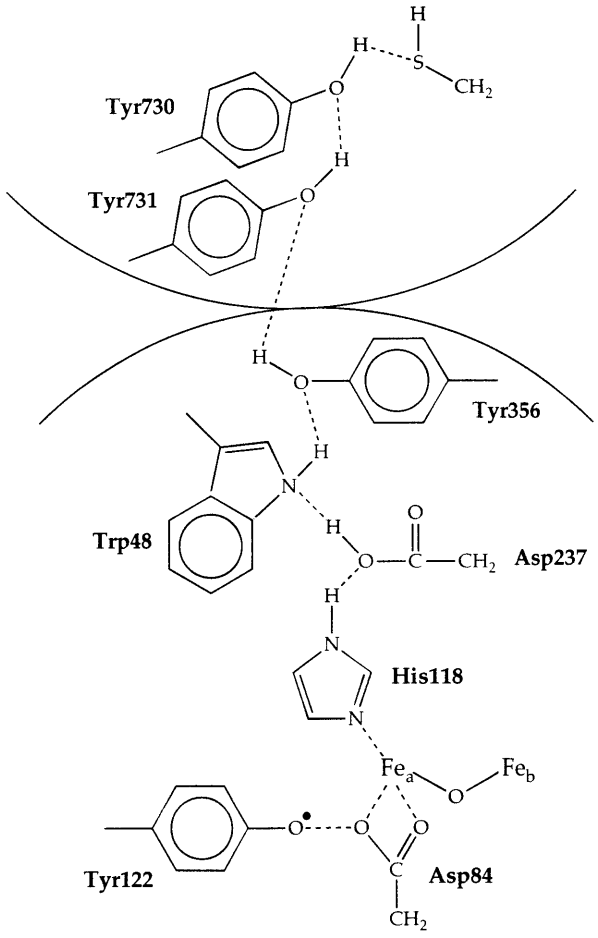

Figure 1. Radical transfer pathway between the tyrosyl radical on protein R2 and Cys439 in the active site of protein R1.

Reversible binding of NO to the tyrosyl free radical

With a stable free radical and an iron center which is also a dioxygen binding site (in the diferrous state), the R2 subunit of class Ia reductases was a priori a likely target for NO. This was shown for the first time in 1991 by electron paramagnetic resonance (EPR) experiments showing a dose-dependent scavenging of the free radical by NO-releasing molecules [7]. That a decrease in the Tyr• EPR signal could also be observed with two other NO donors structurally unrelated to S-nitrosoglutathione was consistent with the actual effector being NO. This was confirmed by the inhibitory effect of HbO_2 on Tyr• scavenging.

The reaction of NO with Tyr• of *E. coli* protein R2 was further explored in another study using short-lived S-nitrosocysteamine derivatives [13]. A key finding was the reappearance of the EPR free radical signal, following the initial decrease, suggesting a regeneration of Tyr• when the NO concentration decreased in the medium. In agreement with this hypothesis, the lag time preceding radical regeneration increased as the concentration of thionitrite increased, or when the experiment was performed under anaerobic conditions in order to stabilize NO. No regeneration was observed if a thionitrite with a longer half-life was used. Other experiments performed with mouse protein R2 incubated with authentic NO gas also indicated a reversibility in Tyr• radical quenching by NO [12]. Several antioxidant compounds, like hydroxyurea and other hydroxamates scavenge the free radical in class I RRs by reducing Tyr• into a normal tyrosine residue [9]. Thorough investigations excluded a reducible action of NO against the free radical of protein R2 [12, 13]. Instead, a radical-radical coupling reaction was proposed [13], generating a relatively unstable Tyr-NO adduct that spontaneously dis-

sociates to regenerate Tyr•. The reversibility of the coupling, which was unprecedented in RR reactivity, was supported by the work of Petrouleas and Diner [6] on PSII (see below) and also by previous reports showing a similar reversible coupling reaction between NO and a variety of stable phenoxyl radical models such as 2,4,6-tri-*tert*-butylphenol and its analogs [28, 29]. In the latter studies, the coupled products included C-nitroso compounds, suggesting C-N coupling at the ortho and para positions. According to Janzen et al. [28], more stable phenoxyl radicals would form a weaker bond with NO and therefore dissociate more rapidly. Thus, a very stable tyrosyl free radical would probably have a tendency to dissociate rapidly from NO. This agrees with our own observations on protein R2.

NO-induced iron release

It is interesting to note that despite numerous examples illustrating the reactivity of NO toward heme and non-heme iron proteins, NO does not bind to the diferric center of protein R2 [13, 30]. However, NO has been used as an O_2 analog to investigate the mode of O_2 binding in reduced protein R2 [30]. When NO was anaerobically bubbled into a solution containing *E. coli* protein redR2, several nitrosylated species were produced. The majority of these species are dinitrosyl complexes where one NO molecule binds to each iron(II). A transfer of electron density from Fe(II) to NO gives rise to Fe(III)-NO^- species. The nitrosylated complex then decays into N_2O and metR2, the oxo bridge in metR2 deriving from bound NO (fig. 3). Despite the authors' suggestion, it is unlikely that the reaction described here would contribute significantly to the effects of NO toward the reductase in vivo, since it apparently requires very high NO levels and seems too slow to outcompete favorably the fast reaction of dioxygen with redR2.

The lack of reactivity of the diferric iron sites in active protein R2 does not necessarily mean that the metal center is not affected by NO. Recent experiments performed with murine protein R2 have shown the labilization of iron(III) upon prolonged incubation with NO [12]. Since an increase in iron release from protein R2 also occurs after reduction of Tyr• by *p*-propoxyphenol [12] or phenylhydrazine [31], it was suggested that the

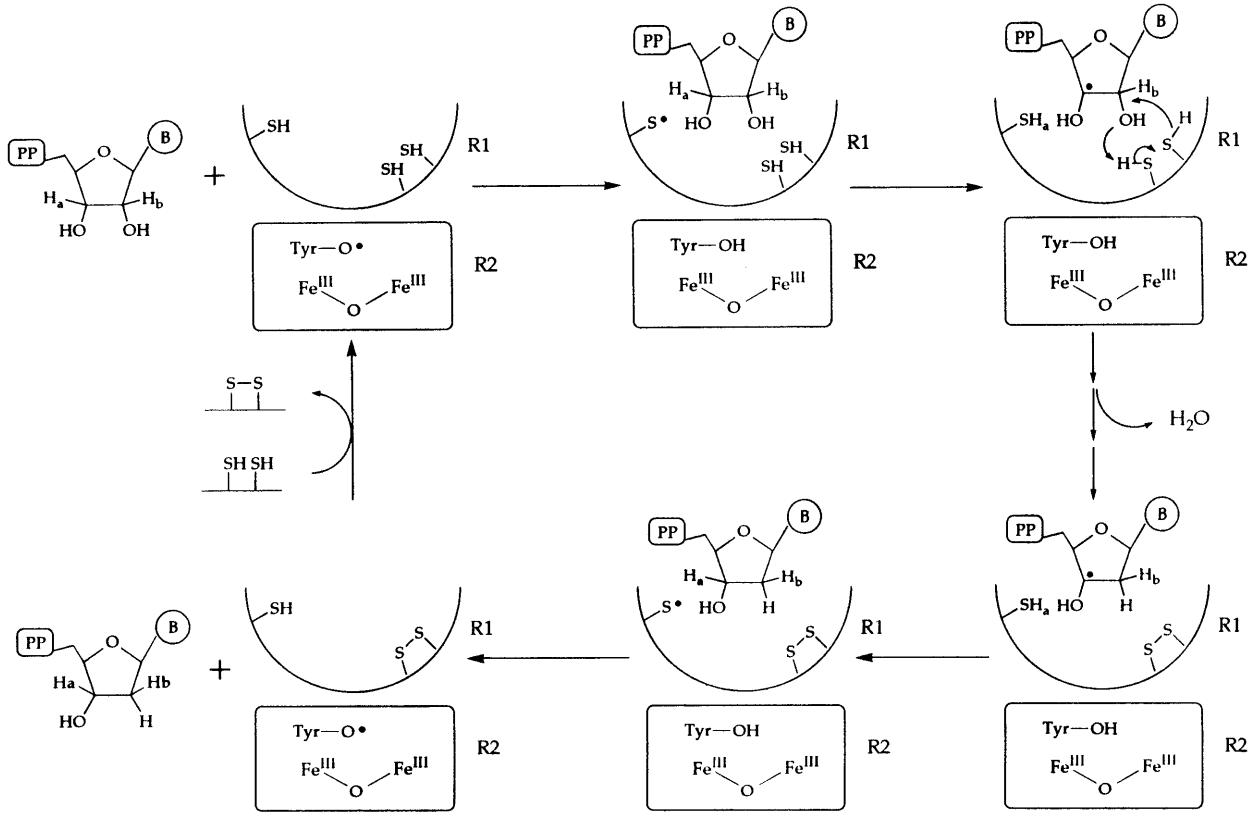

Figure 2. Proposed catalytic mechanism for the reduction of ribonucleotides by *E. coli* class I RR.

ferric iron atoms might be less tightly bound in radical-free mouse metR2 protein than in active R2. Similarly, it seems that the diferric iron core was labilized as a consequence of free radical quenching by NO. A satisfactory explanation for this change in metal stability accompanying the loss of the Tyr• radical is still awaited. No such iron release is induced in the *E. coli* R2 after free radical quenching by NO or hydroxyurea [13]. The diferric iron cofactor in the murine protein is much less stable than its bacterial counterpart [16]. Crystallographic structures of radical-free R2 proteins have revealed a more accessible and less hydrogen bonded environment of the iron sites in mouse R2 that may contribute to the reduced stability of the iron center [19]. There is good evidence from EPR studies that the presence of the tyrosyl free radical influences the structure (and the reactivity) of the diferric metal center, both in *E. coli* and in mouse protein R2 [32, 33]. It is conceivable that the slight but important structural changes induced by loss of the Tyr• radical may further increase the lability of the diiron cluster in the mouse protein, while not significantly modifying the high stability of the metal center in the bacterial R2 subunit. Whatever the mechanism, the net result of prolonged incubation of mouse protein R2 with NO is the formation of a primary radical-free R2Tyr-NO adduct, which slowly evolves into radical-free, iron-free apoR2 (fig. 3).

Proposed mechanisms for the physiological inactivation of RR

Since both the free radical and the iron center are essential for catalysis, NO is expected to inhibit RR activity. Attempts to check this inactivation in vitro have given variable results. The most important inhibitory effects were found when the source of NO, such as a chemical NO donor or crude NO synthase (NOS) activity, was present during the assay for reductase activity [7, 34]. In contrast, experiments with a preincubation protocol were only occasionally successful [12, 35], probably because the dissociation of the Tyr-NO adduct and/or the reconstitution of the metal center could occur after NO treatment. However, there is now a large body of evidence showing both quench-

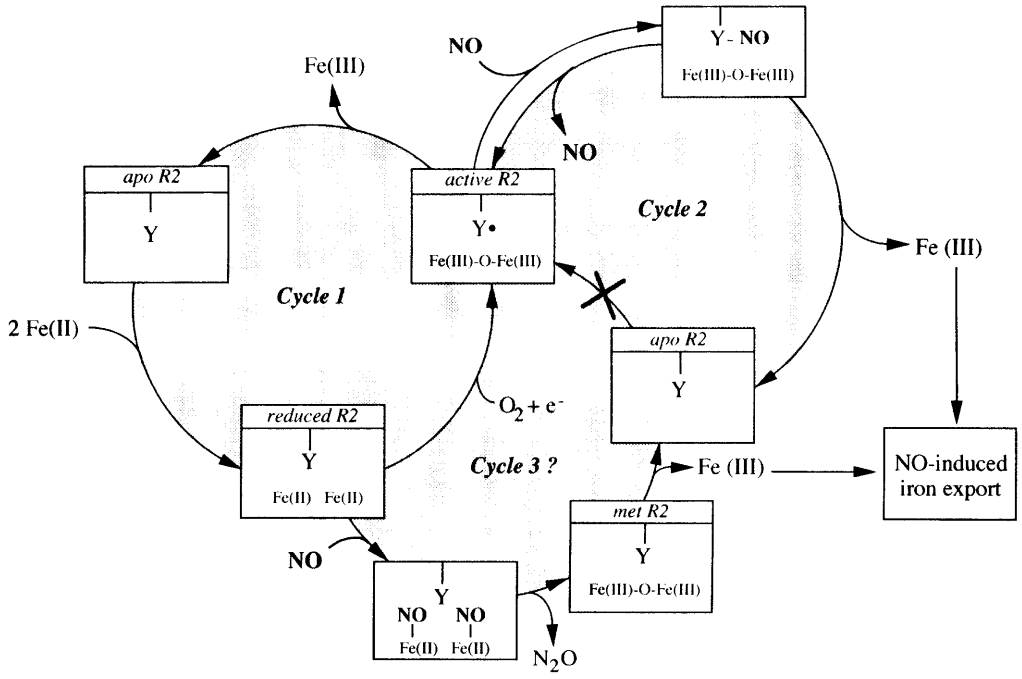

Figure 3. Effects of NO on mouse protein R2. Incorporation of ferrous iron into apoR2 leads to the diferrous reduced R2 state, which upon oxidation is converted into the active, radical-bearing form. This form is converted into the inactive apoprotein through spontaneous efflux of ferric iron (Cycle 1). Coupling of NO to the tyrosyl radical in active R2 generates a reversible radical-free R2-NO adduct, whose iron center is more labile. Thus, long-lasting exposure to NO (for instance via a NO synthase II activity) accelerates formation of apoR2. NO simultaneously induces iron depletion in target cells, which probably precludes recycling of apoR2 into active R2 (Cycle 2). Under anaerobic conditions, high concentrations of NO were shown to oxidize the diferrous center of reduced R2 and, again, promote apoR2 formation (Cycle 3). It is unlikely that this reaction could compete favorably with the very fast binding of O_2 to reduced R2.

ing of $Tyr^{\bullet}$ and inactivation of the reductase in cell culture systems, under conditions where a sustained level of NO is generated over a long period (that is, by NOS II activity). The concentration of R2-centered tyrosyl free radical in a mammary adenocarcinoma cell line was estimated between 0.1 and 0.4 μM, highest in S-phase when protein R2 is expressed [36]. On the basis of a previous estimation, steady-state concentrations of NO required to scavenge 100% of this radical concentration would be of the order of 0.5–3 μM [12]. Both experimental data and modelling studies indicated that this concentration range is readily achieved by NOS II activity [37–39]. Constitutive NOS activities can also produce such high levels of NO, although only transiently. Therefore, because of the reversibility of the coupling reaction, durable scavenging of the tyrosyl free radical of RR by NO is restricted to long-lasting NOS II activity. Inhibition of the reductase can be indirectly demonstrated by the beneficial effect of deoxyribonucleotide supplementation in DNA synthesis. The activity of the enzyme is the rate-limiting step for DNA synthesis when deoxyribonucleotides are synthesized via the de novo metabolic route. If this major pathway is blocked, deoxyribonucleotides can still be synthesized to some extent by a minor salvage pathway using exogenous deoxyribonucleosides as precursors [15]. Ribonucleoside analogs are inefficient if the blockage is at the level of the reductase. This strategy has been used successfully to detect impairments in RR function in murine and human tumor cells incubated with NO-generating macrophages [35, 40], in hepatocytes in which NOS II activity has been induced by interleukine-1β [41], and in a NO-producing kidney cell line infected with vaccinia virus [42]. NO produced by NOS II activity was also shown to scavenge the $Tyr^{\bullet}$ free radical of the reductase in mutant tumor cells overexpressing the R2 subunit for EPR detection of the radical in packed cell pellets [40, 43]. It was not shown that the loss of $Tyr^{\bullet}$ was responsible for the inhibition of the reductase. Yet the two events were observed over the same short time period and a very good correlation was observed between the decreased free radical EPR signal and inhibition of DNA synthesis in NO-injured tumor cells. A substantial but partial regeneration of the free radical was observed in cells withdrawn from NO-producing macrophages, together with a partial resumption in DNA synthesis [40]. Since an active protein can easily be regenerated from apoR2 in mammalian cells, it is not possible to know from these experiments whether the reappearance of $Tyr^{\bullet}$ is due to dissociation of a Tyr-NO adduct, or to reconstitution of the radical/metal cluster from apoR2. Almost complete regeneration of the free radical content in cell pellets supplemented with Fe(II) and dithiothreitol [12] might be indicative of formation of apoR2 protein.

Mouse R2 protein requires a continuous supply of iron to reconstitute the unstable diferric center [16]. Overexpression of recombinant protein R2 elicits in the transfected cells an elevation in gene expression of ferritin H- and L-chains [44], the iron storage protein. Conversely, iron chelators inhibit RR activity by preventing iron incorporation into apoR2 or newly synthesized enzyme [16, 22]. Therefore, intracellular iron availability can regulate protein R2 activity. There is now concrete evidence that NO and other nitrogen oxides can alter iron metabolism significantly. An early study described a time-dependent iron depletion in tumor cells cocultivated with cytotoxic macrophages [45] and later reports have shown that this event was NO dependent. NO and peroxynitrite also modulate the activity of iron-responsive protein 1, a key regulatory protein for intracellular iron homeostasis [46]. NO can also mobilize iron into fairly stable dinitrosyl-dithiol iron complexes, whose formation is coincident with the loss of $Tyr^{\bullet}$ free radicals in tumor cells cocultivated with NO-generating macrophages [40, 43]. All these effects might contribute to restraining iron availability for protein R2 (and other iron-containing proteins). Besides its own reactivity against the radical/metal cluster of protein R2, NO might indirectly inhibit the formation (and maintenance) of the active R2 protein by preventing iron (re)incorporation into it, acting like an iron chelator (fig. 3). Whether the loss of R2-centered free radical in cell culture results mainly from a reversible coupling reaction between NO and $Tyr^{\bullet}$ (and subsequent destruction of the iron center) or from a NO-mediated decrease in iron availability is currently unknown.

Other mechanisms might also explain RR inhibition in NO-injured cells. Nitrosation of cysteines in protein R1 has been described in vitro [13]. The nitrosated protein is inactive. Thiols are now well-recognized targets for NOs in vitro and in vivo [3]. It is possible that nitrosation and inactivation of protein R1 could be a phenomenon of biological relevance. Peroxynitrite, the reaction product of NO and $O_2^{\bullet -}$, nitrates tyrosine residues in protein R2 and irreversibly inactivates the protein in vitro [12]. Certain experiments suggest production of peroxynitrite in vivo [1]. One can therefore speculate that, beside NO, peroxynitrite might also participate in RR inactivation, even if recent investigations in vitro have led to a rejection of this hypothesis [12].

Photosystem II

Two similar tyrosyl radicals with different roles

Photosynthesis in plants involves two discrete, light-harvesting photosystems present in the thylakoid membrane of chloroplasts. Electrons acquired from PSII by PSI are used for $NADP^+$ reduction. The light-driven

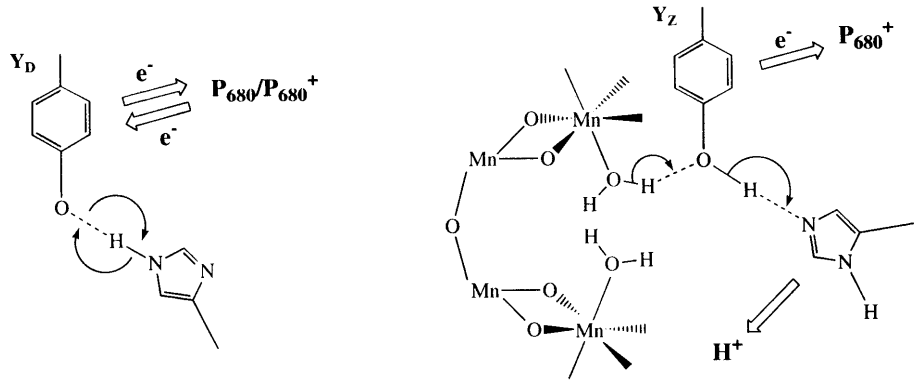

Figure 4. Proton/hydrogen atom abstraction models for $Y_D^{\bullet}$ and $Y_Z^{\bullet}$ tyrosyl free radicals, adapted from Tommos et al. [49] and Force et al. [75] (see text).

oxidation of water into O_2 is performed by PSII in an oxygen-evolving complex consisting of a water-binding tetranuclear manganese cluster and the essential cofactors Ca^{2+} and Cl^- [for reviews, see refs 47, 48, 49]. The four oxidizing equivalents required for oxygen synthesis from water come from a chlorophyll complex, designated $P680^+$ in the oxidized state. Photooxidation of P680 at the resting level generates an excited state P680*, which quickly reacts with an adjacent pheophytin (Ph) to produce the $P680^+Ph^-$ pair. This charge separation step is the key primary event in water oxidation chemistry. Charge recombination in $P680^+Ph^-$ is prevented by rapid transfer of the strong oxidizing power of $P680^+$ to a tyrosyl residue, called Y_Z, which acts as an intermediate between $P680^+$ and the $(Mn)_4$ cluster. The latter accumulates, one by one, four oxidizing equivalents from the one-electron oxidized free radical species $Y_Z^{\bullet}$. The oxygen evolution cycle involves five '*S*' states, S_0–S_4, where the *S* state identifies the number of oxidizing equivalents stored in the metal center. Molecular oxygen is released following the formation of the final S_4 state, and the oxygen-evolving complex then resets to the basal, most reduced S_0 state. In addition to $Y_Z^{\bullet}$ located in the membrane-spanning D1 polypeptide, PSII contains another tyrosyl free radical $Y_D^{\bullet}$ in the D2 protein [50]. The EPR line shapes of the two free radicals are identical, suggesting a similar distribution of radical density and a similar orientation and environment of the phenol ring.

Due to a rapid electron transfer rate between the $(Mn)_4$ cluster and $Y_Z^{\bullet}$ in the intact oxygen-evolving complex (from 30 μs to 1.2 ms, increasing with the *S* number [47]), $Y_Z^{\bullet}$ is not detected in functional, oxygen-evolving preparations. The function of $Y_Z^{\bullet}$ has been recently unravelled, in the light of a hydrogen atom abstraction mechanism operative in other radical enzymes [17]. The current view of the interactions between $Y_Z^{\bullet}$ and the manganese cluster is summarized in figure 4, according to recent reviews [17, 48, 49]. In this model, the oxidized tyrosine $Y_Z^{\bullet}$ is hydrogen-bonded to the substrate water molecule, allowing transfer of a hydrogen atom from the substrate, reduction of $Y_Z^{\bullet}$ and *S* state transition of the $(Mn)_4$ center. Water oxidation thus proceeds by four successive steps, each abstracting a hydrogen atom from the substrate. The H-atom flux is divided at the level of Y_Z into an electron current to the $P680^+$ species and a proton current to the nearby histidine. The model is consistent with the observation that $Y_Z^{\bullet}$ reduction and O_2 release occur at comparable rates [51]. In contrast to $Y_Z^{\bullet}$, $Y_D^{\bullet}$ is stable and is not involved in water oxidation chemistry. Its function is not clearly understood. Like $Y_Z^{\bullet}$, it results from a one-electron oxidation induced by $P680^+$ and it is located in the vicinity of the manganese cluster. A $Y_D^{\bullet}$-lacking mutant is competent in O_2 production but exhibits a reduced growth rate [52]. For this reason, a role in the assembly or in the stabilization of the oxygen-evolving complex has been proposed. Spectroscopic data led to a model in which Y_D, buried in PSII, is isolated from solvent and upon oxidation transfers a proton to a nearby histidine residue (fig. 4) [53]. Reduction of $Y_D^{\bullet}$ to a normal tyrosine residue induces the reverse motion of the well confined proton.

Reversible reactions of NO in PSII

In the course of a study designed to probe the environment of a non-heme iron complexed with a quinone downstream of pheophytin in PSII, Petrouleas and Diner [6] made the incidental observation that the characteristic EPR signal arising from $Y_D^{\bullet}$ was absent in spinach preparations containing 100 μM NO. Elimina-

tion of NO in vacuo restored the EPR signal to practically the control level, indicating that disappearance of the radical was not due to reduction, but probably to an exchange coupling between the two radicals. The binding site of NO was not clearly identified, but the manganese cluster and the non-heme iron were excluded. For the first time, it was suggested that NO might bind reversibly with a protein-centered tyrosyl free radical. A K_d of approximately 3 μM was found for the NO-induced disappearance of the $Y_D^\bullet$ EPR signal under anaerobic conditions, a posteriori consistent with our own observations with RR [12]. Reversible binding of NO to $Y_D^\bullet$ was confirmed by another group [54]. Again, the almost complete recovery of the EPR signal after NO evacuation ruled out chemical reduction of the oxidized residue by NO. When an identical sample was illuminated under conditions that induce high yields of $Y_Z^\bullet$, the radical could also be scavenged by NO. Interestingly, the concentration of free NO could be directly estimated from an EPR signal in the $g = 2$ region. The signal decreased upon illumination to produce $Y_Z^\bullet$ and increased again to its initial level after 15 min of dark incubation. This crucial result clearly demonstrated NO release during the decay of the Tyr-NO adduct. Another important observation was that both the Y_D-NO and Y_Z-NO adducts did not give rise to a new radical species, indicating that these adducts were resistant to oxidation (for example by $P680^+$) under the conditions of these experiments.

In Mn-containing, acetate-treated PSII samples, the oxygen-evolving complex is trapped in a particular *S* state and $Y_Z^\bullet$ is stabilized. A broad EPR signal known as the 'S_3' EPR signal is observed in these inhibited preparations [55, 56]. It has been proposed that this arises from $Y_Z^\bullet$ interacting magnetically with the manganese cluster in the S_2-state [57, 58]. In the elegant study of Szalai and Brudvig [54], NO was used to eliminate $Y_Z^\bullet$, suppressing the coupling between the two components of the S_3 EPR signal and revealing the manganese cluster EPR signal.

From a tyrosyl free radical to an iminoxyl radical

Together, the above experiments on RR and PSII seemed to indicate that the reaction of NO with tyrosyl radicals was essentially reversible. This assumption has been recently questioned by Sanakis et al. [59], who showed that the extent of reversibility appears to depend on the length of incubation with NO. As previously described, they first checked that incubation of a $Y_D^\bullet$-containing PSII preparation with NO for a few minutes, followed by NO evacuation, induced the formation of a fully reversible EPR-silent adduct. However, recovery of the initial EPR signal from $Y_D^\bullet$ was only partial after a 1-h incubation period with NO, and almost negligible after 4 h. Moreover, a new radical species appeared upon illumination (and after NO elimination) in the samples treated with NO for a long time, but not in those briefly treated once. Since illumination generates the oxidizing $P680^+$ chlorophyll state, these results confirmed that the Tyr-NO adduct is resistant to oxidation [6, 54]. They also suggested that, upon prolonged incubation with NO, the initial adduct slowly changed to a more stable species which was still oxidizable. Since the EPR parameters of the new, light-induced signal indicated an iminoxyl radical, it was suggested the non-oxidized precursor was a nitrosotyrosine. A sequence of reactions was therefore presented, starting with the formation of a weak, reversible, EPR-silent adduct between NO and $Y_D^\bullet$ (fig. 5). Cleavage of a C-H bond is then required to produce a C-nitrosotyrosine, and this could be the slow, rate-limiting factor for nitrosotyrosine formation, together with the availability of a hydrogen-bonded proton that should translocate from a nearby base (probably D2-His190) to the phenolic oxygen. Finally one-electron oxidation of the nitrosotyrosine generates the iminoxyl radical. The Mn cluster is not involved in the chemistry of the formation of the iminoxyl radical. However, Mn is released from oxygen-evolving complexes in NO-treated samples. The amount released correlated with the development of the iminoxyl radical EPR signal, suggesting some kind of interaction between the iminoxyl radical and/or nitrosotyrosine and the Mn complex. It was hypothesized that reduction of Mn by nitrosotyrosine would lead to an iminoxyl radical and Mn in a reduced, more labile state. The iminoxyl radical could be reduced back to nitrosotyrosine by NO. This redox cycle is likely to be repeated until all the Mn is reduced and released in the medium. $Y_D^\bullet$ was earlier suspected to stabilize the Mn complex. The findings presented by Sanakis et al. [59] are in line with this hypothesis.

Prostaglandin H synthase

A transient tyrosyl free radical

PGHSs, also called cyclooxygenases, catalyze the two first key steps in the biosynthesis of prostanoids such as prostaglandin and thromboxane [see ref. 60 for review]. These membrane-linked enzymes exhibit heme-dependent cyclooxygenase and peroxidase activities which are responsible for the regio- and stereospecific synthesis of prostaglandin hydroxyendoperoxide PGH_2 from arachidonic acid (fig. 6). As with RR and PSII, PGHSs proceed via radical chemistry. The enzymatic reaction begins with the removal of the 13-*pro*S hydrogen atom of arachidonic acid (AA) to create a carbon-centered radical. A free radical chain transfer reaction then allows the addition of a first molecule of dioxygen on C_{11}.

A subsequent intramolecular cyclization leads to a cyclic endoperoxide. Incorporation of a second molecule of dioxygen in C_{15} followed by the transfer of one hydrogen atom back to the substrate results in the formation of the cyclic hydroperoxyendoperoxide PGG_2. The second step of the enzymatic reaction performed by the peroxidase activity involves a two-electron reduction of PGG_2 to the cyclic hydroxyendoperoxide PGH_2.

Two isozymes have been isolated and characterized: PGHS-1 (COX-1) is a constitutive isoform expressed in almost all tissues, while PGHS-2 (COX-2) can be induced in inflammatory cells in response to growth factors, hormones, lipopolysaccharide, and cytokines. Despite differences in expression and localization, the two isoenzymes share a common structure and catalytic mechanism. Both enzymes are homodimeric, glycosylated proteins and harbor a high-spin iron(III)-protoporphyrin IX cofactor. From a pharmacological point of view, PGHSs are of great importance, since they are the targets of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin [60]. The crystal structures of the two enzymes are very similar and comprise three folding domains: an epidermal-growth-factor-like module, a membrane-binding motif, and a large catalytic domain consisting of two adjacent binding sites. The cyclooxygenase binding pocket appears as a long, narrow hydrophobic channel which can accomodate the substrate AA and also NSAID inhibitors. The peroxidase active site is found in a shallow cleft containing the heme prosthetic group. The involvement of a tyrosyl radical in the catalytic mechanism was first suggested by EPR studies at 90 K [61]. The appearance of a doublet signal when PGHS-1 is incubated with PGG_2 or hydroperoxide has been attributed to a tyrosyl radical by analogy with the tyrosyl radical EPR signals found in RR and PSII. Thorough EPR experiments revealed the appearance of several free radical signals when PGHS-1 or PGHS-2 were reacted aerobically with AA or hydroperoxides [61–63]. These signals have been assigned to one or more tyrosyl residues generated during PGHS catalysis. The different EPR line shapes and line widths may result from different conformations of the phenyl ring in the tyrosyl residues [17, 64]. In contrast with RR, the tyrosyl radical is unstable (half-life 20 s at -12 °C) presumably because

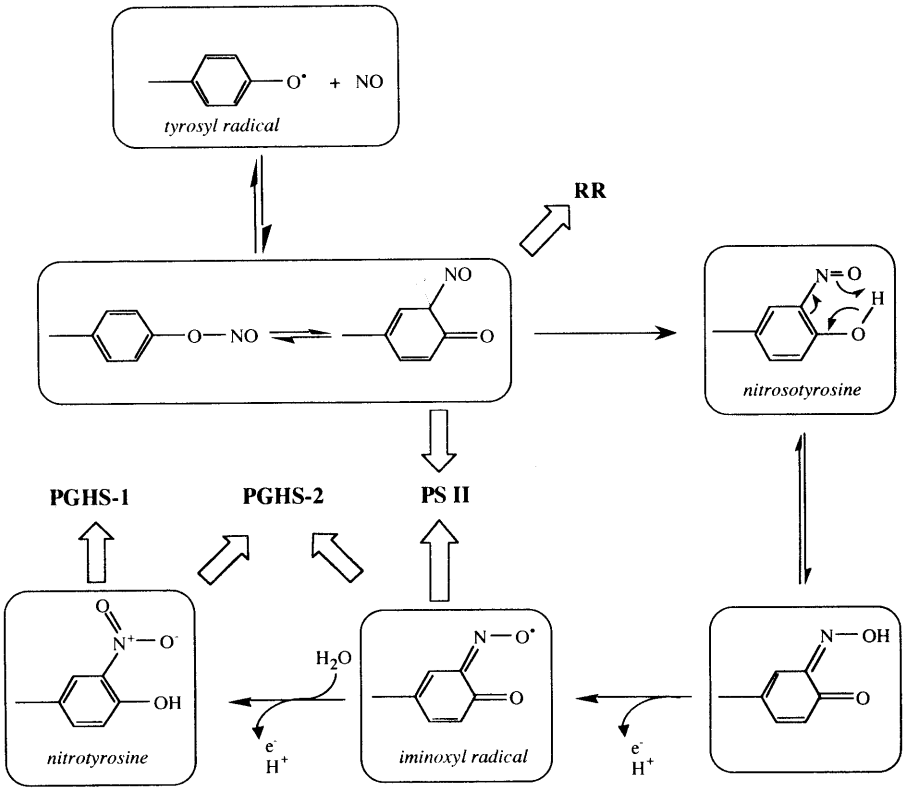

Figure 5. Proposed chemical interactions between NO and tyrosyl free radicals in RR, PSII and PGHS-1 and 2. Large arrows indicate the chemical species identified in the different protein models.

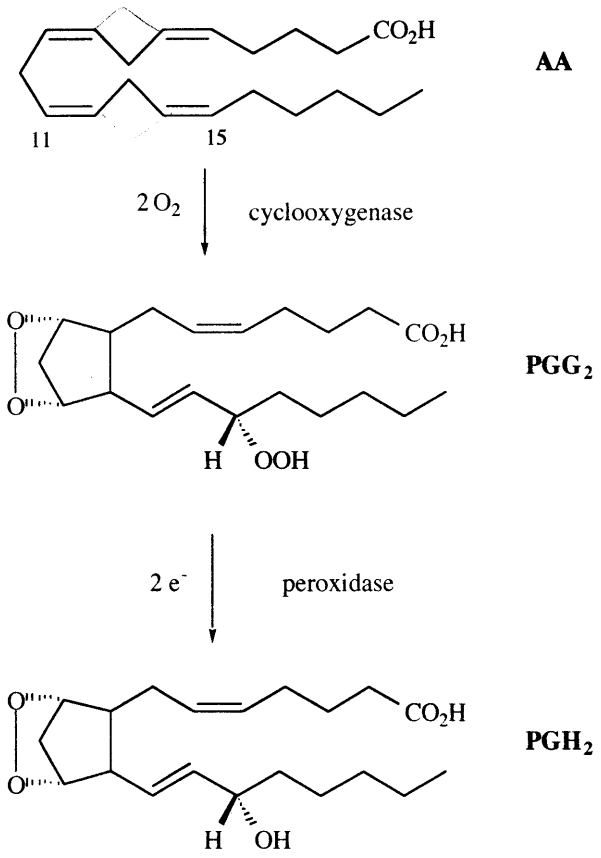

Figure 6. Two-step conversion of arachidonic acid into PGH_2 catalyzed by prostaglandin H synthase.

it is more exposed to the solvent [61, 64]. The tyrosyl radical has been shown to be involved in the initiation of the cyclooxygenase reaction, since it is able to generate an arachidonyl radical from AA under anaerobic conditions [65, 66]. Addition of dioxygen to this substrate-radical-bearing enzyme resulted in the regeneration of the tyrosyl radical, completing cyclooxygenase turnover. The crucial role of Tyr385 in PGHS-1 (Tyr371 in PGHS-2) in the catalytic mechanism was demonstrated using chemical modification of tyrosine residues with tetranitromethane, peptide mapping and also by site-directed mutagenesis experiments [66, 67]. Surprisingly, the reaction of a Y385F mutant with hydroperoxide generated a tyrosyl radical signal similar to the radical produced by the wild-type enzyme [62]. This mutant had lost cyclooxygenase activity but still catalyzed the peroxidation reaction. From crystallographic data it is tempting to think that the residue Tyr385 in PGHS-1 (Tyr371 in PGHS-2), essential for catalysis and suitably located between the heme and the AA binding pocket, is transformed by hydroperoxide into a tyrosyl radical and further oxidizes AA to an arachidonyl radical. However, there is no direct evidence for this putative mechanism. EPR studies have also helped to suggest a role for the iron in the generation of the tyrosyl radical [68]. Several oxidation states for the metal have been characterized by EPR. A ferric atom is present in the resting oxidation state of the enzyme, while higher oxidation intermediates have been identified during turnover. The overall catalytic mechanism proposed to date is summarized in figure 7.

Trapping and identification of the tyrosyl radical by NO

The molecular interactions of PGHSs with NO generated from NONOates were investigated by low-temperature EPR spectroscopy [8, 69]. The tyrosyl radical signal of PGHS-2 observed 4 s after addition of AA to the enzyme was still present after longer incubation at room temperature. In contrast, treatment of PGHS-2 with 500 μM diethylamine diazeniumdiolate, DEA-NO, before addition of the substrate resulted in the rapid formation of a characteristic iminoxyl radical EPR spectrum reminiscent of that observed in PSII (see above). This signal disappeared after a further 1-min incubation [8]. The final reaction mixture contained nitrotyrosine, apparently generated via the iminoxyl radical. For PGHS-1, no iminoxyl radical intermediate was detected after treatment of the enzyme by NO donors [69]. However, as for PGHS-2, formation of nitrotyrosine was established. The addition of NO donors prior to the substrate was absolutely necessary for nitrotyrosine formation. The presence of superoxide dismutase did not modify the nitration of PGHSs by NO, ruling out the involvement of peroxynitrite in the nitration reaction. Altogether, these data suggest that NO reacts with the tyrosyl radicals of PGHS-1 and PGHS-2 to form EPR-silent adducts (nitrosocyclohexadienones) which evolve irreversibly to nitrotyrosines (fig. 5). This is likely to occur via an iminoxyl radical intermediate, even if it has only been detected in PGHS-2. Conversion of a tyrosyl radical into a nitrotyrosine assumes a two-electron oxidation and a proton acceptor. In this way, the peroxidase activity of PGHS may act as an oxidant. The close proximity of Tyr385 and the peroxidase catalytic site support this hypothesis. Another putative oxidant may be $NO_2^{\bullet}$ produced during oxidation of NO under aerobic conditions. The reactivity of NO towards the tyrosyl radical of PGHS-1 was used to localize the tyrosyl radical in the polypeptide chain of PGHS-1. Identification of a single nitrotyrosine, nitroTyr385, was established using peptide mapping [69]. From these experiments, Tyr385 was shown to generate a tyrosyl radical during AA oxidation. The consequence of Tyr385 nitration for PGHS activity was not investigated in these experiments.

Other authors have studied the interaction of NO with PGHS activity using NO gas and several NO generators [8, 69–72]. Under aerobic conditions, activation of the cyclooxygenase and peroxidase activities of PGHS-1 by NO were observed whatever the source of NO [70, 72]. The enzymatic activation was found to be heme-independent, consistent with the weak affinity of NO for the ferric form of PGHS [71]. Interesting results have emerged from the analysis of the nitrosothiol content of PGHS-1 after treatment with NO [70]. Parallel with the dose- and time-dependent formation of a S-nitrosated protein, an increase in PGHS-1 activity was observed. S-nitrosation induced structural modifications of the enzyme as shown by circular dichroism. This is thought to enhance the catalytic efficiency of PGHS either by stimulating heme binding or by facilitating the access of AA to the cyclooxygenase binding site. In summary, several amino acid residues in PGHS are potential targets for NO and probably for other nitrogen oxides like nitrosothiols. The overall effect on the enzyme may depend upon the selective reactivity of NO toward the critical thiols and the tyrosyl radical(s).

Conclusion

The increasing importance of amino acid free radicals in biological catalysis is beginning to be recognized. NO is a neutral and diffusible molecule which can easily gain access to these reactive species, often buried in a hydrophobic environment in the protein core, where they are protected from water reactivity. Evidence has accumulated demonstrating the occurrence of a radical-radical addition reaction involving NO and tryptophanyl or tyrosyl radicals. Considering the high rate constant of the recombination, it is likely that free radicals in proteins represent biological targets for NO that have, until now, been largely overlooked. DNA synthesis is under the control of a radical enzyme, RR. Elimination of the radical and labilization of the nearby iron center by NO have recently been demonstrated. These mechanisms could help rationalize the physiological cytostatic effect of macrophage-like NOS II activity, which has been shown to depend on RR inhibition in several models. In this respect, NO appears to be the first identified natural equivalent of anticancer and antiviral drugs developed as inhibitors of the reductase.

Additional studies on other protein-centered tyrosyl free radicals have brought new insights into chemical aspects of the reaction with NO, albeit used in hundred micromolar concentrations. While the primary product is always an unstable, reversible, EPR-silent adduct, it can subsequently convert into an iminoxyl radical and even into a nitrotyrosine, provided a strong oxidizing species is present in the vicinity of the radical, such as a heme iron in PGHS. If one imagines that such a nitrating reaction could occur at more physiological NO levels, it could be a relevant inhibitory mechanism for the NO-dependent inactivation of PGHS observed in some biological systems. It may also represent an alter-

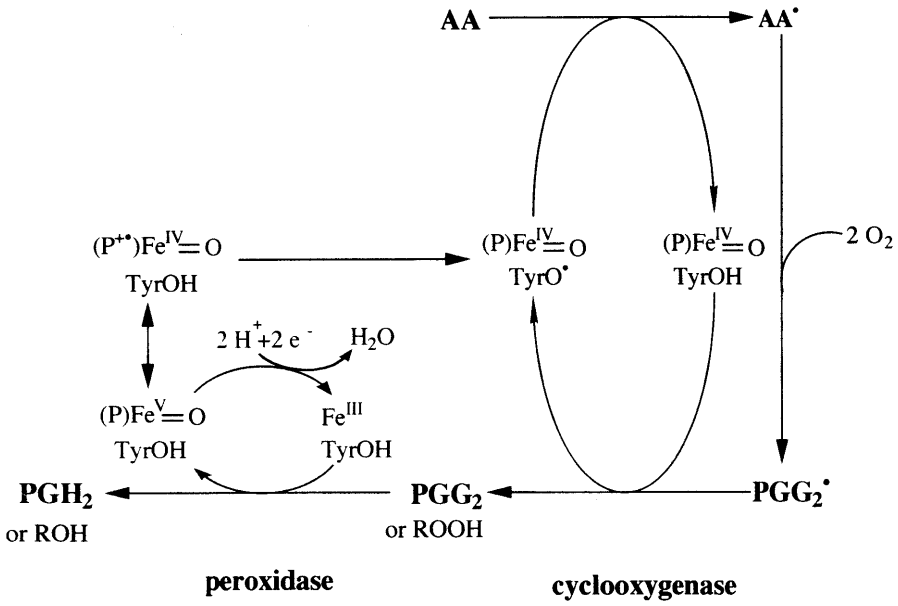

Figure 7. Suggested mechanisms connecting cyclooxygenase and peroxidase activity in PGHS.

native, peroxynitrite-independent pathway to nitration of proteins in vivo.

The efficient neutralization of amino acid free radicals by NO could have biological consequences beyond the context of mammalian enzymology. Tyrosyl free radicals have been implicated as physiological initiators of peroxidation of low-density protein [73]. Along with other protective molecules, NO might serve as an important antioxidant in the vasculature. Furthermore, the recent discovery of NOS-like activities in plants and prokaryotes and the existence of free NO production by denitrifying bacteria [74] opens up the exciting perspective that NO could modulate the function of other radical proteins, such as those specifically expressed in plants and anaerobes [5].

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