Formation of Reactive Nitrogen Species at Biologic Heme Centers: A Potential Mechanism of Nitric Oxide–Dependent Toxicity

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The peroxidase-catalyzed nitration of tyrosine derivatives by nitrite and hydrogen peroxide has been studied in detail using the enzymes lactoperoxidase (LPO) from bovine milk and horseradish peroxidase (HRP). The results indicate the existence of two competing pathways, in which the nitrating species is either nitrogen dioxide or peroxynitrite. The first pathway involves one-electron oxidation of nitrite by the classical peroxidase intermediates compound I and compound II, whereas in the second pathway peroxynitrite is generated by reaction between enzyme-bound nitrite and hydrogen peroxide. The two mechanisms can be simultaneously operative, and their relative importance depends on the reagent concentrations. With HRP the peroxynitrite pathway contributes significantly only at relatively high nitrite concentrations, but for LPO this represents the main pathway even at relatively low (pathophysiological) nitrite concentrations and explains the high efficiency of the enzyme in the nitration. Myoglobin and hemoglobin are also active in the nitration of phenolic compounds, albeit with lower efficiency compared with peroxidases. In the case of myoglobin, endogenous nitration of the protein has been shown to occur in the absence of substrate. The main nitration site is the heme, but a small fraction of nitrated Tyr146 residue has been identified upon proteolytic digestion and high-performance liquid chromatography/mass spectrometry analysis of the peptide fragments. Preliminary investigation of the nitration of tryptophan derivatives by the peroxidase/nitrite/hydrogen peroxide systems shows that a complex pattern of isomeric nitration products is produced, and this pattern varies with nitrite concentration. Comparative experiments using chemical nitrating agents indicate that at low nitrite concentrations, the enzymatic nitration produces a regioisomeric mixture of nitrotryptophanyl derivatives resembling that obtained using nitrogen dioxide, whereas at high nitrite concentrations the product pattern resembles that obtained using peroxynitrite. *Key words:* heme proteins, hemoglobin, hydrogen peroxide, myoglobin, nitrite, peroxidases, tryptophan nitration, tyrosine nitration. *Environ Health Perspect* 110(suppl 5):709–711 (2002).
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Tyrosine nitration has been recently recognized to be linked to nitric oxide metabolism (*1*). This process represents an important mechanism of protein modification because a large number of proteins and enzymes have activities dependent on tyrosine residues. The presence of 3-nitrotyrosine has been associated also with a wide range of human and animal diseases, including neurodegenerative diseases, acute lung injury, atherosclerosis, bacterial and viral infection, and chronic inflammation (*2*). At present the nitrating pathways operating *in vivo* have not been clearly elucidated. It is possible that multiple mechanisms work simultaneously or, alternatively, that different nitrating species operate at different times during the progression of the disease (*2*). 3-Nitrotyrosine is often assumed to result from the action of peroxynitrite, a potent nitrating and oxidizing agent (*3*). *In vivo* this species is formed by the near diffusion-limited reaction of superoxide and nitric oxide (*4*). Peroxynitrite anion can exist in two stable conformers: the *cis* conformer is more stable and less reactive than the *trans* conformer; protonation of the *cis* form allows the reversible isomerization to the *trans* conformer to occur (*5*). Peroxynitrite has been shown to react with heme peroxidases (*6*), and more recently, even a complex of myoglobin (Mb) and peroxynitrite has been detected as an intermediate in the reaction between the oxy form of the protein and nitric oxide (*7*).

Interestingly, the findings of Pfeiffer and Mayer (*8*) reveal an apparent lack of tyrosine nitration by peroxynitrite, generated *in situ* from nitric oxide (NO) and O_2^- donors at physiological pH, even though alkaline solutions of peroxynitrite efficiently nitrate tyrosine under identical conditions. Another nitrating agent that can be involved in biological nitration is nitrogen dioxide (*9*). This species is produced by one-electron oxidation of nitrite, a major product of NO metabolism (*1*), that can be accumulated in conditions when NO is overproduced (*10*). Nitrite oxidation to nitrogen dioxide can be carried out by peroxidases in the presence of hydrogen peroxide (*9*), whereas other oxidative reactions undergone by nitrite in the presence of several biological oxidants lead to nitrate (*1,11*).

We have performed detailed studies on the nitration of phenols related to tyrosine and, more recently, tryptophan derivatives by heme proteins under various conditions. Our studies aim at establishing the mechanisms responsible for nitration of these biologically important substrates, the eventual regiochemical preferences involved in these reactions, and the potential competitive nitrations occurring at protein residues. Two types of heme proteins are being studied: peroxidases, in particular, bovine lactoperoxidase (LPO) and horseradish peroxidase (HRP); and Mb and hemoglobin (Hb), the oxygen storage and carrier proteins.

Peroxidase-Catalyzed Nitration of Phenolics

There have been several recent reports describing the nitration of tyrosine and tyrosyl residues in proteins by peroxidases in the presence of nitrite and hydrogen peroxide (*12–14*). The existence of several pathways for these reactions has been recognized, but no detailed mechanistic studies are available. According to the currently favored mechanism, nitrite undergoes one-electron oxidation by the peroxide-generated enzyme intermediates known as compound I and compound II. In these intermediates the Fe^{3+} center of the native heme group has been oxidized to an Fe^{4+}=O species and a porphyrin or protein cation radical ($P^{\bullet+}$) and an Fe^{4+}=O species, respectively (*15*):

$$P—Fe^{3+} + H_2O_2 \rightarrow P^{\bullet+}—Fe^{4+}=O + H_2O \quad [1]$$

$$P^{\bullet+}—Fe^{4+}=O + NO_2^- \rightarrow P—Fe^{4+}=O + NO_2^{\bullet} \quad [2]$$

$$P—Fe^{4+}=O + NO_2^- + 2H^+ \rightarrow P—Fe^{3+} + NO_2^{\bullet} + H_2O \quad [3]$$

The $NO_2^{\bullet}$ radical generated in this way could either nitrate the phenol, with a reaction stoichiometry of 2:1, or react directly with a peroxidase-generated phenoxy radical:

$$NO_2^{\bullet} + C_6H_5—OH \rightarrow NO_2^- + C_6H_5—O^{\bullet} + H^+ \quad [4]$$

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$$NO_2^{\bullet} + C_6H_5{-}O^{\bullet} \rightarrow O_2N{-}C_6H_5{-}OH \quad [5]$$

An alternative pathway involves a two-electron enzymatic oxidation of nitrite to nitryl cation, a powerful phenol nitrating agent (*16*):

$$P{-}Fe^{3+} + H_2O_2 \rightarrow P^{\bullet+}{-}Fe^{4+}{=}O + H_2O \quad [1]$$

$$P^{\bullet+}{-}Fe^{4+}{=}O + NO_2^- + 2H^+ \rightarrow P{-}Fe^{3+} + NO_2^+ + H_2O \quad [6]$$

It is considered unlikely in view of the extremely rapid reaction of NO_2^+ with water to yield nitrate. A more likely possibility would be the formation of a peroxynitrite active species, through the reaction of hydrogen peroxide with a peroxidase–nitrite complex:

$$P{-}Fe^{3+} + NO_2^- \rightarrow P{-}Fe^{3+}{-}NO_2^- \quad [7]$$

$$P{-}Fe^{3+} + NO_2^- + H_2O_2 \rightarrow P{-}Fe^{3+}{-}OH^- + O{=}N{=}O{=}OH \quad [8]$$

Peroxynitrite can carry out phenol nitration in a single two-electron step without involving intermediate radical species. Our kinetic studies of the peroxidase-catalyzed nitration of various phenolic compounds show that the reaction rates follow saturation behavior with respect to both phenol and nitrite concentrations (*17*), thus indicating that the three substrates must bind simultaneously to the enzyme to give rise to efficient nitration. It is conceivable that the reaction proceeds through a ternary complex between the enzyme, a bound nitrating species, and the phenol. Support for this view comes from examination of the catalytic constants (k_{cat}, K_M^{PhOH} and $K_M^{nitrite}$). Interestingly, the K_M^{PhOH} values for the phenolic substrates, in the range from 0.1 to 1 mM, are one to two orders of magnitude smaller than the K_M values we found for the oxidation of the same phenolic substrates to dimeric coupling products in the classical peroxidase-catalyzed reactions (*18,19*). This observation can be explained considering that a close proximity between the phenol and the heme, which is required for efficient electron transfer to compound I and II in the normal peroxidase reaction, is not needed in the nitration process. Here, the enzyme-generated nitrating species can diffuse and react with the phenol bound near or at the protein surface. The observed kinetic behavior does not allow discrimination between the possible mechanisms, but some evidence indicates that for both LPO and HRP, nitrogen dioxide cannot be the only nitrating species.

Considering the first mechanism, the oxidation of nitrite by compound I in Reaction 2 is much faster than the corresponding reaction involving compound II, according to Reaction 3, which represents the slow step. Oxidation of nitrite by HRP compound II is actually very slow (6.6 ± 0.4 M^{-1} s^{-1}), much slower than the second-order catalytic constant for the nitration ($k_{cat}/K_M^{nitrite} \approx 3 \times 10^2$ M^{-1} s^{-1}) (*17*). Therefore, for HRP this mechanism could be supported only assuming that compound I is reduced by nitrite and compound II is reduced by phenol because the latter reaction is fast [between 10^3 and 10^4 M^{-1} s^{-1}; path (a) in Figure 1]. In this way the two radical species generated by the enzyme intermediates, $NO_2^{\bullet}$ and $C_6H_5{-}O^{\bullet}$, can couple to form the nitrophenol. However, to be efficient, this pathway implies that the phenol binds close to the heme, a necessary condition for rapid electron transfer to compound II, and this contradicts our findings. We believe, at least at high nitrite concentrations, the relatively high nitration rates can be explained by the simultaneous activation of the alternative peroxynitrite-dependent pathway, even though this may involve only a fraction of the enzyme.

With LPO the rate of compound II reduction by nitrite is high (>10^4 M^{-1} s^{-1}) and could support the $NO_2^{\bullet}$ mechanism (Reactions 1–5). But with this enzyme we could obtain direct spectral evidence for an enzyme intermediate different from compounds I or II, even operating at subsaturating nitrite concentrations. When the spectrum of LPO is monitored after the addition of nitrite and hydrogen peroxide in a stopped-flow apparatus, the spectrum of the observed intermediate species (Soret band at 425 nm) corresponds neither to compound I nor to compound II (*17*). We attribute this species to a ferric–peroxynitrite complex, presumably a very powerful nitrating agent, which can account for the much higher efficiency of LPO with respect to HRP as nitrating catalyst, particularly at low (pathophysiological) concentrations of nitrite [path (b) in Figure 1].

In conclusion, our data suggest that both mechanisms using nitrogen dioxide or peroxynitrite can be operative in peroxidase-mediated nitration of phenolics, with a relative importance that depends on reagent concentrations. For the mammalian LPO enzyme the high efficiency observed even at low nitrite concentration indicates that the peroxynitrite pathway is dominating.

Phenol Nitration Mediated by Myoglobin

Myoglobin is able to support the catalytic nitration of phenolics by nitrite and hydrogen peroxide, albeit with much lower efficiency than peroxidases ($k_{cat}/K_M^{nitrite} \approx 2 - 10$ M^{-1} s^{-1}) (*20*). In this case kinetic experiments carried out as for LPO and HRP show that the K_M^{PhOH} values are similar to those found for the corresponding catalytic oxidations of phenols in the presence of hydrogen peroxide (*21*). This indicates that the same binding site for the phenol is maintained in the two types of reactions. However, it is worth noting that the k_{cat} values for the phenol nitration are one order of magnitude larger than those for the peroxidase-like phenol oxidation. It is even surprising that with Mb the rate of reduction of compound II by nitrite (18.6 ± 0.8 M^{-1} s^{-1}) is larger than that with HRP. Preliminary experiments show that Hb is also active in the catalytic nitration of phenolics, with rates and general behavior comparable with those of Mb.

With Mb we have been able to detect and characterize the site of protein nitration occurring when the protein reacts with nitrite and hydrogen peroxide in the absence of exogenous substrate. The first and most important nitration site is the heme; the amount of nitrated heme ranges from 30 to 50% according to the experimental conditions. Upon proteolytic digestion of the protein after treatment with nitrite/hydrogen peroxide and high-performance liquid chromatography (HPLC)/mass spectrometry analysis of the resulting fragments, a secondary protein nitration site has been identified as Tyr146, with a yield of about 5%. Tyr146 is an inner residue that appears to be connected to the heme through a favorable electron transfer pathway to promote tyrosyl radical formation.

Nitration of Tryptophan Derivatives

We have recently undertaken studies on the peroxidase-mediated nitration of tryptophan derivatives. This type of investigation is complicated by the fact that, unlike tyrosine, the aromatic indole ring of tryptophan can be nitrated at different positions and, in general, the nitration of these substrates is more difficult than that of phenolic compounds. Using various chemical nitrating agents (HNO_3/CH_3COOH, gaseous nitrogen dioxide, peroxynitrite), we showed that the regioisomer

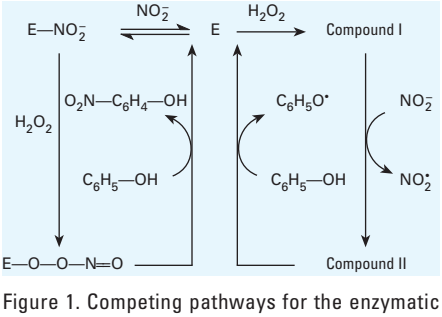

Figure 1. Competing pathways for the enzymatic nitration promoted by heme proteins.

pattern of nitrotryptophanyl derivatives, as determined through coupled HPLC separation and nuclear magnetic resonance analysis, is reagent dependent (*22*). In addition, the product mixture observed for the peroxidase-catalyzed nitration resembles that found using excess nitrogen dioxide when the reaction is carried out at low nitrite concentrations, whereas at high nitrite concentrations the product pattern resembles that obtained using peroxynitrite. This strongly supports our hypothesis of two competing mechanisms for the enzymatic and pseudoenzymatic biological nitration by heme proteins promoted by nitrite and hydrogen peroxide.

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