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**DOES HUMAN NEUROGLOBIN ACT ONLY AS A SCAVENGER? REACTIVITY
AND ENDOGENOUS MODIFICATION BY NITRITE AND HYDROGEN PEROXIDE***

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Running Title: Activation of Nitrite by Neuroglobin and Hydrogen Peroxide

Abbreviations used: NGB, human neuroglobin; Mb, myoglobin; Hb, hemoglobin; ROS, reactive oxygen species; RNS, reactive nitrogen species; NGB_{S-S} and NGB_{SH}, the two forms of NGB with and without the internal disulfide bond, respectively; 4-PDS, 4,4'-dithiodipyridine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; 4-TP, 4-thiopyridone; HPA, 3-(4-hydroxyphenyl)-propionic acid; HMb, human myoglobin; LPO, lactoperoxidase; p-NGBs, modified NGBs at low nitrite and hydrogen peroxide concentrations; h-NGBs, modified NGBs at high nitrite and hydrogen peroxide concentrations; p'-NGB_{SH}, modified NGB_{SH} at low hydrogen peroxide concentrations; h'-NGB_{SH}, modified NGB_{SH} at high hydrogen peroxide concentrations; lpo-NGBs, modified NGBs in the presence of LPO, nitrite and hydrogen peroxide; EIC, extracted ion current; ABTS, 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid); HPLC-ESI-MS/MS, high performance liquid chromatography-electrospray ionization-tandem mass spectrometry; TFA, trifluoroacetic acid; CID, collision induced dissociation; amu, atomic mass unit; Gdn-HCl, guanidinium hydrochloride.

ABSTRACT

Human neuroglobin (NGB), a recently discovered heme protein of the globin family containing a six-coordinated heme, is expressed in the nervous tissue with a yet unclear physiological function. Besides being involved in neuronal oxygen homeostasis, NGB is thought to act as a scavenger of reactive species. Herein we report on the reactivity of metNGB, which can accumulate *in vivo* by reaction of oxyNGB with nitric oxide, towards nitrite and H₂O₂. Nitrite coordination to the heme accounts for the activity of metNGB in the nitration of phenolic substrates. The two metNGB forms, with and without the internal disulfide bond between Cys(46)CD7 and Cys(55)D5, exhibit different reactivity, the former being more efficient in activating NO₂⁻. The kinetics of the reactions, the nitrite-binding studies, and the analysis of the nitrated products from different substrates all support the hypothesis that metNGB is able to generate, at pathophysiological concentration of nitrite and H₂O₂, an active species with the chemical properties of peroxynitrite.

Without external substrates, the targets of the reactive species generated by the metNGB/NO₂⁻/H₂O₂ system are endogenous tyrosine (transformed into 3-nitrotyrosine) and cysteine (oxidized to sulfinic and sulfonic acids) residues. The endogenous modifications were characterized by HPLC-MS/MS analysis of metNGB reacted with nitrite and H₂O₂ in various conditions. The internal S-S bond affects the functional properties of the protein. Therefore, metNGB acts not only as scavenger of toxic species, but also as target of the self-generated reactive species: the protein self-modification may be related or impair its postulated neuroprotective activity.

KEY WORDS: human neuroglobin, peroxynitrite, heme proteins, tyrosine nitration, cysteine oxidation, oxidative stress

INTRODUCTION

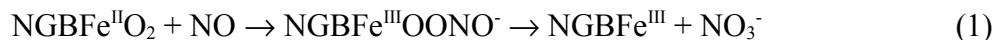
Human neuroglobin (NGB) was first identified in 2000 as a third globin type protein, besides myoglobin (Mb) and hemoglobin (Hb) [1]. NGB is expressed at low levels (in the μM range) in various regions of the brain, but at much higher levels ($\sim 100 \mu\text{M}$) in retinal cells [1,2]. NGB consists of 151 amino acids and shares high sequence similarity with mouse neuroglobin (94 % identity) but little homology with vertebrate Mbs and Hbs (<21 % and <25 % identity, respectively) [1]. It differs from the other vertebrate globins also for the six-coordinated heme (with His(96)F8 and His(64)E7 acting as the fifth/proximal and sixth/distal ligands, respectively), in both the ferrous and ferric forms [3,4] (Figure 1). The presence of a six-coordinated heme reduces the affinity of the protein for ligands with respect to the expected values in the absence of the competitive coordination by the distal histidine [3].

= Figure 1 =

The function of NGB in the human brain remains unclear. In analogy to other globins, several possible physiological roles have been considered [5]; among these, much indirect evidence such as the correlation between NGB expression and O_2 consumption, suggests a role of NGB in neuronal oxygen homeostasis [6]. The oxygen affinity of NGB depends on several factors: the relative binding rates at the heme sixth position of O_2 and the competing distal histidine [3,7], and the redox state of the cell which controls the formation or cleavage of an internal disulfide bond between Cys(46)CD7 and Cys(55)D5 [8,9]. The presence of the S-S bond could perturb the three-dimensional structure of the CD-D region and affect the location of the neighboring E-helix, thus modulating the binding of the endogenous His(64)E7 ligand to the heme [9,10]. As a consequence, in ferrous NGB the distal histidine dissociation rate increases by a factor of 10 with respect to the protein form without the disulfide bond [9,11], thus leading to an effective increase in O_2 affinity by the same factor [12] (Figure 1).

O_2 storage or diffusion to or from the heme of NGB may be assisted by the presence of a large protein matrix cavity (about 120 \AA^3), which is located between the heme distal site and the EF interhelical hinge and connected to the protein surface [10,13]. The cavity may be reshaped upon ligand binding, which may allow trapping of harmful reactive oxygen or nitrogen species (ROS and RNS, respectively) [14-17]. In particular, the rise of NO concentration up to the low μM range occurring under ischemic conditions [18], may be contrasted by its reaction with

oxyNGB, yielding metNGB (i.e. the ferric form of NGB) and NO_3^- , through a heme-bound peroxynitrite intermediate [15]:



Since in the brain a metNGB reductase system has not been identified yet [15], the *in vivo* formation of metNGB through this mechanism may have physiological relevance. Also metNGB reacts with NO [19], generating the stable $\text{NGBFe}^{\text{II}}\text{NO}$ species by reductive nitrosylation; this form of NGB is a good scavenger of peroxynitrite, which oxidizes the protein back to metNGB, according to the following reactions [19]:



In contrast to Mb and Hb, the protein does not generate the ferryl form upon reaction with H_2O_2 or ONOO^- [20]. No reaction was either observed upon addition of nitrite [19].

Thus, even if the Fe^{III} center of metNGB seems to be protected from attack of oxidizing species by the coordinated distal His, the competitive coordination of exogenous ligands could account for the activation of alternative reactions. Herein we report on the reactivity of human metNGB in the presence of nitrite and hydrogen peroxide, whose concentrations *in vivo* increase under conditions of oxidative stress [21]. The activation of $\text{NO}_2^-/\text{H}_2\text{O}_2$ by metNGB was assayed in the nitration of phenolic substrates [22-24], obtaining an unexpectedly high reactivity. Moreover, the two forms of the protein, with and without the internal disulfide bond (metNGB_{S-S} and metNGB_{SH}, respectively), exhibit significantly different efficiency in the activation of NO_2^- and H_2O_2 . The problem of the endogenous modification of metNGB by reaction with NO_2^- and H_2O_2 , even at pathophysiological concentrations, in the absence of external substrates was also addressed, since the identification of protein residues modified by ROS and RNS is essential for the understanding of the mechanisms of development of various pathologies [25-29] and the postulated neuroprotective function of NGB. The endogenous targets of the reactive species generated by the protein are tyrosine and cysteine residues, with a more extensive pattern of modifications than previously observed with HMb [24].

EXPERIMENTAL

Reagents - All the buffer solutions were prepared with deionized Milli-Q water. Hydrogen peroxide (30 % solution), 3-(4-hydroxyphenyl)-propionic acid (HPA), 4-hydroxybenzotrile and phenylacetic acid were obtained from Aldrich, whereas trypsin was from Sigma. The other reagents were obtained at the best grade available. The concentration of hydrogen peroxide solutions was controlled by monitoring the formation of the ABTS radical cation according to a standard enzymatic method [30]. Recombinant human NGB was expressed and purified as previously reported [12]. In order to obtain a homogeneous sulfur-bridged protein (i.e. NGB_{S-S}), the protein solution was dialyzed overnight at 4 °C against 50 mM phosphate buffer pH 7.5. To reduce the intramolecular disulfide bond (obtaining NGB_{SH}), the NGB solution was dialyzed at 37 °C against 1 mg/ml DTT dissolved in degassed 50 mM Tris-HCl/0.5 mM EDTA buffer, pH 7.5, under anaerobic conditions. After 30 min reduction, three exchanges of the same degassed buffer (50 mM Tris-HCl/0.5 mM EDTA, pH 7.5) were made to eliminate DTT. Protein dialysis was always performed at 4 °C. In all the experiments described below, when not explicitly stated, the proteins (NGB_{S-S} and NGB_{SH}) were utilized in their met form. All spectrophotometric measurements were performed on a Hewlett Packard HP 8452A diode array spectrophotometer.

Kinetic Studies of Phenol Nitration Catalyzed by NGB_{S-S} and NGB_{SH} - The kinetic experiments were carried out in 200 mM phosphate buffer pH 7.5 using a quartz cuvette with path length of 1 cm, thermostated at 25.0 ± 0.1 °C and equipped with a magnetic stirrer. The initial solution containing protein and variable substrate and nitrite concentrations (final volume 1600 µL) was obtained by mixing solutions of appropriate concentration of the reagents in the buffer. The reaction was started by the addition of the H₂O₂ solution and was followed by monitoring the absorbance change at 450 nm during the initial 10-15 s. The conversion of the rate data from absorbance s⁻¹ into [nitrophenol] s⁻¹ was done by using the extinction coefficient of 3-(4-hydroxy-3-nitrophenyl)-propionic acid at 450 nm, ε = 3350 M⁻¹ cm⁻¹ [22]. The kinetic parameters were obtained from fitting the plots of experimental rates at different substrate/nitrite concentrations to the appropriate equations (see *Results*).

For each substrate, the rate dependence on the various reactant concentrations was studied through three series of steps: (1) finding a suitable [H₂O₂] maximizing the rate but avoiding unwanted excess of the oxidant, and then using this [H₂O₂] (2) study the dependence of

the rate *vs.* [HPA], and (3) study the dependence of the rate *vs.* [NO₂⁻], following the iterative procedure previously described in detail [23]. The protein (NGB_{S-S} or NGB_{SH}) concentration was 1 μM in all the reactions, while the concentration of the other reactants were as follows: (1) optimization of the peroxide concentration: using NGB_{S-S}, [HPA] = 1 mM, [NO₂⁻] = 0.3 M, [H₂O₂] = 0.09–0.8 M; using NGB_{SH}, [HPA] = 0.4 mM, [NO₂⁻] = 1.0 M, [H₂O₂] = 0.09–0.8 M; (2) dependence of the rate *vs.* HPA concentration: using NGB_{S-S}, [H₂O₂] = 0.6 M, [NO₂⁻] = 0.3 M, [HPA] = 0.031–1.2 mM; using NGB_{SH}, [H₂O₂] = 0.6 M, [NO₂⁻] = 1.0 M, [HPA] = 0.0096–0.96 mM; (3) dependence of the rate *vs.* nitrite concentration: using NGB_{S-S}, [H₂O₂] = 0.6 M, [HPA] = 0.4 mM, [NO₂⁻] = 0.0062–0.44 M; using NGB_{SH}, [H₂O₂] = 0.6 M, [HPA] = 0.3 mM, [NO₂⁻] = 0.037–2.5 M.

The reaction rates observed in the absence of the protein (noncatalytic reaction) or without hydrogen peroxide are completely negligible.

Binding of Nitrite - To a solution of NGB_{S-S} (4.8 μM, 1600 μL) or NGB_{SH} (5.2 μM, 1600 μL) in 200 mM phosphate buffer pH 7.5, increasing quantities of a concentrated nitrite solution in the same buffer (final concentration from 0 to 0.42 M for NGB_{S-S} and from 0 to 0.64 M for NGB_{SH}) were added in a quartz cuvette with path length of 1 cm, thermostated at 25.0 ± 0.1 °C, and UV-Vis spectra were recorded after each addition. Blank spectra were recorded in the same way but in the absence of protein. After subtracting the corresponding blank to each spectrum, the resulting spectra were corrected for dilution and then transformed into difference spectra by subtracting the native protein spectrum. A plot was constructed with the difference between the absorbance changes at 414 nm and at 434 nm, the wavelengths at which the difference spectra exhibit the maximum variations, *vs.* the ligand concentration.

For the binding of NO₂⁻ to NGB_{S-S}, the constants K₁ and K₂ were obtained by interpolation of the absorbance data with the binding isotherm for low affinity binding of two consecutive ligands: $\Delta A = (\Delta A_{\infty,1} \times K_1 \times [\text{NO}_2^-]_{\text{tot}} + \Delta A_{\infty,2} \times K_1 \times K_2 \times [\text{NO}_2^-]_{\text{tot}}^2) / (1 + K_1 \times [\text{NO}_2^-]_{\text{tot}} + K_1 \times K_2 \times [\text{NO}_2^-]_{\text{tot}}^2)$ (where ΔA_{∞,1} and ΔA_{∞,2} represent, respectively, the absorbance changes due to the binding of one and two ligand molecules, and [NO₂⁻]_{tot} the total nitrite concentration, free plus bound). For the binding of NO₂⁻ to NGB_{SH}, the constant K_B was obtained by interpolation of the absorbance data with the binding isotherm for low affinity binding of a single ligand: $\Delta A = \Delta A_{\infty} \times K_B \times [\text{NO}_2^-]_{\text{tot}} / (1 + K_B \times [\text{NO}_2^-]_{\text{tot}})$ (where ΔA_∞ represents the absorbance change consequent to the binding of NO₂⁻, and [NO₂⁻]_{tot} the total nitrite concentration).

HPLC Analysis of the Nitration Products of 4-Hydroxybenzointrile and Phenylacetic Acid - The product mixtures derived from the nitration of 4-hydroxybenzointrile and phenylacetic acid promoted by the NGB/NO₂⁻/H₂O₂ system, were analyzed by HPLC using a Jasco MD-1510 instrument with diode array detection equipped with a SupelcosilTM LC18 reverse-phase semipreparative column (5 μm, 250×10 mm). Elution was carried out using 0.1 % TFA in distilled water (solvent A) and 0.1 % TFA in acetonitrile (solvent B), with a flow rate of 5 ml/min. Elution started with 100 % solvent A for 5 min, followed by a linear gradient from 100 % A to 100 % B in 25 min. Spectrophotometric detection of the eluate was performed in the range 200-600 nm.

The catalytic nitrations of 4-hydroxybenzointrile and phenylacetic acid were studied under the following experimental conditions: [NGB_{S-S}] = 1 μM, [substrate] = 1 mM, [H₂O₂] = 0.6 M, [NO₂⁻] = 0.01, 0.05, or 0.15 M, in 200 mM phosphate buffer pH 7.5. The reaction mixtures were allowed to react for 10 min at room temperature, and then were analyzed by HPLC as described above. The retention time of 4-hydroxybenzointrile was 15.7 min, while the corresponding nitration product, 4-hydroxy-3-nitrobenzointrile, was absent in all the conditions. Its retention time (17.4 min) was established through the LPO-catalyzed nitration carried out in the following conditions: [LPO] = 80 nM, [phenol] = 1 mM, [H₂O₂] = 1 mM, [NO₂⁻] = 0.01 M, in 200 mM phosphate buffer pH 7.5. The HPLC chromatograms of the mixtures obtained from the nitration of phenylacetic acid showed, besides unreacted substrate at 16.8 min, two main peaks at 16.0 and 16.2 min and two minor peaks at 14.5 and 14.7 min (obtained all both at low and high NO₂⁻ concentrations).

HPLC analysis showed that no modification of 4-hydroxybenzointrile and phenylacetic acid occurred in the absence of the protein or without hydrogen peroxide.

Modification of NGB in the Presence of NO₂⁻ and H₂O₂ - Samples of modified NGB_{S-S} and NGB_{SH} proteins were prepared by adding to the proteins solutions (60 μM, in 50 mM phosphate buffer pH 7.5 for NGB_{S-S}, and in 50 mM Tris-HCl/0.5 mM EDTA buffer pH 7.5 for NGB_{SH}) sodium nitrite and hydrogen peroxide to the concentrations described below. a) pathophysiological conditions [21]: for p-NGB_{S-S} and p-NGB_{SH}, 0.1 mM NO₂⁻ and 0.15 mM H₂O₂ (divided in 5 aliquots of 30 μM each); b) harsh conditions: for h-NGB_{S-S} and h-NGB_{SH}, 0.1 M NO₂⁻ and 1.0 mM H₂O₂ (divided in 5 aliquots); c) modifications without nitrite: for p'-NGB_{SH} and h'-NGB_{SH} 0.15 mM H₂O₂ (divided in 5 aliquots of 30 μM each) and 1.0 mM H₂O₂ (divided

in 5 aliquots), respectively (no nitrite); d) LPO catalyzed modification: for lpo-NGB_{S-S} and lpo-NGB_{SH}, 0.25 M NO₂⁻ and 0.15 mM H₂O₂ (divided in 5 aliquots), and in addition a solution of LPO (80 nM) was added.

The proteins were allowed to react at 20 °C for 10 min. Excess nitrite and oxidant were removed by overnight dialysis against 20 mM phosphate buffer pH 7.5 for NGB_{S-S} derivatives and degassed 20 mM Tris-HCl/0.2 mM EDTA buffer pH 7.5 for NGB_{SH} derivatives.

Analysis of Protein Fragments and Heme Modification - For the analysis of protein fragments, a portion of each sample of the unmodified proteins NGB_{S-S} and NGB_{SH}, and the derivatives p-, h-, and lpo-NGB_{S-S}, and p-, h-, p'-, h', and lpo-NGB_{SH} (about 0.5 mg) was transformed into the apo-protein by standard hydrochloric acid/2-butanone method [31] and subsequently hydrolyzed by trypsin. Digestion was performed with 1:50 (w/w) trypsin at 37 °C for 3 h in 20 mM ammonium bicarbonate buffer pH 8.0. Prior to the HPLC-MS/MS analysis, the samples were reacted with 5 mM DTT for 15 min at 37 °C since Cys containing peptides generally couple with one other during digestion. The reduction of the disulfide bonds was necessary for the quantification of the unreacted cysteine residues in NGB derivatives.

The heme modification was studied by direct HPLC-MS/MS analysis on solutions of NGB derivatives in 20 mM ammonium bicarbonate buffer pH 8.0 after acidification with HCl to pH ~ 1.

LC-MS and LC-MS/MS data were obtained using an LCQ ADV MAX ion trap mass spectrometer equipped with an ESI ion source and controlled by Xcalibur software 1.3 (Thermo-Finnigan, San Jose, CA, USA). ESI experiments were carried out in positive ion mode under the following constant instrumental conditions: source voltage 5.0 kV, capillary voltage 46 V, capillary temperature 210 °C, tube lens voltage 55 V. The system was run in automated LC-MS/MS mode and using a Surveyor HPLC system (Thermo Finnigan, San Jose, CA, USA) equipped with a BioBasic™ C18 column, 5 µm, 150×2.1 mm. The elution was performed using 0.1 % HCOOH in distilled water (solvent A) and 0.1 % HCOOH in acetonitrile (solvent B), with a flow rate of 0.2 ml/min. Elution started with 98 % solvent A for 5 min, followed by a linear gradient from 98 % to 55 % A in 65 min for the analysis of tryptic digests, and by a two-steps linear gradient (from 98 % to 50 % A in 5 min, and then from 50 % to 20 % A in 40 min) for the analysis of the non-digested protein solutions. MS/MS spectra obtained by CID were performed

with an isolation width of 2 Th (m/z), the activation amplitude was around 35 % of ejection RF amplitude of the instrument.

For the analysis of protein fragments derived from NGB derivatives the mass spectrometer was set such that one full MS scan was followed by zoomscan and MS/MS scan on the most intense ion from the MS spectrum. To identify the modified residues, the acquired MS/MS spectra were automatically searched against protein database for human neuroglobin using the SEQUEST[®] algorithm incorporated into Bioworks 3.1 (ThermoFinnigan, San Jose, CA).

Guanidinium hydrochloride denaturation assay – The stability to denaturation of NGB_{SH}, p-NGB_{SH}, and h-NGB_{SH} was determined by monitoring the absorbance variation of the Soret band of the protein (about 7 μ M) upon addition of increasing amounts of a 8 M guanidinium hydrochloride (Gdn-HCl) solution (up to 4.5 M) in degassed 50 mM Tris-HCl/0.5 mM EDTA buffer, pH 6.0, to the protein solution in the same buffer. Data were corrected for dilution by the Gdn-HCl addition. The Gdn-HCl concentration at 50 % unfolding of the proteins was evaluated from the curves of absorbance versus denaturant concentration according to a standard method [32].

RESULTS

The Two Forms of NGB, with and without the Disulfide Bond - The purification of human NGB provides the protein in its met form, since *in vitro* the oxygenated form of NGB is unstable and rapidly autoxidizes to the ferric form ($t_{1/2} = 11$ min) [7]. This is confirmed by the UV-Vis spectrum, which is characteristic of metNGB (Figure 2) and clearly distinguishable in the visible region from the spectrum of the Fe^{II}-O₂ form (for metNGB, $\lambda_{max} = 532$ nm with a shoulder at 554 nm; for oxyNGB, $\lambda_{max} = 543, 576$ nm) [7,12]. The molar extinction coefficient of the Soret band of metNGB in the disulfide-bridged form (metNGB_{S,S}), obtained from the standard pyridine hemochrome assay [33] and used in the determination of the protein concentration, was 1.29×10^5 M⁻¹ cm⁻¹ at 414 nm (in phosphate buffer pH 7.5). The phosphate buffer used in our experiments is believed to promote the disulfide bond formation (Figure 1) [12]. Actually, the number of accessible thiol groups per heme measured by the 4,4'-dithiodipyridine (4-PDS) assay [12,34] was slightly lower than 1.0. This result is consistent with the presence of the single Cys(120)G19 in the reduced form and with the almost quantitative oxidation of the other two

cysteines to S-S bond [9]. These disulfide bonds are thought to play a significant role in the protein [9,35].

= Figure 2 =

Treatment of NGB_{S-S} with DTT in anaerobic conditions leads to the partial reduction of the disulfide bridge; NGB_{SH} is stable to oxidation when kept in degassed Tris-EDTA buffer at 4 °C. Different experimental conditions (temperature and reaction time) were examined to maximize the S-S bond reduction, obtaining at most 2.4 cysteines per heme titrated in the protein reacted with DTT for 30 min at 37 °C. It should be noted that the 4-PDS assay underestimates the number of thiol groups, since the initial fast reduction of 4-PDS to 4-thiopyridone by the CysSH groups overlaps with the subsequent slow formation of the latter product by reaction with some reducing species that can be present in solution; therefore, it can be assumed that in NGB_{SH} almost all Cys residues are in the reduced form.

The UV-Vis spectra of the met forms of NGB_{S-S} and NGB_{SH} are identical ($\lambda_{\text{max}} = 414, 532$ and 554sh nm) and characteristic of a six-coordinated ferric heme (Figure 2). Actually, even if the ratio between the high-spin and low-spin forms increases upon formation of the disulfide bridge, the amount of high-spin form remains low (less than 5 % at pH 7.5 [36], and less than 10 % at pH 5 [37]): the only species detectable in the UV-Vis spectra is the predominant six-coordinated species, for both NGB_{S-S} and NGB_{SH}.

Phenol Oxidation - In the presence of hydrogen peroxide, heme proteins such as Mb in their met form exhibit peroxidase-like activity towards phenolic substrates, oxidizing them to phenoxy radicals, which give rise to dimers in solution [38,39]. The formation of these species can be followed spectrophotometrically through the characteristic absorption around 300 nm. Moreover, a protein ferryl intermediate (named compound II) can be detected in the reactions of both Mb and peroxidases upon addition of the oxidant. This is characterized by a red-shift of the protein Soret band, from 408 to 424 nm for Mb [40], 403 to 420 nm for horseradish peroxidase [41], and 412 to 422 nm for LPO [42]. In contrast, no spectral change was observed upon mixing NGB (1 μM) with H₂O₂ (up to 100 mM) in the absence or presence of a phenol like HPA. Indeed, also Herold et al. reported that, in contrast to Mb and Hb, NGB does not generate the ferryl form of the protein, since its reactivity is strongly limited by the coordinated distal histidine [19]. In fact, when NGB is reacted with H₂O₂ and HPA, the UV-Vis spectral features

characteristic of phenolic dimers do not develop, indicating that the reactivity of NGB with hydrogen peroxide is negligible.

Phenol Nitration Catalyzed by NGB_{S-S} and NGB_{SH} - Surprisingly, NGB activates nitrite in the presence of H₂O₂ and nitrates the phenolic substrate HPA in *ortho* position to the hydroxyl group. An analogous catalytic activity was reported for peroxidases [21,22,43,44] and the heme proteins Mb [23,24,45,46] and Hb [46]. The investigation of the kinetics of phenol nitration catalyzed by NGB (both the NGB_{S-S} and NGB_{SH} forms) is not simple, since the reaction rate depends on the concentration of all the reagents. In order to get insight into the mechanism of nitration by the metNGB/NO₂⁻/H₂O₂ system, it was necessary to investigate the reaction varying the concentration of the reagents in a large range, beyond the values that can be encountered *in vivo*. Following the approximations and procedures that we previously followed for the enzymatic nitration reactions [22] it was possible to obtain the kinetic parameters k_{cat} , K_M^{Ph-OH} , and k_{cat}/K_M^{Ph-OH} , from the rate dependence on the HPA concentration, through the following equation:

$$rate = \frac{k_{cat} \cdot [NGB] \cdot [HPA]}{K_M^{Ph-OH} + [HPA]} \quad (5)$$

and k_{cat} , $K_M^{nitrite}$, and $k_{cat}/K_M^{nitrite}$ from the rate dependence on [NO₂⁻], through the following equation:

$$rate = \frac{k_{cat} \cdot [NGB] \cdot [NO_2^-]}{K_M^{nitrite} + [NO_2^-]} \quad (6)$$

The data are collected in Table 1; for comparison purposes, also the corresponding data for the nitration of HPA catalyzed by human myoglobin (HMb) are reported [24]. The rate constant k_{cat} represents the maximum turnover number of the proteins, K_M^{Ph-OH} and $K_M^{nitrite}$ give an indication of the dissociation constants of HPA and NO₂⁻, respectively, from the complexes with the proteins, and k_{cat}/K_M^{Ph-OH} and $k_{cat}/K_M^{nitrite}$ give the dependence of the reaction rates on HPA and NO₂⁻, respectively, at low reagent concentrations.

It is worth noting that the *overall* catalytic efficiency of NGB (expressed by k_{cat} and both k_{cat}/K_M^{Ph-OH} and $k_{cat}/K_M^{nitrite}$) is only slightly lower than that of Mb. This is surprising, considering that NGB contains a six-coordinate heme, because the reactivity of the protein in the nitration

reaction was expected to be negligible, as found for the phenol oxidation. Since the fraction of five-coordinated high-spin metNGB is less than 5 %, its intrinsic reactivity must be very high, much more than that of Mb. The interpretation of the kinetic parameters is complicated by the presence of the heme-distal His dissociation equilibrium, since the reported value of rate constant for His release, $k_{\text{H}} \sim 1 \text{ s}^{-1}$ [36], is of the same order of magnitude of the NGB turnover rate, k_{cat} . It follows that the high-spin/low-spin equilibrium is neither slow nor fast, so that, during the kinetic studies, the steady state is not reached in the initial phase and the predominant form of the protein that is present in solution tends to change with time.

The most interesting information emerges from the catalytic activity studied as a function of nitrite concentration, since the disulfide-bridged $\text{NGB}_{\text{S-S}}$ exhibits a higher affinity for NO_2^- with respect to the thiol form NGB_{SH} ($K_{\text{M}}^{\text{nitrite}} = 43$ and 220 mM , respectively). For ferrous NGB_{SH} , the distal histidine dissociation rate is nearly one order of magnitude smaller than that of $\text{NGB}_{\text{S-S}}$, thus leading to an effective decrease in O_2 affinity [9]. A similar effect of the S-S bond can be considered for the metNGBs on the iron affinity towards exogenous ligands such as nitrite. Therefore, the larger $K_{\text{M}}^{\text{nitrite}}$ value observed for NGB_{SH} is in agreement with the requirement of nitrite coordination to the iron of NGB in order to promote nitrating activity.

The catalytic efficiency at low $[\text{NO}_2^-]$ (i.e. $k_{\text{cat}}/K_{\text{M}}^{\text{nitrite}}$) is similar for the two proteins $\text{NGB}_{\text{S-S}}$ and HMb, since the lower turnover rate of the former is compensated by its higher affinity for nitrite.

Binding of Nitrite to $\text{NGB}_{\text{S-S}}$ and NGB_{SH} - Small but significant changes can be observed in the spectrum of $\text{NGB}_{\text{S-S}}$ upon addition of excess nitrite to a solution of the protein. Difference spectra from optical titration clearly show both a smoothing and a slight shift towards higher energy of the Soret band during the first part of the binding process (at $[\text{NO}_2^-]$ below 50 mM), and a slight decrease in the intensity of the band in the second part of the titration (at $[\text{NO}_2^-]$ above 50 mM). The fitting of the changes of the Soret band as a function of $[\text{NO}_2^-]$ is consistent with low affinity binding of two ligands, yielding the constants $K_1 = (51 \pm 15) \text{ M}^{-1}$ and $K_2 = (5 \pm 2) \text{ M}^{-1}$ (Figure 3). The former constant probably refers to an electrostatic interaction of a nitrite ion with charged amino acid residues of the protein and the latter constant to the coordination of a nitrite ion to the iron center. As nitrite has to replace the distal histidine from the sixth

coordination site, the affinity of NGB_{S-S} for nitrite results about one order of magnitude lower with respect to that previously obtained for HMb in the same conditions ($K_B = 76 \text{ M}^{-1}$) [24].

The nitrite binding experiment performed with NGB_{SH} shows a different behavior with respect to NGB_{S-S}, since only the initial spectral changes are similar for the two proteins. Indeed, the data could be fitted with a single binding isotherm, giving the association constant $K_B = (48 \pm 5) \text{ M}^{-1}$, very similar to the K_1 value obtained with NGB_{S-S} (Figure 3). By analogy, also in this case the process can be associated to the electrostatic interaction of nitrite to NGB_{SH}. It was impossible for NGB_{SH} to obtain a reliable binding constant for the direct coordination of nitrite to the heme iron, thus confirming the lower affinity of the thiol form of the protein for exogenous ligands with respect to the disulfide-bridged NGB, as it emerged also from the kinetic studies of phenol nitration.

= Figure 3 =

The interaction of NGB with nitrite was also investigated by the group of Herold, but at the low $[\text{NO}_2^-]$ employed (below 1 mM) the binding of nitrite was not observed [19]. Since metNGB is active in promoting the nitration reaction also at low nitrite concentration (pathophysiological values), a small and hardly detectable fraction of the heme iron must be accessible to the ligand.

Nitration of 4-Hydroxybenzointrile and Phenylacetic Acid - The substrates phenylacetic acid and 4-hydroxybenzointrile were used as mechanistic probes for the nitration catalyzed by the system NGB/ $\text{NO}_2^-/\text{H}_2\text{O}_2$. The reaction could proceed through the formation of the same nitrating species involved in the reactions promoted by peroxidases and Mb, i.e. nitrogen dioxide (NO_2) or peroxynitrite (ONOO^-), depending on nitrite concentration [22-24]. Phenylacetic acid reacts with ONOO^- generating nitrated and/or hydroxylated compounds, while it is unreactive to NO_2 ; hence it is a good probe for peroxynitrite. The reactivity of 4-hydroxybenzointrile is totally different, since it reacts with NO_2 generating 4-hydroxy-3-nitrobenzointrile, while ONOO^- is a poor nitrating agent for this substrate [22] (Figure 4).

= Figure 4 =

4-Hydroxybenzointrile was then reacted with NGB and H_2O_2 at different nitrite concentrations (from 10 to 150 mM) and the reaction mixtures were analyzed by HPLC. The nitrated product was absent in all the conditions, indicating that NO_2 was not formed, or formed in negligible amounts, by NGB in these conditions. In contrast, NGB/ $\text{NO}_2^-/\text{H}_2\text{O}_2$ induced the

modification of phenylacetic acid and by HPLC it was possible to separate four different products, independently of NO_2^- concentration. These derivatives shares spectral features and retention times with the products obtained in the reaction of phenylacetic acid with peroxyxynitrite [22]. These results suggest that the nitration catalyzed by NGB in the presence of nitrite and H_2O_2 proceeds through the formation of a reactive species with the chemical properties of peroxyxynitrite (Figure 4).

Tandem Mass Analysis of NGB_{S-S} and NGB_{SH} Modified by H₂O₂ and NO₂⁻ - To investigate the endogenous modifications undergone by NGB in the presence of nitrite and hydrogen peroxide, the protein (both in the NGB_{S-S} and NGB_{SH} forms) was reacted with various amounts of NO_2^- and H_2O_2 in the absence of external substrate. Depending on the concentration of the reagents, a series of protein derivatives was prepared: *i*) under very mild conditions, similar to those that can occur under pathophysiological conditions [21], NGB_{S-S} and NGB_{SH} were reacted with 0.1 mM NO_2^- and 0.15 mM H_2O_2 , obtaining p-NGB_{S-S} and p-NGB_{SH}, respectively; *ii*) under more harsh conditions, NGB_{S-S} and NGB_{SH} were reacted with 100 mM NO_2^- and 1 mM H_2O_2 , obtaining h-NGB_{S-S} and h-NGB_{SH}, respectively; *iii*) the reactivity of the cysteine residues in the presence of hydrogen peroxide alone was studied by reacting NGB_{SH} with H_2O_2 at low (0.15 mM) and high (1 mM) oxidant concentration, obtaining p'-NGB_{SH} and h'-NGB_{SH}, respectively.

Moreover, the derivatization of NGB by NO_2^- and H_2O_2 was performed in the presence of LPO as an external source of the nitrating agent. The protein derivatives obtained in these conditions were labeled lpo-NGB_{S-S} and lpo-NGB_{SH}.

The UV-Vis spectra of the p-NGB derivatives are basically indistinguishable from that of native metNGB, while for the h-NGB and lpo-NGB derivatives both a slight broadening of the Soret band and the appearance of a shoulder around 440-450 nm are observed. These spectral features reflect the color change of the protein solutions from brown to greenish-brown accompanying the formation of h-NGB_{S-S}, h-NGB_{SH}, lpo-NGB_{S-S} and lpo-NGB_{SH} derivatives and are associated with heme nitration [24]. All the protein derivatives were analyzed by HPLC-ESI-MS/MS with the aim of investigating the endogenous modifications; the results are reported in Tables 2 and 3. The potential targets of the oxidizing and nitrating species are, besides the heme prosthetic group, the protein tyrosine residues (Tyr(44)CD3, Tyr88, Tyr(115)G14, and Tyr(137)H12) and cysteine residues (Cys(46)CD7, Cys(55)D5, and Cys(120)G19 in NGB_{SH}; only Cys(120)G19 in NGB_{S-S}) (Figure 5).

= Figure 5 =

The modification of the prosthetic group can be detected by direct HPLC-MS/MS analysis of the acidified protein solution, since the heme group is released from the protein. The modified heme was detected only in the NGB derivatives obtained upon reaction with high levels of NO_2^- and H_2O_2 , i.e. h-NGB_{S-S} and h-NGB_{SH}. In particular, the ions with *m/z* 616 and 661, corresponding to free heme and heme modified with a NO_2 group [23,24], respectively, were identified. The modification probably occurs at one of the heme vinyl groups, in analogy to the heme nitration observed upon treatment Mb with a large excess of nitrite at pH 5.5 [47]. Integration of the peaks in the EIC chromatograms indicated that heme nitration occurred with 38 % yield in h-NGB_{S-S}, 14 % in h-NGB_{SH}, 25 % in lpo-NGB_{S-S}, and 27 % in lpo-NGB_{SH} (Table 2).

The modifications at the cysteine and tyrosine residues were characterized on the polypeptide fragments resulting from tryptic digestion of the apoNGB derivatives. The HPLC-ESI-MS/MS analysis showed the presence of many modified peptide fragments (the molecular weights and *m/z* values as bicharged ions are reported in Supplementary Table S1). The analysis of the MS/MS spectra with the SEQUEST[®] algorithm allowed the assignment of the modifications to the nitration of the tyrosines to 3-nitrotyrosine [23] and the oxidation of the cysteines to sulfinic (RSO_2H) and sulfonic (RSO_3H) acids. As an example of MS results, the MS/MS spectrum of the 68-94 peptide nitrated at Tyr88 obtained by the analysis of h-NGB_{S-S}, is shown in Figure 6 (with the y and b ion series).

= Figure 6 =

The percent of residue modifications is reported in Tables 2 and 3, and was obtained by comparing, in the EIC chromatograms, the areas of the peaks corresponding to the derivatized peptides with those of the corresponding peptides in the starting proteins. The greater efficiency of NGB_{S-S} with respect to NGB_{SH} in promoting self-nitration of both the heme and the tyrosine residues by NO_2^- and H_2O_2 clearly appears from the amount of endogenous modifications obtained for h-NGB_{S-S} and h-NGB_{SH} (Table 2). This is in agreement with the kinetic parameters for the phenol nitration (Table 1), since NGB_{S-S} exhibits a significantly higher catalytic efficiency in terms of $k_{\text{cat}}/K_M^{\text{nitrite}}$ with respect to NGB_{SH}.

A complete picture of the cysteine derivatizations is provided by the modification of NGB_{SH} under different conditions, giving rise to the derivatives h-, p-, h'- and p'-NGB_{SH} (Table

3). All the cysteine residues were oxidized to sulfinic and sulfonic acids; the amount of the modification induced by NO_2^- and H_2O_2 increases with the concentration of the reagents (compare p-NGB_{SH} with h-NGB_{SH}) and is significantly lower in the absence of nitrite (h'-NGB_{SH} vs. p'-NGB_{SH}), thus indicating that the NGB/ NO_2^- / H_2O_2 system generates a distinctive oxidant species.

The mechanism of protein modification was explored by inducing the derivatization in the presence of an external catalyst. The enzyme LPO was chosen, since it reacts with H_2O_2 and NO_2^- generating nitrating species with a rate two orders of magnitude larger with respect to NGB [22]. Therefore, when NGB is reacted with nitrite at low peroxide concentration in the presence of LPO, the reactive species is generated by the enzyme, making negligible the NGB self-promoted derivatization. This is confirmed by the similarity in the extent of nitration (both at the heme and the tyrosine residues) obtained for the LPO-promoted modification of NGB_{S-S} and NGB_{SH} (Table 2).

From the analysis of the derivatives lpo-NGB_{S-S} and lpo-NGB_{SH}, it emerges that the peroxidase promoted NGB tyrosine nitration and cysteine oxidation occur to a lower extent with respect to the modifications self-promoted by NGB. In contrast, for both lpo-NGBs the heme is the favored modification site (Tables 2 and 3). These results indicate that the prosthetic group is the most accessible target for the LPO-generated reactive species, while the NGB-generated reactive species exhibits a preference for intramolecular rather than intermolecular reactivity.

Guanidinium hydrochloride denaturation assay – The unfolding midpoint [D_0] in the presence of Gdn-HCl for NGB_{SH}, p-NGB_{SH}, and h-NGB_{SH}, obtained from the curves of absorbance vs. denaturant concentration, was estimated to be in the range of 4.1-4.2 M for both the proteins. These values can be compared with those obtained in the same conditions for sperm whale Mb, [D_0] = 1.46 M [32], and horse heart Mb, [D_0] = 1.32 M (unpublished data). Therefore, NGB exhibits a considerably higher stability to Gdn-HCl than myoglobins. Moreover, the derivatization of the protein residues (nitration of tyrosines and oxidation of cysteines) in the h-NGB_{SH} derivative has very little effect on the stability of the protein. Treatment of NGB with pathophysiological nitrite and H_2O_2 concentrations, yielding p-NGB_{SH}, induces completely negligible effects on protein stability to Gdn-HCl denaturation. A similar negligible effect toward denaturation is observed for horse heart Mb upon nitration of the tyrosine residues in the presence of NO_2^- and H_2O_2 ([D_0] = 1.35 M vs. 1.32 M for the native protein). A more detailed

characterization of the unfolding properties of NGB and its derivatives will be carried out separately.

DISCUSSION

In Vivo NGB Species - Since NGB possesses a bis-histidine, six-coordinated heme, the reactivity of the protein towards exogenous ligands is regulated by the dissociation rate of the distal His residue. Nevertheless, NGB in its ferrous state shows significant reactivity towards small ligands such as NO, O₂ and CO. The competition between these external molecules and the histidine residue for the sixth coordination site of the Fe^{II} center emerges from the kinetic and thermodynamic constants so far reported [3,4,48]. It follows that in spite of the high intrinsic affinity of NGB for both O₂ and CO, the binding of these ligands is suggested to be slow *in vivo* [7]. Furthermore, the influence of the internal disulfide bridge on the affinity of ferrous NGB_{S-S} for molecular oxygen is clearly demonstrated [9].

In the reducing conditions existing within living cells, NGB would be present in the ferrous NGB_{SH} form, saturated with O₂ at ~ 12 % [12]. Although the ferric form studied here may not be the most physiologically relevant form of the protein, the presence of hydrogen peroxide and nitrite would rapidly convert ferrous or oxyNGB to the met form. Furthermore, metNGB is the product of the fast NO scavenging by oxyNGB, that in the absence of a metNGB reductase system would accumulate metNGB (according to reaction 1) [15].

MetNGB Promoted Nitration of Phenolic Compounds - Currently, the only data concerning the behavior of metNGB with hydrogen peroxide indicate an absence of reactivity [19]. The results reported here show an interesting pseudo-enzymatic activity of metNGB in the presence of H₂O₂ and nitrite. Even if at first the mechanism of the NGB reaction could be considered similar to that of peroxidases, this contrasts with the absence of activity of metNGB in the oxidation of phenolic compounds in the presence of hydrogen peroxide alone. In fact, in these conditions metNGB does not generate the ferryl species compound I and II, that are the characteristic intermediates of the catalytic cycle of peroxidases. Nevertheless, in analogy to peroxidases, metNGB promotes nitration of phenolic substrates by activation of NO₂⁻ and H₂O₂. This result is not in contradiction with the absence of reaction between NGB and H₂O₂, since besides the peroxidase-like mechanism of nitration through NO₂, an alternative mechanism for the nitration of phenolic substrates emerged in our previous studies on peroxidases and Mb in the

presence of NO_2^- and H_2O_2 [22,23]. This mechanism involves the initial binding of nitrite to the heme iron, followed by the reaction of hydrogen peroxide with the coordinated NO_2^- , to produce an iron-bound peroxynitrite ($\text{NGBFe}^{\text{III}}\text{-N(O)OO}$), which acts as the nitrating active species. In the case of peroxidases and Mbs, the ONOO^- pathway becomes important only at relatively high nitrite concentrations [22-24], while with NGB our results indicate that this is the only mechanism active in the nitration of exogenous and endogenous phenolic groups (i.e. tyrosine residues), even in the pathophysiological concentration range of NO_2^- and H_2O_2 (Figure 7).

= Figure 7 =

The Peroxynitrite Pathway - The kinetic parameters for HPA nitration reported in Table 1 show the importance of nitrite in discriminating the reactivity of the two forms $\text{metNGB}_{\text{S-S}}$ and $\text{metNGB}_{\text{SH}}$. As expected from the influence of the internal S-S bond on the binding rate of exogenous ligands to the iron center, the kinetic parameters for the two forms of the protein in the rate dependence on $[\text{NO}_2^-]$ are significantly different, supporting the hypothesis of direct coordination of NO_2^- to the Fe^{III} center to promote the catalysis. In particular, $\text{metNGB}_{\text{S-S}}$ exhibits larger values both for nitrite affinity extrapolated from kinetic data ($1/K_{\text{M}}^{\text{nitrite}}$) and reactivity at low $[\text{NO}_2^-]$ ($k_{\text{cat}}/K_{\text{M}}^{\text{nitrite}}$) with respect to $\text{metNGB}_{\text{SH}}$, thus indicating that the interaction of NO_2^- with the catalytic site regulates the efficiency of nitration at low concentrations of the reagent, in conditions that can occur *in vivo*. The higher affinity of nitrite for $\text{metNGB}_{\text{S-S}}$ with respect to $\text{metNGB}_{\text{SH}}$ was confirmed by nitrite binding studies (Figure 3).

The peroxynitrite pathway proposed for the nitrating activity of metNGB gains an important support by the reactions with the substrates 4-hydroxybenzointrile and phenylacetic acid, that are mechanistic probes of the nitrating species NO_2 and peroxynitrite, respectively. The lack of reactivity of hydroxybenzointrile on one hand, and the presence of several products deriving from nitration and/or hydroxylation of phenylacetic acid on the other hand, are in agreement with the formation of an active species with the chemical properties of ONOO^- by metNGB (Figure 4). It follows that the formation of a protein-bound peroxynitrite intermediate is a reasonable step for the nitration catalyzed by metNGB in the presence of NO_2^- and H_2O_2 even at rather low concentration of the reagents.

NGB Endogenous Modifications – Further support to the generation and activity of the oxidant species generated by metNGB were extrapolated by the pattern of protein modifications obtained with LPO as an external catalyst, in comparison with the metNGB -promoted

endogenous modifications (Tables 2 and 3). At the high nitrite concentration (250 mM) employed for the preparation of the lpo-NGB_{S-S} and lpo-NGB_{SH} derivatives, the LPO promoted nitration proceeds through the formation of the peroxyxynitrite active species [22]. The differences in the pattern of modified residues by the LPO-generated peroxyxynitrite in comparison with those self induced by NGB, indicate that in the latter case peroxyxynitrite reacts intramolecularly. Moreover, the peroxyxynitrite reactive species is probably responsible for the oxidation of cysteinyl residues to the corresponding sulfinic and sulfonic acids: not only it can promote this type of modifications, as observed for instance in the oxidations of bovine serum albumin and HMb [24,49], but the high reactivity of this species also accounts for the lower extent of derivatization observed in the h'-NGB_{SH} and p'-NGB_{SH} derivatives (obtained by H₂O₂ only) in comparison with the h-NGB_{SH} and p-NGB_{SH} derivatives, respectively. The “harsh” conditions employed in the preparation of the h-NGB_{SH} and h-NGB_{S-S} derivatives, forcing the extent of endogenous modifications, allow the investigation of the competitive reactivity of the protein residues. Both the presence of three reactive cysteines (which may scavenge the RNS) in NGB_{SH} and its poorer catalytic efficiency in the nitration of phenolic compounds with respect to NGB_{S-S}, may account for the lower degree of tyrosine nitration in h-NGB_{SH} with respect to h-NGB_{S-S}.

From the point of view of the physiological relevance of NGB endogenous modifications, of particular interest is the analysis of p-NGB_{SH}, since this was obtained from NGB_{SH}, presumed to be the predominant form *in vivo* [12], with low concentrations of NO₂⁻ and H₂O₂. Among the protein residues, cysteine residues showed the highest reactivity towards the peroxyxynitrite active species, and their reaction prevents nitration of tyrosine residues and the heme prosthetic group. It is possible to calculate that ~36% of H₂O₂ oxidizing equivalents introduced were used for oxidation of cysteines to sulfinic and sulfonic acids. Oxidation of cysteines to sulfinic and sulfonic acid derivatives has been often observed [50,51], together with other types of oxidations that can be reversed by reaction with biological thiols [52]. It is worth noting that, among the tyrosine residues, the only one undergoing nitration in both p-NGB_{S-S} and p-NGB_{SH} is Tyr88, the phenolic OH group of which points toward the large NGB cavity connected to the heme distal site [10]. This may indicate that the peroxyxynitrite reactive species endogenously generated at the heme distal site can easily diffuse through the cavity and nitrate Tyr88.

Does Human NGB Act Only as a Scavenger? – In the globins, cysteine residues often play specific roles, modulated by the formation of intra- or intermolecular disulfide bonds [9]. In

the case of NGB, the oxidation state of Cys46 and Cys55 is relevant because it is linked to the peculiar six-coordination of the heme. The present study confirms the importance of the internal S-S bond in controlling the reactivity of metNGB towards nitrite and hydrogen peroxide. It also shows that the protein can be involved in other activities. In fact, NGB is thought to act as scavenger of toxic species generated *in vivo* under conditions of oxidative stress. But the ability of the metNGB form generated by this activity to activate NO_2^- and H_2O_2 means that, besides acting as a scavenger, NGB can be the source of RNS reactive towards protein residues, thus affecting its physiological activity. A significant fraction of the nitrating and oxidizing species (~36% in the conditions used to prepare p-NGB_{SH}) does not escape from the protein but is consumed in the self-modification of NGB. The easy oxidation of cysteines to sulfinic and sulfonic acids may be one way used by NGB to scavenge part of the generated RNS. To gain a better understanding of the physiological relevance of these reactions, it would be important to analyze the state of endogenous modification of samples of the protein isolated after *in vivo* exposure to oxidative stress conditions.

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Table 1. Kinetic Data for HPA Nitration Catalyzed by NGB and HMb. Steady state kinetic parameters for the NGB and HMb dependent HPA nitration by $\text{NO}_2^-/\text{H}_2\text{O}_2$ in 200 mM phosphate buffer, pH 7.5, at 25 °C. ^aData from ref. 24.

Protein	K_M^{PhOH} (mM)	k_{cat} (s ⁻¹)	$k_{cat}/K_M^{\text{PhOH}}$ (M ⁻¹ s ⁻¹)	K_M^{nitrite} (M)	k_{cat} (s ⁻¹)	$k_{cat}/K_M^{\text{nitrite}}$ (M ⁻¹ s ⁻¹)
NGB_{s,s}	0.078±0.008	0.177±0.004	2300±200	0.043±0.006	0.140±0.007	3.3±0.3
NGB_{SH}	0.058±0.008	0.132±0.005	2300±200	0.22±0.03	0.139±0.005	0.63±0.06
HMb^a	0.12±0.02	0.67±0.03	5600±900	0.24±0.04	1.03±0.08	4.3±0.5

Table 2. Heme and Tyrosine Modifications in NGB Derivatives. Nitration (expressed in %) of heme and tyrosine residues in NGB derivatives obtained in the following conditions: for p-NGBs, [NGB] = 6×10^{-5} M, $[\text{NO}_2^-]$ = 0.1 mM, $[\text{H}_2\text{O}_2^-]$ = 0.15 mM; for h-NGBs, [NGB] = 6×10^{-5} M, $[\text{NO}_2^-]$ = 0.1 M, $[\text{H}_2\text{O}_2^-]$ = 1 mM; for lpo-NGBs, [LPO] = 8×10^{-8} M, [NGB] = 6×10^{-5} M, $[\text{NO}_2^-]$ = 0.25 M, $[\text{H}_2\text{O}_2^-]$ = 0.15 mM. All reactions were performed in 200 mM phosphate buffer, pH 7.5.

Protein derivatives	Heme-NO ₂	Y44-NO ₂	Y88-NO ₂	Y115-NO ₂	Y137-NO ₂
p-NGB _{S-S}	0	0	0.6	0	0
p-NGB _{SH}	0	0	1	0	0
h-NGB _{S-S}	38	50	11	65	38
h-NGB _{SH}	14	11	7	36	14
lpo-NGB _{S-S}	25	5	1.3	3.5	15
lpo-NGB _{SH}	27	2.3	0.9	7	11

Table 3. Modification of Cysteines in NGB Derivatives. Oxidation (expressed in %) of cysteine residues in NGB derivatives obtained by protein modification in the following conditions: for p-NGBs, [NGB] = 6×10^{-5} M, $[\text{NO}_2^-]$ = 0.1 mM, $[\text{H}_2\text{O}_2^-]$ = 0.15 mM; for h-NGBs, [NGB] = 6×10^{-5} M, $[\text{NO}_2^-]$ = 0.1 M, $[\text{H}_2\text{O}_2^-]$ = 1 mM; for lpo-NGBs, [LPO] = 8×10^{-8} M, [NGB] = 6×10^{-5} M, $[\text{NO}_2^-]$ = 0.25 M, $[\text{H}_2\text{O}_2^-]$ = 0.15 mM; for p'-NGB_{SH}, [NGB] = 6×10^{-5} M and $[\text{H}_2\text{O}_2^-]$ = 0.15 mM; for h'-NGBs, [NGB] = 6×10^{-5} M and $[\text{H}_2\text{O}_2^-]$ = 1 mM. All reactions were performed in 200 mM phosphate buffer, pH 7.5. ^aThe residues C46 and C55 are involved in the S-S bond.

Protein derivatives	C46-SO ₂ H	C46-SO ₃ H	C55-SO ₂ H	C55-SO ₃ H	C120-SO ₂ H	C120-SO ₃ H
NGB _{S-S}	a	a	a	a	1.7	1.5
NGB _{SH}	0.3	0.9	0.6	1.7	2	2
p-NGB _{S-S}	a	a	a	a	2	2
p-NGB _{SH}	2.3	4.5	2.5	7	10	9
p'-NGB _{SH}	1	2	0.5	2	2	1.5
h-NGB _{S-S}	a	a	a	a	5	8
h-NGB _{SH}	21	14	4	14	18	16
h'-NGB _{SH}	0.9	4	0.7	4	5	6
lpo-NGB _{S-S}	a	a	a	a	6	7
lpo-NGB _{SH}	22	8	3	7	10	5

FIGURE LEGENDS

Figure 1. Cysteine-oxidation states and coordination equilibria in metNGB. Schematic representation of the low-spin/high-spin equilibria for metNGB_{S-S} (on the left) and metNGB_{SH} (on the right). The disposition of the side chains of the proximal and distal histidine residues (His(96)F8 and His(64)E7, respectively) is shown. The length of the vertical arrows indicates that even if the coordination equilibrium is always shifted towards the six-coordinated species, in the case of metNGB_{S-S} the amount of high-spin form is larger with respect to metNGB_{SH}.

Figure 2. UV-Vis spectra of NGB_{S-S} and NGB_{SH}. UV-Vis spectra of metNGB_{S-S} (6.7 μM, solid line) and metNGB_{SH} (6.2 μM, dashed line) in 0.2 M phosphate buffer, pH 7.5. The wavelength maxima are shown.

Figure 3. Binding of Nitrite to NGB_{S-S} and NGB_{SH}. Plot of the difference between the absorbance changes at 414 and 434 nm in the UV-Vis spectra of NGB_{S-S} and NGB_{SH} upon addition of nitrite, vs. the ligand concentration. The absorbance data were fitted with the binding isotherm for low affinity binding of two ligands for NGB_{S-S} and a single ligand for NGB_{SH}.

Figure 4. Reactivity of 4-hydroxybenzoxonitrile and phenylacetic acid with RNS. Schematic representation of the reactivity of 4-hydroxybenzoxonitrile and phenylacetic acid with the nitrating agents NO₂ and ONOO⁻, and the catalytic system metNGB/NO₂/H₂O₂.

Figure 5. Structure of NGB. Structure of the C46G/C55S/C120S mutant of NGB (according to ref. 10). The disposition of the side chains of the tyrosines (Y44, Y88, Y115, Y137), and the cysteines (C46, C55, and C120) present in the wild type protein are shown.

Figure 6. Collision-induced dissociation spectrum of the modified 68-94 peptide. MS/MS spectrum of the *m/z* 1477.6 peak (mass of 2953.3 amu) assigned to the 68-94 peptide in a double-charged state and with the residue of Tyr88 modified to 3-nitrotyrosine. The assignment of the y and b ion series is shown. Above the spectrum, the sequence of the 68-94 peptide, with

the modified residue in bold and with the summary of the y and b ions found in the spectrum, is reported.

Figure 7. Mechanism of metNGB promoted nitration of phenolic compounds. Schematic representation of the activation of NO_2^- and H_2O_2 by the high-spin metNGBs (metNGB_{S-S} or metNGB_{SH}) through the peroxyxynitrite pathway, involving the initial binding of NO_2^- to the heme iron, followed by the reaction with H_2O_2 , to produce an iron-bound peroxyxynitrite ($\text{NGBFe}^{\text{III}}\text{-N(O)OO}$) nitrating active species. Nitration of the phenol occurs at the ortho position to its hydroxyl group. The upper part of the scheme shows the absence of reactivity of metNGB with hydrogen peroxide.

Figure 1

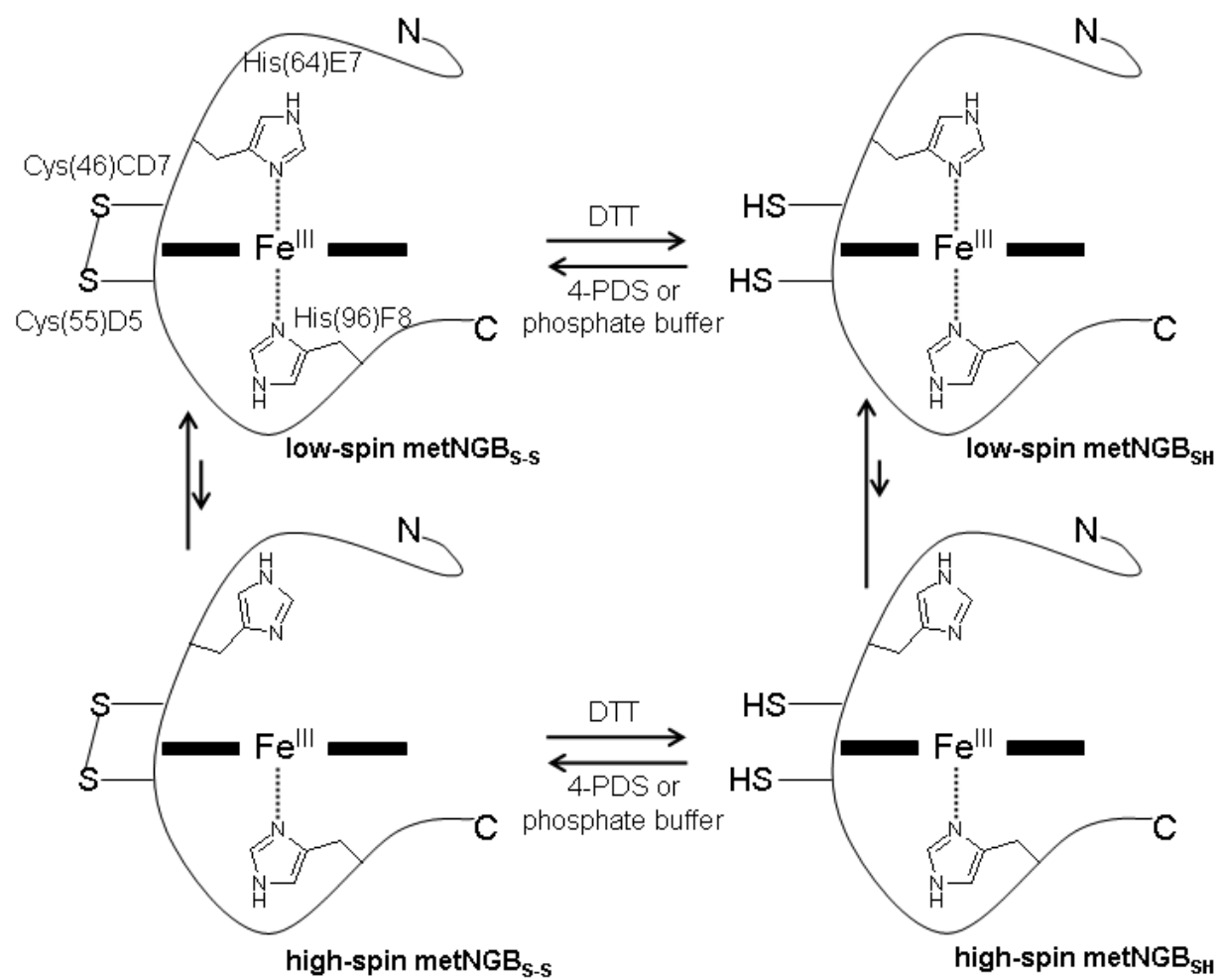


Figure 2

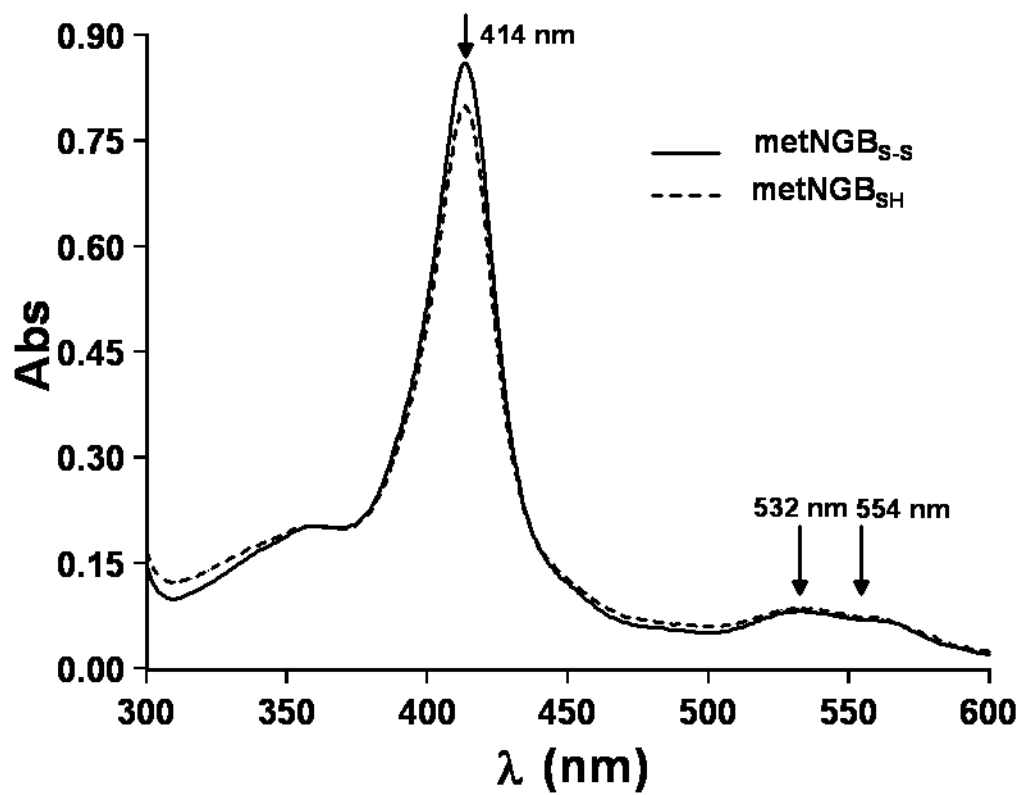


Figure 3

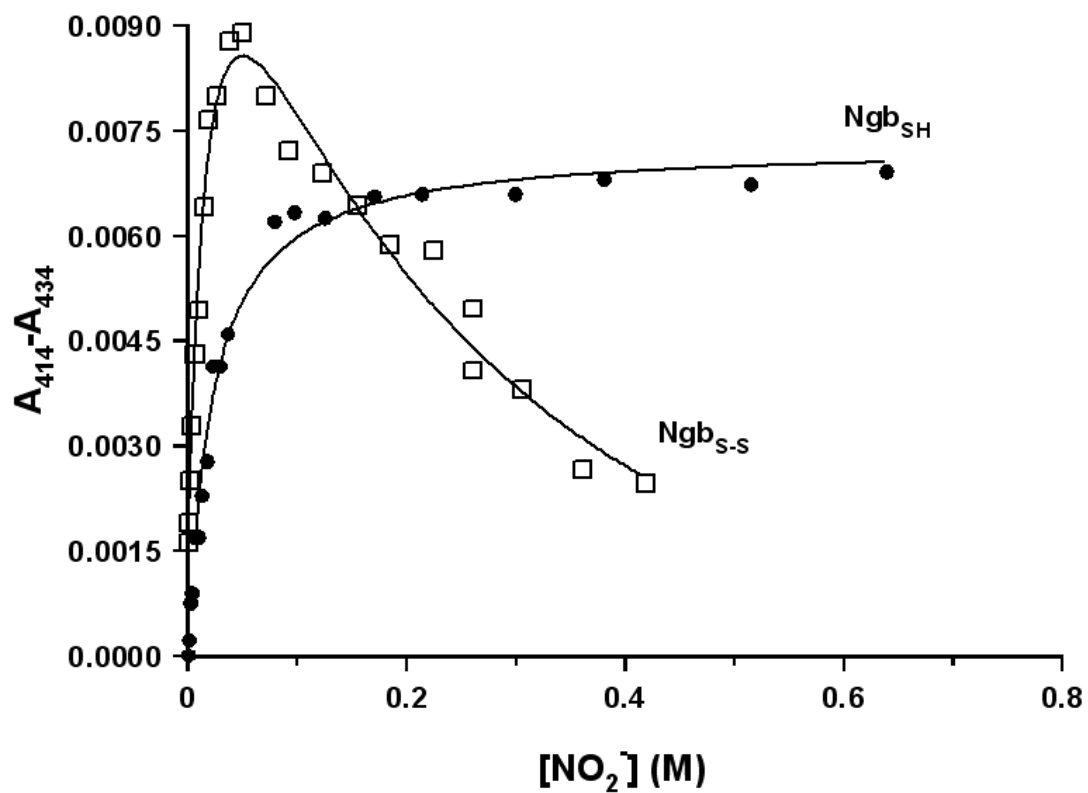


Figure 4

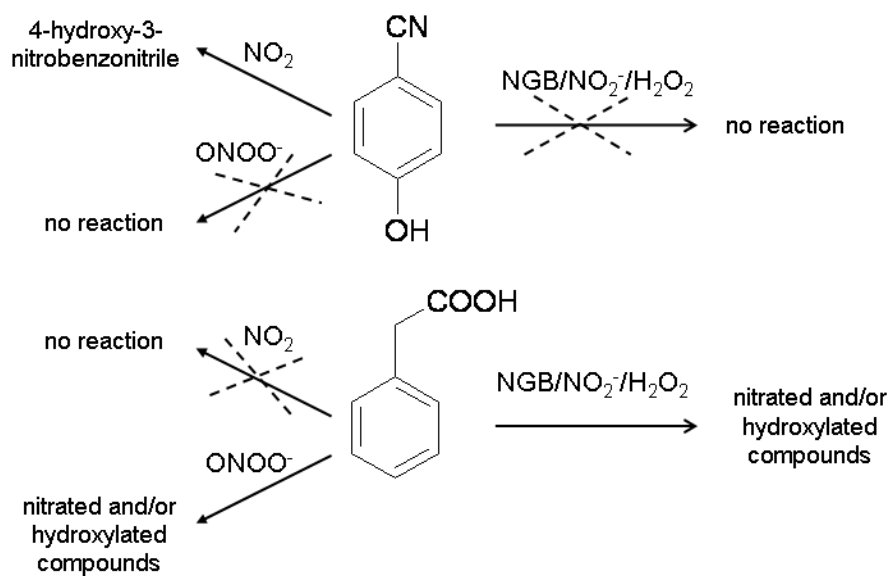


Figure 5

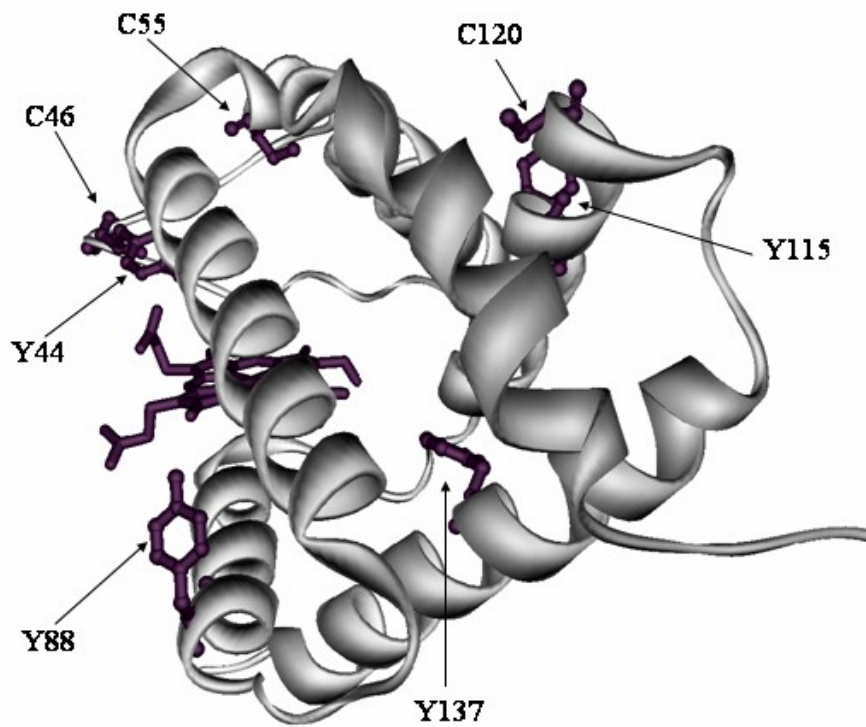


Figure 6

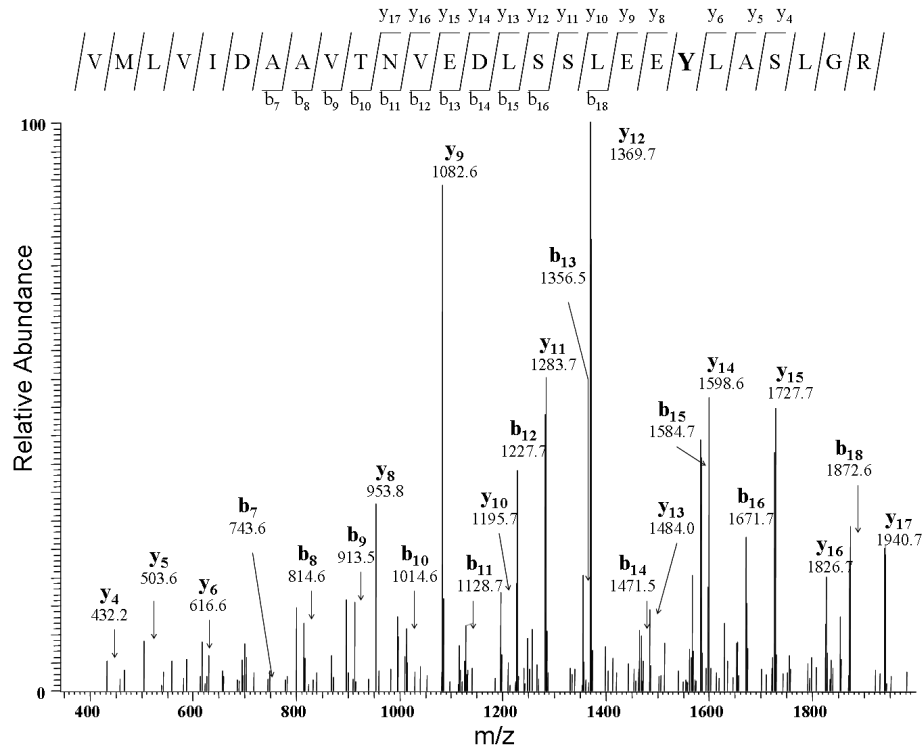


Figure 7

