

# Nitric Oxide and Myoglobins

Jens K. S. Møller and Leif H. Skibsted\*

Food Chemistry, Department of Dairy and Food Science, Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C., Denmark

Received May 31, 2001

## Contents

I. Introduction	1167
II. Mechanism of Formation of Nitrosylmyoglobins	1168
A. Nitric Oxide Binding	1168
B. Reduction of Nitrite and Nitrate	1169
C. Oxidation of Nitrogen–Hydrogen Compounds	1169
D. Formation during Meat Curing	1170
E. Bacterial Formation	1171
III. Structure and Properties of Nitrosylmyoglobins	1172
A. Nitric Oxide Binding and Heme/Protein Structure	1172
B. Thermodynamics of Nitric Oxide Binding	1173
C. Radical Scavenging of Nitrosylmyoglobins	1173
IV. Reactions of Nitric Oxide and Myoglobin Complexes	1174
A. Ligand Exchange Reactions	1174
B. Thermal Oxidation of Nitrosylmyoglobins	1174
B. Chemically Induced Oxidation of Myoglobin Complexes	1175
D. Photooxidation of Nitrosylmyoglobins	1176
V. Conclusion and Biological Perspectives	1176
VI. References	1177

## I. Introduction

The primary function of myoglobin has normally been considered as oxygen storage in muscles. Other functions of myoglobin seem, however, now to have been identified, since oxymyoglobin ( $\text{MbFe}^{\text{II}}\text{O}_2$ ), the oxygenated iron(II) myoglobin, readily reacts with nitric oxide (NO) and may prevent NO from inhibiting cytochrome *c* oxidase.<sup>1</sup> The viability of myoglobin knockout mice may even raise critical questions about the general importance of oxygen storage by myoglobin.<sup>1</sup> The scavenging of NO by  $\text{MbFe}^{\text{II}}\text{O}_2$  makes the heme protein a protector of cellular respiration through this function, where the otherwise slow oxidation of NO to nitrate is catalyzed efficiently.<sup>2,3</sup> In this context, it has been hypothesized that myoglobin may protect the parasite, which is responsible for Chagas disease in man, preferentially colonizing cardiomyocytes in the host, and here benefits from the NO scavenging action of  $\text{MbFe}^{\text{II}}\text{O}_2$ .<sup>4</sup> Similarly, flavohemoglobins found in bacteria and yeast act as a NO dioxygenase, thereby providing the cells with resistance toward NO toxicity.<sup>5,6</sup> Myoglobin on the other hand binds NO in an iron(II) complex, which only slowly reacts with oxygen, but which may scavenge radicals and accordingly have antioxidative

properties.<sup>7</sup> The binding of small molecules such as NO, CO, and  $\text{O}_2$  to myoglobin followed by electron transfer or reaction with activated oxygen species has even led to the suggestion that myoglobin should be classified as an allosteric enzyme,<sup>8</sup> despite the fact that myoglobin is a heme monomer.

The reactions between cytosolic NO and myoglobins are thus central in regulation of the level of the important transmitter substance NO. Nitrosylmyoglobin ( $\text{MbFe}^{\text{II}}\text{NO}$ ), the NO complex of iron(II) myoglobin, as formed in meat products, has now also been detected in vivo in rats.<sup>9</sup>  $\text{MbFe}^{\text{II}}\text{NO}$  thus seems important in controlling radical processes associated with oxidation.

The thermodynamics and kinetics of the reaction between NO and the myoglobins are accordingly becoming of increasing importance to understand the mechanisms of regulatory processes in muscle tissue and in relation to optimized protection of foods against oxidative deterioration. Other functions of myoglobin than oxygen storage and related to NO and nitrogen metabolism may even be of comparable importance for the muscle myoglobins.<sup>1</sup> NO thus binds to iron in other heme proteins such as guanylate cyclase, cytochromes, and hemoglobin, and NO also interacts with catalytically active cysteine in alcohol dehydrogenase.<sup>10,11</sup> In addition, a complex of NO and leghemoglobin (found in  $\text{N}_2$  fixing leguminous plants with sequence homology close to that of myoglobin) has been detected in soybean nodules.<sup>12</sup> Studies of NO binding to myoglobins and of electron-transfer processes within NO complexes of myoglobins may thus also provide mechanistic information of relevance for other metalloproteins active in blood pressure regulation, nitrogen fixation, and NO oxidation.

The current interest in interaction between NO and heme proteins is emphasized by the recent publication of two comprehensive reviews on the subject.<sup>13,14</sup> The following text will focus mainly on the reactivity of NO complexes of myoglobin, but some examples may concern interaction of NO with hemoglobin or porphyrin model molecules, which also has been studied intensively. To limit the scope of this text, the review covers mainly literature dated later than 1992, and for a discussion of the electronic structure of NO complexes of myoglobin, older literature or recent handbooks may be consulted.<sup>15</sup> The chemistry of myoglobin and NO in relation to meat curing has



Jens K. S. Møller graduated with an M.Sci. degree (food science) in 1996 from The Royal Veterinary and Agricultural University in Copenhagen. Subsequently, he worked for 3 years in applied research of meat products and packaging technology at the Danish Meat Research Institute and Danish Technological Institute, respectively. In 1998 he returned to The Royal Veterinary and Agricultural University to work as a research assistant in the Department of Dairy and Food Science, where he studied light-induced degradation of cured meat pigment at low oxygen tension and the effect of various myoglobin derivatives on oxidative processes. Currently, he is working on his Ph.D. degree, focusing on the chemical changes of myoglobin and oxidative status of cured meat products during processing.



Leif H. Skibsted graduated with an M.Pharm. degree from the Royal Danish School of Pharmacy in 1972 and obtained a Ph.D. in inorganic chemistry in 1976 after studying with Jannik Bjerrum at the University of Copenhagen. He has been with the Royal Veterinary and Agricultural University since 1974 and was in 1978–79 on a sabbatical leave to study photochemistry with Peter C. Ford at the University of California at Santa Barbara. Following mechanistic studies of iron-catalyzed oxidation reactions in meat systems and of light-induced degradation of carotenoids and other plant pigments, he was appointed Professor of Food Chemistry in 1992. His current research interests include high-pressure effects on proteins, the mechanism of antioxidant interaction as studied by ESR and laser-flash spectroscopy, and the radical chemistry of meat pigments. Among the several awards, he has received, are the Niels and Ellen Bjerrum Award and Gold Medal in Chemistry, Carlsberg Award in Agricultural Science, and Ole Roemer Research Award.

been reviewed more specifically,<sup>16</sup> and literature dated before 1992 was also addressed here.

Comparison of interaction between different diatomic molecules and myoglobin is important, and the interactions of myoglobin with CO and O<sub>2</sub> have been reviewed, including theories for the ability to discriminate between the two ligands.<sup>17</sup> NO and CO both bind stronger to myoglobin than O<sub>2</sub>. An important difference is, however, that NO does not replace O<sub>2</sub> in a ligand exchange reaction, but becomes

oxidized by coordinated O<sub>2</sub> upon entering the heme cavity.

## II. Mechanism of Formation of Nitrosylmyoglobins

The interaction of diatomic ligands and myoglobins has been studied extensively due to the biological relevance of the reactions and as a model for macromolecular interactions with small ligands. The high reactivity of NO toward heme iron in myoglobin and the high stability of the iron(II) complex have been used in the mechanistic studies of formation of NO.<sup>18</sup> Nitrosylporphyrins are formed from several NO sources,<sup>15</sup> whereas formation of nitrosylmyoglobins depends on direct nitrosylation with NO gas or is the result of reduction of nitrate/nitrite by external reductants or of oxidation of nitrogen–hydrogen compounds by the heme iron.

### A. Nitric Oxide Binding

For ligand binding to myoglobins several reaction steps have been recognized, which include (i) movement of the ligand into the heme pocket, (ii) displacement of a water molecule, (iii) in-plane movement of the Fe atom to form a hexacoordinate complex, and (iv) Fe–ligand bond formation and stabilization.<sup>19,20</sup> The discrimination of myoglobins toward NO, O<sub>2</sub>, and CO has until recently mainly been attributed to steric hindrance in the heme cleft. However, electrostatic interactions between the Fe atom and the ligand and/or conformational changes of the amino acid residues in the distal heme pocket or related to the proximal imidazole are also involved.<sup>19</sup> The bond formation between ferrous iron and NO (step iv) has been described as having almost no “inner quantum barrier”. The rate of binding of NO to native deoxymyoglobin (MbFe<sup>II</sup>) is entirely controlled by ligand movement into the distal pocket.<sup>15,20</sup> A pathway for entry into and exit from myoglobin has recently been described for O<sub>2</sub>,<sup>21</sup> while a similar attempt has not been reported for NO, although possible differences may be of relevance for the dioxygenase function of myoglobin and the autoreduction mechanism of nitrosylmetmyoglobin (MbFe<sup>III</sup>NO).

Bonding of NO to iron(II) in myoglobin results in a charge transfer, and NO attains partly anionic character (nitroxyl anion). In this respect NO is intermediate to the ligands O<sub>2</sub> and CO. Like NO, the ligand O<sub>2</sub> is also bent, and due to the charge distribution, MbFe<sup>II</sup>O<sub>2</sub> has been described as a superoxide ferric complex (Fe(III),O<sub>2</sub><sup>-</sup>). In contrast, the C–O bond in ferrous myoglobin is perpendicular to the porphyrin plane and there is no net charge transfer between the ligand and heme iron.<sup>20</sup> Also with respect to the importance of hydrogen bonding NO is intermediate between O<sub>2</sub> and CO. The bent structure and charge distribution of MbFe<sup>II</sup>NO (and linearity of Fe–N–O in MbFe<sup>III</sup>NO) are rationalized within the Enemark–Feltham notation.<sup>15</sup>

Until recently, mutagenesis studies of myoglobin complexes of NO and other small ligands have led to the conclusion that steric hindrance caused by distal amino acid residues determines the variation in

myoglobin affinity for binding various ligands.<sup>19</sup> Alternatively, discrimination between different ligands has been assigned to differences in electrostatic interaction between the distal amino acid residue and the ligand.<sup>19</sup> Myoglobin mutants, in which distal His64(E7) has been substituted with an apolar amino acid, e.g., Ala, Val, Ile, and Phe, show little variation in NO affinity compared to wild-type myoglobin. Mutants with enhanced rotation of His64(E7), the amino acid residue that forms a hydrogen bond to water, yields a dramatic drop in NO affinities. The myoglobin mutant, in which His64(E7) is substituted with Leu, deviates from mutants with other apolar amino acids at the E7 position in that the binding affinities for NO and CO increase by factors of 7 and 40, respectively.

Several studies indicate that binding of NO to ferrous iron in myoglobin weakens the bond to the proximal histidine.<sup>22</sup> Imidazole derivatives can bind as a proximal ligand with affinities depending on the ability to form hydrogen bonds with the proximal amino acid residues.<sup>23</sup> In addition, lowering of the pH will further cause weakening or breaking of the bond of the proximal base coordinated to heme iron in MbFe<sup>II</sup>NO.<sup>24,25</sup>

Apart from the high binding constants for myoglobin, NO possesses another unique feature compared to O<sub>2</sub> or CO, namely, the capability to bind to ferric heme iron.<sup>26</sup> The affinity of NO for binding to ferric heme is significantly lower than for binding to ferrous heme,<sup>27,28</sup> but it is uncertain whether the binding of NO to iron(III) is of biological significance in vivo. Nevertheless, reductive nitrosylation of ferric myoglobin may play an important role in the color formation of cured meat.<sup>29</sup> The reversible binding of NO to metmyoglobin (MbFe<sup>III</sup>) in aqueous solution has an equilibrium constant of  $1.3 \times 10^4 \text{ M}^{-1}$  at pH 6.1 (eq 1),



and at higher pH binding becomes irreversible, due to base-catalyzed reduction of MbFe<sup>III</sup>NO to MbFe<sup>II</sup>NO.<sup>28</sup> A more recent study determines an even smaller equilibrium constant ( $2.4 \times 10^3 \text{ M}^{-1}$ ) under similar conditions.<sup>30</sup>

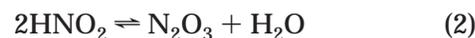
In the presence of glutathione, MbFe<sup>III</sup>NO reacts to give nitrosogluthathione and MbFe<sup>II</sup> in a relatively slow reaction (rate constant of  $47 \text{ M}^{-1} \text{ s}^{-1}$ ), and subsequently MbFe<sup>II</sup> forms a complex with NO.<sup>31</sup> The transfer of NO in the "opposite" direction resulting in formation of MbFe<sup>II</sup>NO from nitrosogluthathione has been observed in isolated hearts during ischemia.<sup>32</sup> ESR spectroscopy studies of photoinduced intermediates of various MbFe<sup>III</sup>NO mutants (ESR spectra originating from ferric Fe ( $s = 5/2$ ) and trapped NO ( $s = 1/2$ )), in which the distal amino acid residues His64(E7) and Val68(E11) are substituted with other amino acids, show that the mobility of NO in the distal heme pocket depends on the size and polarity of the amino acid residue at position 68 rather than position 64.<sup>33</sup> Calculations suggest that the torsion of proximal His93 is important for the energy of the MbFe<sup>III</sup>NO.<sup>34</sup> Vibrational spectroscopy further provides evidence for differences between

binding of NO to ferric and ferrous iron in myoglobin.<sup>35–37</sup> For instance, it has been shown that the Fe–N–O bond in ferrous and ferric myoglobin is bent and linear, respectively, relative to the porphyrin plane.<sup>25,38</sup>

Linkage isomers exist for NO complexes of ferrous iron porphyrins.<sup>15</sup> These are called side-on, reverse or normal binding of the NO molecule. In a recent study of five-coordinated iron porphyrins a linkage isomer with binding to the O atom in NO was found to be photoinduced.<sup>39</sup> The different reaction stoichiometry and the different product distribution observed for photooxidation compared to thermal oxidation of MbFe<sup>II</sup>NO may be understood on the basis of such isomers.<sup>40,41</sup> Moreover, recombination of myoglobin and NO following photolysis of MbFe<sup>II</sup>NO shows more than one rate.<sup>42–44</sup> The multiple phases seen for the geminate NO rebinding have, however, also been assigned to differing degrees of movement away from and back to the iron atom.<sup>42,43</sup>

## B. Reduction of Nitrite and Nitrate

Synthesis of MbFe<sup>II</sup>NO from MbFe<sup>III</sup> and nitrite using various reductants such as ascorbate is a standard method.<sup>45</sup> Nitrate may replace nitrite, but is then reduced to nitrite by either chemical or microbial means. Nitrous acid (eq 2) is reduced by ascorbate to yield NO (eq 3).



Reduction of MbFe<sup>III</sup> occurs by either direct reaction with ascorbate or reductive nitrosylation with NO under anaerobic or aerobic conditions,<sup>45,46</sup> and ascorbate seems to reduce both MbFe<sup>III</sup> and nitrite; for nitrite a transient ascorbyl radical was detected by ESR spectroscopy.

NADH was also found to reduce MbFe<sup>III</sup> and nitrite under both anaerobic and aerobic conditions.<sup>46</sup> NADH reduces nitrite, and NAD• radicals may react with MbFe<sup>III</sup> (eqs 4–6).



Synthesis of MbFe<sup>II</sup>NO with stoichiometric amounts of MbFe<sup>III</sup> and reductant (dithionite) is recommended followed by immediate anaerobic chromatographic purification of MbFe<sup>II</sup>NO, not allowing time for side products to be formed in the reaction mixture.<sup>47</sup>

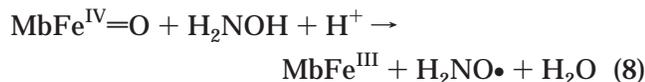
Cyclic voltametry of myoglobin in a surfactant film has shown that myoglobin itself may catalyze reduction of nitrite to NO in aqueous solution, as MbFe<sup>II</sup>NO can be detected, while no evidence of nitrosylated ferric myoglobin was seen.<sup>48,49</sup>

## C. Oxidation of Nitrogen–Hydrogen Compounds

When MbFe<sup>II</sup>O<sub>2</sub> is reacted with hydroxylamine derivatives or with hydroxyurea, MbFe<sup>III</sup> is formed.

Hydroxylamines and hydroxyurea produce an intermediate nitroxide radical and the aminocarbonyl-aminooxyl radical, respectively, as detected by ESR spectroscopy. Relative rates of MbFe<sup>III</sup> formation increase in the order hydroxylamine > methylhydroxylamine > dimethylhydroxylamine > hydroxyurea, and MbFe<sup>III</sup> is further converted to MbFe<sup>II</sup>NO.<sup>50</sup> Similar observations have been made for oxyhemoglobin in isolated erythrocytes, where addition of the hydroxylamine derivatives or hydroxyurea led to short-lived radicals and subsequent formation of a NO complex of hemoglobin, plus a change in membrane fluidity and release of iron.<sup>51</sup> Hydroxylamine may due to its release of NO following oxidation by oxyheme pigments be a potent vasodilative drug,<sup>52</sup> and can also be used to treat sickle cell anemia by increasing the level of fetal hemoglobin in patients.<sup>53</sup>

Hydroxylamine, but not the less reactive methyl derivatives, can transfer MbFe<sup>III</sup> to MbFe<sup>II</sup>NO in the presence of H<sub>2</sub>O<sub>2</sub> in a process which involves formation of hypervalent perferrylmyoglobin (•MbFe<sup>IV</sup>=O) (eq 7).<sup>52</sup> This perferryl pigment or the ferryl compound, MbFe<sup>IV</sup>=O, oxidizes hydroxylamine to a nitroxyl intermediate (HNO/NO<sup>-</sup>) or to NO (eqs 8 and 9), which subsequently binds to myoglobin. Hydroxylamine has dose-dependent vasodilatory properties in rats in agreement with its oxidation to NO.



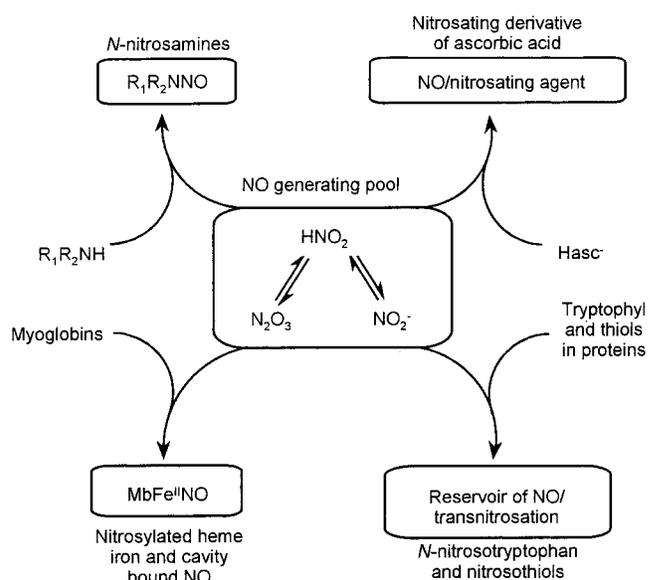
Ferric iron porphyrins ( $E_{1/2} = -0.29$  to  $-1.07$  V) fairly easily undergo reductive nitrosylation, a process which is coupled to the disproportionation of hydroxylamine, thereby yielding the nitrosylporphyrin and ammonia (eq 10).<sup>54</sup> However, it is not known whether this reaction also is relevant for MbFe<sup>III</sup> ( $E_{1/2} = 0.0$  V in neutral solution).



A recent study of reaction of various iron heme proteins with hydroxyurea confirms that nitrosyl complexes of heme iron are formed.<sup>53</sup> MbFe<sup>II</sup>O<sub>2</sub> reacts faster with hydroxyurea to yield MbFe<sup>II</sup>NO than both normal and sickle cell hemoglobin and hemin. H<sub>2</sub>O<sub>2</sub> accelerated the overall reaction, and the reaction of hydroxyurea with hemoglobins has been suggested to be explored for treatment of sickle cell anemia.

#### D. Formation during Meat Curing

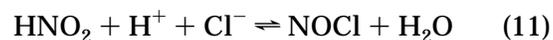
The chemistry of meat curing has been the subject of several comprehensive reviews over the past 10 years.<sup>16,55,56</sup> Historically, the discovery of nitrite as the active component in the curing process of meat dates back to the late 19th century, and subsequently Haldane was the first to prepare the pigment nitrosylhemoglobin in 1901 by adding nitrite to hemo-



**Figure 1.** Pools of nitrite-modified compounds present in cured meat. Nitric oxide radicals interchange rapidly between pools through transnitrosation reactions.

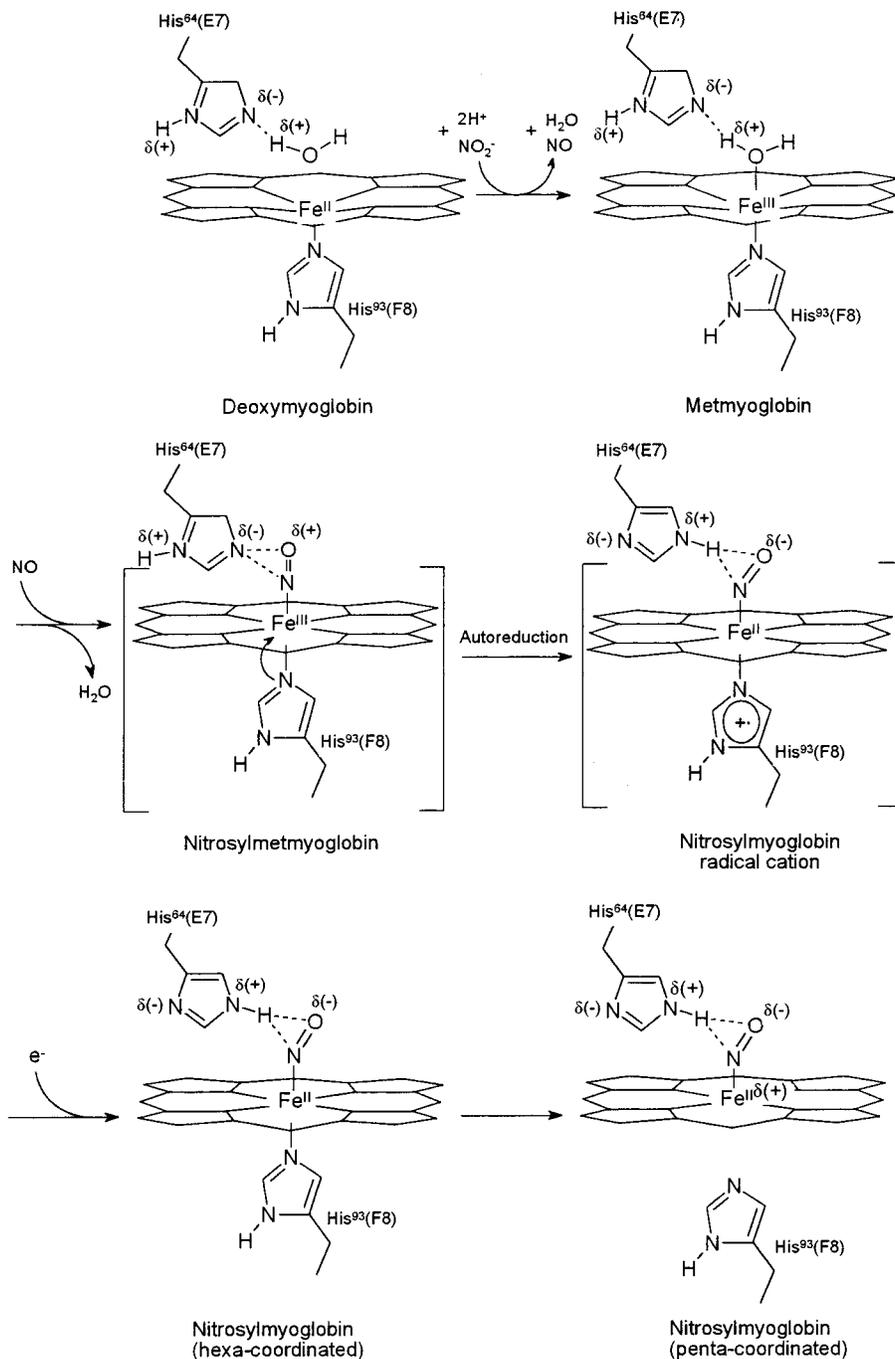
globin.<sup>56</sup> Hoagland confirms this observation and suggests that the reduction of nitrate/nitrite to NO by bacterial or enzymatic action accounts for the cured pigment. Figure 1 shows various pools of nitrite-modified compounds present in cured meat, in which transnitrosation accounts for transfer of NO radicals.<sup>16</sup>

Nitrite does not act directly as a nitrosylating agent in meat, but reacts to form N<sub>2</sub>O<sub>3</sub>, a compound that in the presence of reducing substances yields NO. Cured meat always contains sodium chloride, and the chloride accelerates the rate of formation of MbFe<sup>II</sup>-NO, as nitrosyl chloride (eq 11) is a more reactive nitrosylating agent than N<sub>2</sub>O<sub>3</sub>.<sup>16</sup>



Nitrite will oxidize MbFe<sup>II</sup>O<sub>2</sub> to MbFe<sup>III</sup>, and in the meat matrix, MbFe<sup>III</sup>NO is generally believed to undergo reduction by endogenous reductants such as NADH or by added exogenous reductants such as ascorbate or erythorbate to yield MbFe<sup>II</sup>NO.<sup>57</sup> An alternative mechanism has been suggested in which autoreduction of the intermediate yields an imidazole-centered protein radical that can be reduced by electron donation from a reducing group in the protein (Figure 2).<sup>29</sup> It is, however, not clear, how this autoreduction may occur at low pH, where the proximal histidine will be protonated and autoreduction will involve pentacoordinate NO-heme. At high pH, the hydroxide attack mechanism will dominate.<sup>28</sup>

Parma ham is traditionally produced using only sodium chloride without addition of nitrate or nitrite and develops a deep red color, which is stable also on exposure to air. The identity of the pigment of Parma ham has not been established, but bacterial activity has been explored as responsible for transformation into nitrosylated heme pigments.<sup>58</sup> In one study, the stability of the pigment isolated from two different types of dry-cured ham (made with or



**Figure 2.** Proposed reaction mechanism for the formation of nitrosylmyoglobin in cured meat (adapted from refs 15 and 29).

without nitrite) was compared to that of the NO derivative of myoglobin formed by bacterial activity. Heme pigment from Parma ham made without nitrite was more stable against oxidation than the pigment from dry-cured ham with added nitrite.

Heme pigments extracted from Parma ham and a bacterial (*Staphylococcus xylosus*) formed NO–heme derivative had similar spectral characteristics (UV/vis spectra and ESR).<sup>58</sup> ESR spectroscopy of heme pigment isolated from salami inoculated with bacteria had NO in a predominant pentacoordinate NO–heme environment, whereas MbFe<sup>II</sup>NO, formed from nitrite and ascorbate, exclusively showed hexacoordinated iron,<sup>59</sup> a difference which could be due to the decrease in pH during fermentation.

## E. Bacterial Formation

NO is an intermediate in the metabolism of denitrifying bacteria.<sup>60</sup> Bacteria vary in their tolerance toward NO, as the molecule is a natural metabolite for some bacteria, but is toxic to other species.<sup>61</sup> A bacterial NO synthase (NOS) has been isolated and characterized from a *Nocardia* species,<sup>62</sup> and a bacterial strain with more relevance to meat products, *Lactobacillus fermentum*, also possesses NOS activity.

Various bacteria have been shown to modify oxidized heme pigments in laboratory media, and these modifications yield either MbFe<sup>II</sup>O<sub>2</sub> or MbFe<sup>II</sup>NO. In a comprehensive screening of 1550 bacterial isolates,

Arihara and co-workers found two bacterial strains able to convert  $\text{MbFe}^{\text{III}}$  into  $\text{MbFe}^{\text{II}}\text{O}_2$  and a single strain capable of forming  $\text{MbFe}^{\text{II}}\text{NO}$  from  $\text{MbFe}^{\text{III}}$ .<sup>63</sup>

For bacterial NOS activity  $N^G$ -hydroxy-L-arginine has been shown as a reaction intermediate, and optimal conditions for enzyme activity have been established as 30 °C for a pH range between 7.0 and 7.5.<sup>62</sup> In cultures of *L. fermentum* IFO3956, it has been found that the incorporation of  $^{15}\text{N}$  into nitrite/nitrate from guanidino- $^{15}\text{N}$ -labeled L-arginine increased when various reducing cofactors such as NADPH, tetrahydrobiopterin, FAD, and FMN were present,<sup>64</sup> as an indication of bacterial NOS activity in this species, although NO production is observed in the absence of added L-arginine or reducing cofactors. NO has also been detected by an assay in which  $\text{MbFe}^{\text{III}}$  is converted to  $\text{MbFe}^{\text{II}}\text{NO}$ , a reaction not directly related to NOS activity. Notably, heme proteins such as horseradish peroxidase, hemoglobin, and catalase are capable of catalyzing the conversion of  $N^G$ -hydroxy-L-arginine to citrulline and NO,<sup>65</sup> where the heme-NO adducts in some cases have been found.

Unlike mammals, in which NO is involved in cell to cell signaling or pathogen-host response, no such action is likely in bacteria given their unicellular status and the biological significance of NO production in bacteria such as the *Nocardia* species is unknown.<sup>66</sup> Findings from the closely related *Rhodococcus* sp. suggest that NO via binding to a non-heme Fe center may be involved in regulation of an enzyme, nitrile hydratase, catalyzing hydration of nitriles to the corresponding amides.<sup>67</sup>

### III. Structure and Properties of Nitrosylmyoglobins

$\text{MbFe}^{\text{II}}\text{NO}$  has been extensively used as a model compound for biological NO signaling, e.g., activation of guanylate cyclase or feedback inhibition of NO synthase. Direct evidence for Fe-NO binding in nitrosylated ferrous heme in myoglobin (and hemoglobin) has been provided by electrospray ionization mass spectrometry.<sup>68</sup> From the X-ray crystal structure of  $\text{MbFe}^{\text{II}}\text{NO}$  bond distances between the central ferrous atom and the proximal histidine and the ligand NO are 2.18 and 1.89 Å, respectively.<sup>38</sup> ESR spectroscopy provides a sensitive method for detection of the paramagnetic Fe-NO complex,<sup>69</sup> and has been used to detect nitrosylated hemoglobin in tissue.<sup>11</sup>

#### A. Nitric Oxide Binding and Heme/Protein Structure

The binding of NO to myoglobin has been studied using a kinetic approach, determining the rate for association and dissociation of CO, O<sub>2</sub>, and NO.<sup>24,70</sup> The effect of varying the pH is interpreted as various "open" or "closed" states of myoglobin, and may be studied depending on the degree of protonization. At low pH, the proximal histidine does not bind to the heme iron and the recombination is very fast.<sup>70</sup> For a NO complex of a myoglobin mutant (His93Gly), which is pentacoordinated even at neutral pH, the

pentacoordinated state is found to be 3-fold more stable than the wild-type myoglobin, in which NO is bound to hexacoordinated iron.<sup>22</sup> When the pH effects on binding of the trans ligand in NO complexes of horse heart myoglobin and minimyoglobin (in which the peptide chain has been digested except from residues 32–139) are compared, it is evident that both residues 1–31 and 139–153 play an important role in controlling solvent accessibility to the heme pocket.<sup>71</sup> A unique feature of the NO complex of minimyoglobin is the presence of both an acid and alkaline hexa- to pentacoordination transition with apparent  $pK_a$  values of 6.6 and 7.8, respectively. In ordinary nitrosylmyoglobins only the acid transition can be observed with a  $pK_a \approx 4.7$ , whereas the value for a solvent-exposed histidyl residue ( $pK_a = 6.8$ ) is very close to the one found for NO minimyoglobin.

In addition, the presence or absence of various anions alters the coordination state of  $\text{MbFe}^{\text{II}}\text{NO}$  in the pH range 4.5–9.0.<sup>72</sup> Thus, anions such as phosphate, acetate, and sulfate have a cooperative effect with protons in transition from an axial hexacoordinated to a rhombic pentacoordinated complex of  $\text{MbFe}^{\text{II}}\text{NO}$ . At pH 6.0, addition of chloride acts synergistically with other anions, thereby promoting the transformation to a pentacoordinated complex. Likewise, inorganic phosphate has been shown to rupture the proximal binding to His(F8) at pH 6.5, stabilizing nitrosylhemoglobin in the T state with a pentacoordinated Fe atom.<sup>73</sup> In addition, compounds such as inositol hexaphosphate (IHP) and bezafibrate have been shown to perturb the coordination state of tetrameric nitrosylhemoglobin to varying degrees,<sup>74</sup> whereas IHP, ATP, and 2,3-bisphosphoglycerate have no effect on the coordination state of  $\text{MbFe}^{\text{II}}\text{NO}$ .<sup>72</sup> Even the iron-heme geometry in nitrosylated heme bound to human serum albumin is altered by bezafibrate and clofibrate, which are used as anticoagulant and antihyperlipoproteinemic drugs.<sup>75</sup> Furthermore, the presence of detergents such as sodium dodecyl sulfate and hexadecyltrimethylammonium bromide has been shown to modify the coordination state of  $\text{MbFe}^{\text{II}}\text{NO}$  from hexa- to pentacoordination at alkaline pH.<sup>15,76</sup> The rupture of the proximal histidine bond leads to release of the heme-NO from the heme cavity and an encapsulation inside the aqueous micelles.<sup>76</sup> The importance of the coordination number of iron for the properties of  $\text{MbFe}^{\text{II}}\text{NO}$  is not clear, e.g., in cured meat, where chloride and phosphates are added. However, other nitrosylated heme proteins change properties depending on the coordination number of the central Fe atom, e.g., guanylate cyclase.<sup>77</sup>

The protein conformation also affects the association and dissociation rates of ligands, as helices at the distal side will adjust by 0.5 Å to facilitate the binding/transportation of a diatomic ligand.<sup>78</sup> The "packaging defect" or cavities in the interior of the myoglobin have been identified as important for ligand diffusion and binding.<sup>79</sup> Molecular simulation estimates a pattern of cavities in the protein that fluctuate and interconnect, whereby the accessibility to the iron atom is greatly affected.<sup>78</sup> Rather detailed information about NO motion inside myoglobin was

**Table 1. Free Energy of Binding of Imidazole Derivatives to Nitrosylmyoglobin Mutants at 25 °C**

myoglobin mutant	$\Delta G^\circ$ (kJ/mol) for binding				ref
	imidazole	<i>N</i> -methylimidazole	4-methylimidazole	2-methylimidazole	
H93G	<b>-18</b>	<b>-16</b>	<b>-19</b>	<b>-8</b>	22
S92A/H93G	<b>-6</b>	<b>-11</b>	<b>-10</b>		23
S92T/H93G	<b>-19</b>	<b>-17</b>	<b>-20</b>		23

**Table 2. Free Energy of Binding of Nitric Oxide to Ferrous/Ferric Iron Porphyrin Complexes at 25 °C**

iron porphyrin complex	$\Delta G^\circ$ (kJ/mol)	ref	iron porphyrin complex	$\Delta G^\circ$ (kJ/mol)	ref
Fe <sup>II</sup> (TPPS) <sup>a</sup>	<b>-51</b>	27	metmyoglobin	<b>-24/-18</b>	27/30
Fe <sup>III</sup> (TPPS)	<b>-17</b>	84	reduced cytochrome <i>c</i>	<b>-31</b>	27
Fe <sup>III</sup> (TMPS) <sup>b</sup>	<b>-21</b>	84	oxidized cytochrome <i>c</i>	<b>-24</b>	27
deoxymyoglobin	<b>-64</b>	84	catalase	<b>-30</b>	27

<sup>a</sup> TPPS = tetra(4-sulfonatophenyl)porphine. <sup>b</sup> TMPS = tetra(sulfonatomesityl)porphine.

obtained experimentally, results which together with simulation served to model oxygen migration. Oxygen has been shown to migrate away from the heme iron into such cavities in myoglobin,<sup>21</sup> whereas no information is available for NO. In relation to meat products, unfolding of myoglobin caused by lowering the pH can be avoided by increasing the ionic strength and denatured myoglobin can be partially refolded by addition of salt.<sup>80</sup> Circular dichroism spectra of MbFe<sup>II</sup>NO at pH 4 in the visible and near-UV regions suggest, apart from a full transition to pentacoordination, a major conformational change in the protein structure, even though the tertiary structure is almost fully preserved.<sup>24</sup> NO may be trapped noncovalently in such cavities both in heme pigment and in albumin in a manner similar to that of nitrous oxide, which does not bind to heme.<sup>81</sup> However, NO or O<sub>2</sub> binding in the cavities seems to be too weak to be significant at equilibrium. These cavities are only important as transient kinetic intermediates.

Spin–lattice relaxation rates have been determined by ESR spectroscopy for native, lyophilized, and denatured MbFe<sup>II</sup>NO,<sup>82</sup> where the latter exhibits the highest rate of spin–lattice relaxation followed by lyophilized and native MbFe<sup>II</sup>NO. These findings indicate that drastic changes in the globin structure occur upon denaturation of MbFe<sup>II</sup>NO and various degrees of dehydration seem responsible for the variation in the relaxation rate.

## B. Thermodynamics of Nitric Oxide Binding

The importance of a *trans*-imidazole for binding of diatomic molecules to porphyrin has been demonstrated by molecular simulations. The energies of binding of O<sub>2</sub>, CO, and NO to the iron atom in porphyrin in the absence of a *trans*-imidazole are 37.7, 108.8, and 146.4 kJ/mol, respectively, whereas in the presence of imidazole, the values are shifted up to 62.8, 146.4, and 150.6 kJ/mol, respectively.<sup>83</sup> The binding at 295 K for various ligands to penta-coordinated MbFe<sup>II</sup>NO His93Gly mutant has been studied experimentally,<sup>22,23</sup> and imidazole and the 4-methyl derivative are found to have matching binding affinities with  $\Delta G^\circ$  values of -18 and -19 kJ/mol, respectively (Table 1). The binding of imid-

azole to MbFe<sup>II</sup>NO His93Gly mutant is found to have  $\Delta H^\circ = -34$  kJ/mol and  $\Delta S^\circ = -55$  kJ/mol.<sup>22</sup>

From rate data for the association and dissociation of NO to various model porphyrins, ferrous/ferric myoglobins, and cytochromes,  $\Delta G^\circ$  for NO binding may be estimated (Table 2). Especially MbFe<sup>II</sup>NO has a high  $\Delta G^\circ$  compared to both model porphyrins and other biological macromolecules.<sup>84</sup>

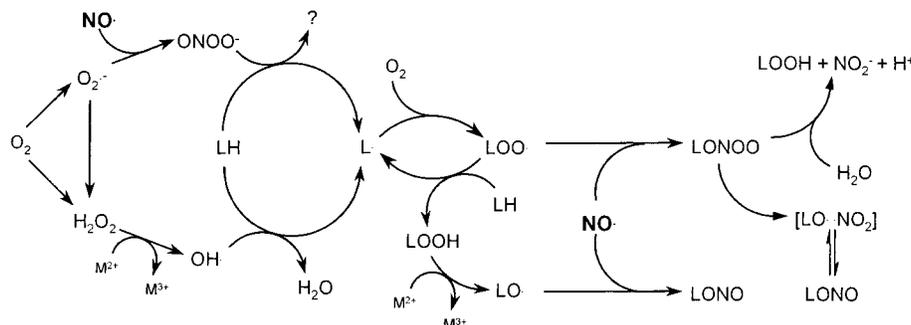
Activation parameters, including the volume of activation from pressure dependence, of reversible NO binding to ferric myoglobin have also been determined,  $\Delta H_{on}^\ddagger = 65$  kJ/mol,  $\Delta S_{on}^\ddagger = 60$  J/mol/K, and  $\Delta V_{on}^\ddagger = 20$  cm<sup>3</sup>/mol, whereas the same parameters for the “off” reaction are  $\Delta H_{off}^\ddagger = 76$  kJ/mol,  $\Delta S_{off}^\ddagger = 41$  J/mol/K, and  $\Delta V_{off}^\ddagger = 20$  cm<sup>3</sup>/mol.<sup>30</sup> The binding of NO to MbFe<sup>III</sup> thus decreases with temperature, but is surprisingly insensitive to pressure ( $\Delta V^\circ = 0$  cm<sup>3</sup>/mol).

## C. Radical Scavenging of Nitrosylmyoglobins

In biological systems NO may have antioxidative or prooxidative effects depending mainly on the concentration of NO, but also on the presence of other components.<sup>60,85–87</sup> For meat production, it is evident that addition of nitrite results in a prolonged oxidative stability and prevents rancidity. Moreover, NO may also play a role in metal-catalyzed, oxidative processes promoted by heme compounds such as myoglobin in living organisms.<sup>88</sup>

The lifetime of NO in biological systems has been reported to be in the range 5–15 s, and this combined with the very high diffusion constant allows it to travel a distance of 150–300 nm in biological systems.<sup>89</sup> NO acts as a reductant or as a weak oxidant, yielding either nitrosonium (NO<sup>+</sup>) or nitroxyl anion (NO<sup>-</sup>), respectively. During lipid peroxidation, NO may react with lipid peroxy radical and the addition product LOONO may break down to yield an alkoxy radical and nitrogen dioxide, or the intermediate isomerizes to give the more stable LONO<sub>2</sub> (Scheme 1).<sup>90</sup>

MbFe<sup>II</sup>NO (15  $\mu$ M) effectively retards carotene–linoleate peroxidation in comparison to both MbFe<sup>II</sup>-O<sub>2</sub> and MbFe<sup>III</sup>, whereas a lower concentration of MbFe<sup>II</sup>NO (2  $\mu$ M) only slows the peroxidation rate.<sup>91</sup> Equimolar amounts (30  $\mu$ M) of either MbFe<sup>III</sup> or MbFe<sup>II</sup>NO affect membrane lipid peroxidation op-

**Scheme 1. Involvement of Nitric Oxide in Propagation and Termination of Lipid Peroxidation<sup>a</sup>**

<sup>a</sup> Modified from refs 60, 87, and 90.

positely, as MbFe<sup>III</sup> increases oxidation by a factor of 2 and MbFe<sup>II</sup>NO decreases oxidation by a factor of 2.<sup>91</sup> It is accordingly obvious that NO may act as an antioxidant via reductive nitrosylation of MbFe<sup>III</sup>, and although this mechanism is operative in cured meat products, the relevance of this reaction pathway under physiological conditions is speculative.

NO is capable of reducing activated MbFe<sup>IV</sup>=O to MbFe<sup>III</sup>.<sup>88,92</sup> In model systems consisting of rhodamine as oxidation substrate and MbFe<sup>III</sup> as initiator, equal amounts of superoxide anion, H<sub>2</sub>O<sub>2</sub>, and NO (all 1 nM) promote oxidation, whereas NO levels above 1 nM significantly reduce substrate oxidation.<sup>93</sup> When human cell cultures are exposed to oxidative stress from Fe<sup>2+</sup> (20 μM), NO in the range 0.4–1.8 μM exhibits a concentration-dependent inhibition of oxygen consumption.<sup>94</sup> The antioxidative mechanism of NO during “Fenton generation” of radicals may be binding of Fe<sup>2+</sup> ions, but also scavenging of hydroxyl radicals, yielding nitrite.

Depletion of glutathione in combination with hypoxia can lead to the formation of hypervalent heme compounds mediating oxidative stress in the endothelium. NO may also here play a role as antioxidant, since addition of L-arginine resulted in reduction of ferryl formation.<sup>95</sup>

#### IV. Reactions of Nitric Oxide and Myoglobin Complexes

Physiological roles of myoglobin, other than the role as oxygen carrier in muscle tissue, have now been identified. One such role of myoglobin is protection of cytochrome *c* oxidase in mitochondria by NO scavenging of MbFe<sup>II</sup>O<sub>2</sub> or by binding of NO to ferrous heme.<sup>1–3</sup> Furthermore, NO may react with the thiol group found in human myoglobin,<sup>96</sup> and similar reactions of NO and thiols in hemoglobin may account for transport/metabolism of NO in the blood stream.<sup>97,98</sup> The presence of several cavities in myoglobin next to the heme cavity may further facilitate reaction between small molecules trapped temporarily in the cavities.<sup>8</sup>

##### A. Ligand Exchange Reactions

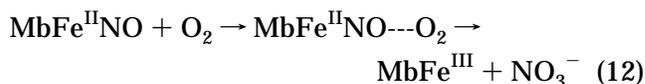
Exchange of NO from manganese to iron tropocoronand complexes is spontaneous at room temperature and occurs with  $k_{\text{obs}} = 2.9 \times 10^{-3} \text{ s}^{-1}$ .<sup>99</sup> The exchange between free NO and NO coordinated in MbFe<sup>II</sup>NO occurs with  $k_{\text{obs}} = 8.2 \times 10^{-5} \text{ s}^{-1}$  at 40

°C.<sup>100</sup> This ligand exchange has a low activation barrier,  $\Delta H^\ddagger = 47 \text{ kJ/mol}$ ,<sup>100</sup> in comparison to the activation barrier of autoxidation of MbFe<sup>II</sup>NO ( $\Delta H^\ddagger = 110 \text{ kJ/mol}$ ).<sup>40</sup>

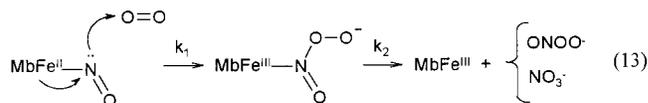
The effect of the coordination state of heme iron on the kinetics of NO dissociation has been studied for penta- and hexacoordinated complexes,<sup>101</sup> e.g., guanylate cyclase, myoglobin, and hemoglobin. However, no systematic effect of the coordination state on the dissociation rate of NO is evident, since the rate constant varies between  $1.7 \times 10^{-5}$  and  $1.3 \times 10^{-3} \text{ s}^{-1}$  for both penta- and hexacoordinated heme proteins.

##### B. Thermal Oxidation of Nitrosylmyoglobins

Oxidation of MbFe<sup>II</sup>NO yields MbFe<sup>III</sup> and nitrate in a slow process with a first-order rate constant of  $5.1 \times 10^{-4} \text{ s}^{-1}$  at 30 °C (eq 12).<sup>40</sup> In this reaction, the stoichiometry of MbFe<sup>II</sup>NO and oxygen is 1:1 and oxygen has been suggested to bind in the heme cavity prior to electron transfer.

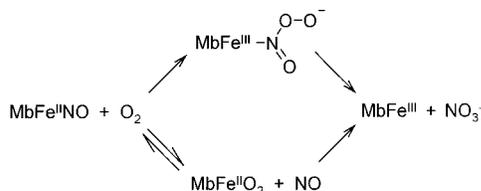


Arnold and Bohle have suggested a mechanism for the autoxidation in which a short-lived intermediate myoglobin species is present. This mechanism is based on kinetic data for MbFe<sup>II</sup>NO autoxidation, which is described as two consecutive reactions with  $k_1 = 1.34 \times 10^{-3} \text{ s}^{-1}$  and  $k_2 = 2.81 \times 10^{-3} \text{ s}^{-1}$  at 37 °C.<sup>47</sup> This reaction mechanism involves an electrophilic attack of molecular oxygen on nitrogen in nitrosyl, forming a direct nitrogen–oxygen bond in a complex of MbFe<sup>III</sup> and peroxyxynitrite (eq 13). Dissociation of this complex yields the highly reactive peroxyxynitrite or nitrate formed by rearrangement.



In another study, in which myoglobin was submitted to repeated cycles of oxygenation, reduction, and nitrosylation, the oxidation product of NO was found to be nitrate, which emerges following a significant induction period, providing further proof for intermediates forming during MbFe<sup>II</sup>NO autoxidation.<sup>102</sup> A methemoglobin peroxyxynitrite complex is also ob-

### Scheme 2. Two Limiting Reaction Mechanisms for the Oxidation of Nitrosylmyoglobin by Oxygen



served at high pH in the NO mediated oxidation of oxyhemoglobin.<sup>103</sup> These findings suggest an alternative pathway for generation of peroxynitrite in biological systems other than the reaction between superoxide anion and NO.<sup>11</sup>

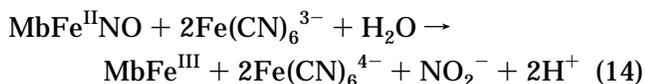
The pathway for oxidation of MbFe<sup>II</sup>NO by O<sub>2</sub> involving binding of O<sub>2</sub> and a possible formation of coordinated peroxynitrite has been criticized and an alternative mechanism presented.<sup>104</sup> The observation of a very similar rate for oxidation and dissociation of NO from MbFe<sup>II</sup>NO (wild type and various single mutants of recombinant myoglobin included) leads thus to the conclusion that NO is replaced by O<sub>2</sub> prior to reaction of NO with MbFe<sup>II</sup>O<sub>2</sub> to yield MbFe<sup>III</sup> and NO<sub>3</sub><sup>-</sup>. NO exchange in MbFe<sup>II</sup>NO is on the other hand faster than dissociation, suggesting at least some degree of association or protein binding of the incoming ligand, NO or O<sub>2</sub>.<sup>40</sup> Scheme 2 presents two alternative reaction mechanisms suggested for the oxidation of MbFe<sup>II</sup>NO by O<sub>2</sub>.

The autoxidation rate of MbFe<sup>II</sup>NO decreases as the hydrostatic pressure is increased (up to 350 MPa investigated). The effect of high pressures cannot be explained by protein denaturation, as autoxidation rates become faster in the presence of the denaturant urea at ambient pressure.<sup>105</sup> The positive volume of activation ( $\Delta V^\ddagger = 8 \text{ mL/mol}$ ) can be explained by rate-determining formation of the peroxynitrite complex or by a rate-determining ligand dissociation.

One oxygenation/reduction/nitrosylation cycle of myoglobin produces low-spin ferric hemichromes (dihistidylheme), and the intensity of the hemichrome ESR spectra increases upon repetition of up to five cycles.<sup>102</sup> Apart from the low-spin ferric hemichrome another low-spin ferric species can be detected after a second cycle. These reactions may have physiological implications for the formation of hemichrome (known as Heinz bodies).

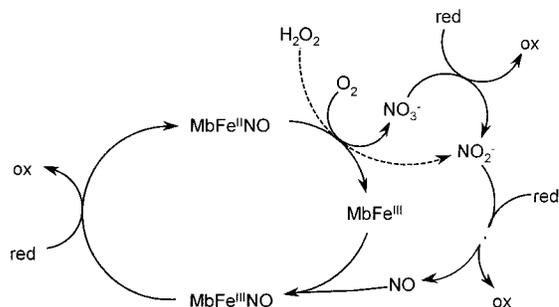
### B. Chemically Induced Oxidation of Myoglobin Complexes

MbFe<sup>II</sup>NO will be degraded by H<sub>2</sub>O<sub>2</sub>, yielding MbFe<sup>III</sup> and nitrite as reaction products.<sup>106</sup> Ferricyanide will also oxidize MbFe<sup>II</sup>NO,<sup>107</sup> possible also with MbFe<sup>III</sup> and nitrite as products (eq 14).



The myoglobin derivative isolated from Italian Parma ham once again behaves differently, as it is not oxidized by ferricyanide.<sup>108</sup> Scheme 3 shows the cycle of NO complexes of myoglobin in cured meat, and it is notable that, as long as the meat system contains

### Scheme 3. Transformation of Nitric Oxide Complexes of Myoglobin in Meat



reducing capacity, the antioxidant MbFe<sup>II</sup>NO may be regenerated from the oxidation products MbFe<sup>III</sup> and nitrite or nitrate from the oxidation by H<sub>2</sub>O<sub>2</sub> or O<sub>2</sub>, respectively.

The reactions between nitrogen oxides and MbFe<sup>II</sup>O<sub>2</sub> have initially been studied due to their biological relevance to model the interaction between oxyhemoglobin and NO (plus other nitrogen oxides) present in the blood stream.<sup>109</sup>

Significant differences are found when MbFe<sup>II</sup>O<sub>2</sub> reacts with NO in comparison to both NO<sub>2</sub> and NO<sub>2</sub><sup>-</sup> in an anaerobic environment. Where both NO<sub>2</sub> and NO<sub>2</sub><sup>-</sup> react in a second-order reaction, NO has been suggested to react in two steps. On the other hand, the rates become similar for the various nitrogen oxides under aerobic conditions, and MbFe<sup>III</sup> and nitrate were detected as products as shown in eq 15 for NO.<sup>109</sup> NO has been detected during nitrite-induced oxidation of MbFe<sup>II</sup>O<sub>2</sub>, and a one-electron reduction of nitrite is suggested to produce NO.<sup>110</sup>



In a more recent study an intermediate peroxynitro complex of myoglobin, only observed at high pH values, was characterized during NO-induced oxidation of MbFe<sup>II</sup>O<sub>2</sub>. However, no evidence is found regarding the formation of free peroxynitrite;<sup>111</sup> e.g., the marker 3-nitrotyrosine in myoglobin is practically absent even after 10 reaction cycles. Peroxynitrite is thus not formed when NO reacts with MbFe<sup>II</sup>O<sub>2</sub>.

From a comprehensive study of NO-induced oxidation of the oxygenated form of mutants of myoglobin, in which amino acid residues have been substituted in the distal heme pocket, a correlation close to one between the rates for NO binding and NO-induced oxidation is evident.<sup>112</sup> In contrast to other studies, no spectral evidence for an intermediate MbFe<sup>III</sup> complex is found. Peroxynitrite is concluded, due to its size, polarity, and poor affinity for ferric iron, to be quickly expelled from the protein. The second-order rate constant at 20 °C for NO-induced oxidation of wild-type MbFe<sup>II</sup>O<sub>2</sub> is found to be  $34 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  at pH 7.0.<sup>112</sup> MbFe<sup>II</sup>O<sub>2</sub> has recently been shown to act as a scavenger of NO in isolated hearts from either wild-type or myoglobin knockout mice.<sup>1</sup> The reaction occurs in vivo, when NO is directly administered (5 nM to 25 μM) or when endogenous production is stimulated (10 nM to 2 μM), and MbFe<sup>III</sup> formation is dose-dependent in hearts isolated from wild-type mice, whereas hearts lacking



oxyheme groups are oxidized or the deoxyheme groups contain bound NO.

Both NO and derived peroxynitrite inhibit mitochondrial respiration, and NO binds to the active site of cytochrome *c* oxidase at low concentration, while the action of peroxynitrite is less specific.<sup>122</sup> In addition, NO will bind reversibly and inhibit catalase with  $K_i = 0.18 \mu\text{M}$ .<sup>123</sup> The reactivity of NO toward  $\text{MbFe}^{\text{II}}$  and  $\text{MbFe}^{\text{II}}\text{O}_2$  is comparable, and thus these reactions may control NO levels and protect mainly cytochromes from inactivation by NO.<sup>1-3</sup>

## VI. References

- Flogel, U.; Merx, M. W.; Godecke, A.; Decking, U. K. M.; Schrader, J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 735.
- Brunori, M. *Trends Biochem. Sci.* **2001**, *26*, 209.
- Brunori, M. *Trends Biochem. Sci.* **2001**, *26*, 21.
- Ascenzi, P.; Salvati, L.; Brunori, M. *FEBS Lett.* **2001**, *501*, 103.
- Liu, L.; Zeng, M.; Hausladen, A.; Heitman, J.; Stamler, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 4672.
- Gardner, P. R.; Gardner, A. M.; Martin, L. A.; Dou, Y.; Li, T.; Olson, J. S.; Zhu, H.; Riggs, A. F. *J. Biol. Chem.* **2000**, *275*, 31581.
- Kanner, J.; Harel, S.; Shagalovich, J.; Berman, S. *J. Agric. Food Chem.* **1984**, *32*, 512.
- Frauenfelder, H.; McMahon, B. H.; Austin, R. H.; Chu, K.; Groves, J. T. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 2370.
- Kohno, M.; Masumizu, T.; Mori, A. *Free Radical Biol. Med.* **1995**, *18*, 451.
- Salerno, J. C. In *Nitric Oxide: Principles and Actions*; Lancaster, J. R. J., Ed.; Academic Press: San Diego, CA, 1996; Chapter 2, p 83.
- Radi, R. *Chem. Res. Toxicol.* **1996**, *9*, 828.
- Mathieu, C.; Moreau, S.; Frendo, P.; Puppo, A.; Davies, M. J. *Free Radical Biol. Med.* **1998**, *24*, 1242.
- Hoshino, M.; Laverman, L. E.; Ford, P. C. *Coord. Chem. Rev.* **1999**, *187*, 75.
- Cooper, C. E. *Biochim. Biophys. Acta* **1999**, *1411*, 290.
- Cheng, L.; Richter-Addo, G. B. In *The Porphyrin Handbook—Biochemistry and Binding: Activation of Small Molecules*; Kadish, K. M., Smith, S. M., Guillard, R., Eds.; Academic Press: San Diego, CA, 2000; Chapter 33, p 219.
- Skibsted, L. H. In *The Chemistry of Muscle-based Foods*; Johnston, D. E., Knight, M. K., Leward, D. A., Eds.; The Royal Society of Chemistry: Cambridge, U.K., 1992; p 266.
- Springer, B. A.; Sligar, S. G.; Olson, J. S.; Phillips, G. N. *Chem. Rev.* **1994**, *94*, 699.
- Poderoso, J. J.; Peralta, J. G.; Lisdero, C. L.; Carreras, M. C.; Radisic, M.; Schopfer, F.; Cadenas, E.; Boveris, A. *Am. J. Physiol.: Cell Physiol.* **1998**, *43*, C112.
- Olson, J. S.; Phillips, G. N. *J. Biol. Inorg. Chem.* **1997**, *2*, 544.
- Olson, J. S.; Phillips, G. N. *J. Biol. Chem.* **1996**, *271*, 17593.
- Scott, E. E.; Gibson, Q. H.; Olson, J. S. *J. Biol. Chem.* **2001**, *276*, 5177.
- Decatur, S. M.; Franzen, S.; DePillis, G. D.; Dyer, R. B.; Woodruff, W. H.; Boxer, S. G. *Biochemistry* **1996**, *35*, 4939.
- Decatur, S. M.; Belcher, K. L.; Rickert, P. K.; Franzen, S.; Boxer, S. G. *Biochemistry* **1999**, *38*, 11086.
- Duprat, A. F.; Traylor, T. G.; Wu, G. Z.; Coletta, M.; Sharma, V. S.; Walda, K. N.; Magde, D. *Biochemistry* **1995**, *34*, 2634.
- Tomita, T.; Hirota, S.; Ogura, T.; Olson, J. S.; Kitagawa, T. *J. Phys. Chem. B* **1999**, *103*, 7044.
- Traylor, T. G.; Sharma, V. S. *Biochemistry* **1992**, *31*, 2847.
- Hoshino, M.; Ozaka, K.; Seki, H.; Ford, P. C. *J. Am. Chem. Soc.* **1993**, *115*, 9568.
- Hoshino, M.; Maeda, M.; Konishi, M.; Seki, H.; Ford, P. C. *J. Am. Chem. Soc.* **1996**, *118*, 5702.
- Killday, K. B.; Tempesta, M. S.; Bailey, M. E.; Metral, C. J. *J. Agric. Food Chem.* **1988**, *36*, 909.
- Laverman, L. E.; Wanat, A.; Oszajca, J.; Stochel, G.; Ford, P. C.; van Eldik, R. *J. Am. Chem. Soc.* **2001**, *123*, 285.
- Reichenbach, G.; Sabatini, S.; Palombari, R.; Palmerini, C. A. *Nitric Oxide Biol. Chem.* **2001**, *5*, 395.
- Konorev, E. A.; Joseph, J.; Kalyanaraman, B. *FEBS Lett.* **1996**, *378*, 111.
- Hori, H.; Masuya, F.; Dou, Y.; Ikeda-Saito, M. *J. Inorg. Biochem.* **2000**, *82*, 181.
- Keseru, G. M.; Menyhard, D. K. *Biochemistry* **1999**, *38*, 6614.
- Zhao, X. J.; Sampath, V.; Caughey, W. S. *Biochem. Biophys. Res. Commun.* **1994**, *204*, 537.
- Miller, L. M.; Pedraza, A. J.; Chance, M. R. *Biochemistry* **1997**, *36*, 12199.
- Rich, A. M.; Armstrong, R. S.; Ellis, P. J.; Lay, P. A. *J. Am. Chem. Soc.* **1998**, *120*, 10827.
- Brucker, E. A.; Olson, J. S.; Ikeda-Saito, M.; Phillips, G. N. *Proteins: Struct., Funct., Genet.* **1998**, *30*, 352.
- Cheng, L.; Novozhilova, I.; Kim, C.; Kovalevsky, A.; Bagley, K. A.; Coppens, P.; Richter-Addo, G. B. *J. Am. Chem. Soc.* **2000**, *122*, 7142.
- Andersen, H. J.; Skibsted, L. H. *J. Agric. Food Chem.* **1992**, *40*, 1741.
- Møller, J. K. S.; Bertelsen, G.; Skibsted, L. H. *Meat Sci.* **2002**, *60*, 421.
- Ikeda-Saito, M.; Dou, Y.; Yonetani, T.; Olson, J. S.; Li, T. S.; Regan, R.; Gibson, Q. H. *J. Biol. Chem.* **1993**, *268*, 6855.
- Gibson, Q. H.; Regan, R.; Elber, R.; Olson, J. S.; Carver, T. E. *J. Biol. Chem.* **1992**, *267*, 22022.
- Shreve, A. P.; Franzen, S.; Simpson, M. C.; Dyer, R. B. *J. Phys. Chem. B* **1999**, *103*, 7969.
- Kamarei, A. R.; Karel, M. *J. Food Sci.* **1982**, *47*, 682.
- Nakamura, M.; Nakamura, S. *Biochim. Biophys. Acta* **1996**, *1289*, 329.
- Arnold, E. V.; Bohle, D. S. *Methods Enzymol.* **1996**, *269*, 41.
- Lin, R.; Bayachou, M.; Greaves, J.; Farmer, P. J. *J. Am. Chem. Soc.* **1997**, *119*, 12689.
- Farmer, P. J.; Lin, R.; Bayachou, M. *Comments Inorg. Chem.* **1998**, *20*, 101.
- Stolze, K.; Nohl, H. *Biochem. Pharmacol.* **1995**, *49*, 1261.
- Stolze, K.; Dadak, A.; Liu, Y.; Nohl, H. *Biochem. Pharmacol.* **1996**, *52*, 1821.
- Taira, J.; Misik, V.; Riesz, P. *Biochim. Biophys. Acta* **1997**, *1336*, 502.
- Rupon, J. W.; Domingo, S. R.; Smith, S. V.; Gummadi, B. K.; Shields, H.; Ballas, S. K.; King, S. B.; Kim-Shapiro, D. B. *Biochem. Chem.* **2000**, *84*, 1.
- Choi, I. K.; Liu, Y. M.; Wei, Z. C.; Ryan, M. D. *Inorg. Chem.* **1997**, *36*, 3113.
- Cornforth, D. In *Nitric Oxide: Principles and Actions*; Lancaster, J. R. J., Ed.; Academic Press: San Diego, CA, 1996; Chapter 8, p 259.
- Pegg, R. B.; Shahidi, F. *Nitrite Curing of Meat. The N-Nitrosamine Problem and Nitrite Alternatives*; Food & Nutrition Press: Trumbull, CT, 2000.
- Pegg, R. B.; Shahidi, F. *Crit. Rev. Food Nutr.* **1997**, *37*, 561.
- Morita, H.; Niu, J.; Sakata, R.; Nagata, Y. *J. Food Sci.* **1996**, *61*, 1021.
- Morita, H.; Sakata, R.; Nagata, Y. *J. Food Sci.* **1998**, *63*, 352.
- Eiserich, J. P.; Patel, R. P.; O'Donnell, V. B. *Mol. Aspects Med.* **1998**, *19*, 221.
- Cammack, R.; Joanou, C. L.; Cui, X.-Y.; Martines, C. T.; Maraj, S. R.; Hughes, M. N. *Biochim. Biophys. Acta* **1999**, *1411*, 475.
- Chen, Y.; Rosazza, J. P. N. *J. Bacteriol.* **1995**, *177*, 5122.
- Arihara, K.; Kushida, K.; Kondo, H.; Itoh, Y.; Luchansky, M. *J. Food Sci.* **1993**, *58*, 38.
- Morita, H.; Yoshikawa, H.; Sakata, R.; Nagata, Y.; Tanaka, H. *J. Bacteriol.* **1997**, *179*, 7812.
- Boucher, J. L.; Genet, A.; Vadon, S.; Delaforge, M.; Mansuy, D. *Biochem. Biophys. Res. Commun.* **1992**, *184*, 1158.
- Cutruzzola, F. *Biochim. Biophys. Acta* **1999**, *1411*, 231.
- Odaka, M.; Fujii, K.; Hoshino, M.; Noguchi, T.; Tsujimura, M.; Nagashima, S.; Yohda, M.; Nagamune, T.; Inoue, Y.; Endo, I. *J. Am. Chem. Soc.* **1997**, *119*, 3785.
- Upmacis, R. K.; Hajjar, D. P.; Chait, B. T.; Mirza, U. A. *J. Am. Chem. Soc.* **1997**, *119*, 10424.
- Cammack, R.; Shergill, J. K.; Inalsingh, V. A.; Hughes, M. N. *Spectrochim. Acta, Part A* **1998**, *54*, 2393.
- Tian, W. D.; Sage, J. T.; Champion, P. M. *J. Mol. Biol.* **1993**, *233*, 155.
- De Sanctis, G.; Falcioni, G.; Polizio, F.; Desideri, A.; Giardina, B.; Ascoli, F.; Brunori, M. *Biochim. Biophys. Acta* **1994**, *1204*, 28.
- Polizio, F.; De Sanctis, G.; Ascenzi, P.; Coletta, M. *J. Biol. Inorg. Chem.* **1998**, *3*, 458.
- Bellelli, A.; Brunori, M. *Methods Enzymol.* **1994**, *232*, 56.
- De Sanctis, G.; Priori, A. M.; Polizio, F.; Ascenzi, P.; Coletta, M. *J. Biol. Inorg. Chem.* **1998**, *3*, 135.
- Mattu, M.; Vannini, A.; Coletta, M.; Fasano, M.; Ascenzi, P. *J. Inorg. Biochem.* **2001**, *84*, 293.
- Das, T. K.; Mazumdar, S.; Mitra, S. *J. Chem. Soc., Chem. Commun.* **1993**, *18*, 1447.
- Sharma, V. S.; Magde, D. *Methods* **1999**, *19*, 494.
- Carlson, M. L.; Regan, R. M.; Gibson, Q. H. *Biochemistry* **1996**, *35*, 1125.
- Brunori, M. *Biophys. Chem.* **2000**, *86*, 221.
- Bismuto, E.; Sirangelo, I.; Irace, G. *Arch. Biochem. Biophys.* **1992**, *298*, 624.
- Sampath, V.; Zhao, X. J.; Caughey, W. S. *J. Biol. Chem.* **2001**, *276*, 13635.
- Wajnberg, E.; Alves, O. C. *J. Magn. Reson., Ser. B* **1996**, *113*, 119.

- (83) Rovira, C.; Kunc, K.; Hutter, J.; Ballone, P.; Parrinello, M. *Int. J. Quantum Chem.* **1998**, *69*, 31.
- (84) Ford, P. C.; Bourassa, J.; Miranda, K.; Lee, B.; Lorkovic, I.; Boggs, S.; Kudo, S.; Laverman, L. E. *Coord. Chem. Rev.* **1998**, *171*, 185.
- (85) Hernandez, P.; Navarro, P.; Toldra, F. *Meat Sci.* **1999**, *51*, 123.
- (86) Hogg, N.; Kalyanaraman, B. *Biochim. Biophys. Acta* **1999**, *1411*, 378.
- (87) Rubbo, H.; Freeman, B. A. *Methods Enzymol.* **1996**, *269*, 385.
- (88) Dee, G.; Rice-Evans, C.; Obeyesekera, S.; Meraji, S.; Jacobs, M.; Bruckdorfer, K. R. *FEBS Lett.* **1991**, *294*, 38.
- (89) Lancaster, J. R. J. *Nitric Oxide Biol. Chem.* **1997**, *1*, 18.
- (90) Patel, R. P.; Levonen, A. L.; Crawford, J. H.; Darley-Usmar, V. M. *Cardiovasc. Res.* **2000**, *47*, 465.
- (91) Kanner, J. *Methods Enzymol.* **1996**, *269*, 218.
- (92) Herold, S.; Rehmann, F. J. K. *J. Biol. Inorg. Chem.* **2001**, *6*, 543.
- (93) Jourdeuil, D.; Mills, L.; Miles, A. M.; Grisham, M. B. *Nitric Oxide Biol. Chem.* **1998**, *2*, 37.
- (94) Kelley, E. E.; Wagner, B. A.; Buettner, G. R.; Burns, C. P. *Arch. Biochem. Biophys.* **1999**, *370*, 97.
- (95) D'Agnillo, F.; Wood, F.; Porras, C.; Macdonald, V. W.; Alayash, A. I. *Biochim. Biophys. Acta* **2000**, *1495*, 150.
- (96) Witting, P. K.; Douglas, D. J.; Mauk, A. G. *J. Biol. Chem.* **2000**, *275*, 20391.
- (97) Gladwin, M. T.; Shelhamer, J. H.; Schechter, A. N.; Pease-Fye, M. E.; Waclawiw, M. A.; Panza, J. A.; Ognibene, F. P.; Cannon, R. O. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 11482.
- (98) Gladwin, M. T.; Ognibene, F. P.; Pannell, L. K.; Nichols, J. S.; Pease-Fye, M. E.; Shelhamer, J. H.; Schechter, A. N. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9943.
- (99) Franz, K. J.; Lippard, S. J. *Inorg. Chem.* **2000**, *39*, 3722.
- (100) Andersen, H. J.; Johansen, H. S.; Shek, C. K.; Skibsted, L. H. *Z. Lebensm.-Unters.-Forsch.* **1990**, *191*, 293.
- (101) Kharitonov, V. G.; Sharma, V. S.; Magde, D.; Koesling, D. *Biochemistry* **1997**, *36*, 6814.
- (102) Arnold, E. V.; Bohle, D. S.; Jordan, P. A. *Biochemistry* **1999**, *38*, 4750.
- (103) Herold, S. *FEBS Lett.* **1998**, *439*, 85.
- (104) Eich, R. F. Reactions of nitric oxide with myoglobin. Ph.D. Dissertation, Rice University, Houston, TX, 1997; p 100.
- (105) Bruun-Jensen, L.; Skibsted, L. H. *Meat Sci.* **1996**, *44*, 145.
- (106) Kanner, J.; Harel, S.; Granit, R. *Arch. Biochem. Biophys.* **1991**, *289*, 130.
- (107) Sakata, R.; Honikel, K.; Morita, H.; Nagata, Y. *Fleischwirtschaft* **1996**, *76*, 1148.
- (108) Sakata, R.; Morita, H.; Norimatsu, T.; Niu, J. *Jpn. J. Swine Sci.* **1999**, *36*, 124.
- (109) Wade, R.; Castro, C. E. *Chem. Res. Toxicol.* **1996**, *9*, 1382.
- (110) Tomoda, A.; Murakami, E.; Shibuya, T. *Tohoku J. Exp. Med.* **1997**, *182*, 61.
- (111) Herold, S.; Exner, M.; Nauser, T. *Biochemistry* **2001**, *40*, 3385.
- (112) Eich, R. F.; Li, T. S.; Lemon, D. D.; Doherty, D. H.; Curry, S. R.; Aitken, J. F.; Mathews, A. J.; Johnson, K. A.; Smith, R. D.; Phillips, G. N.; Olson, J. S. *Biochemistry* **1996**, *35*, 6976.
- (113) Exner, M.; Herold, S. *Chem. Res. Toxicol.* **2000**, *13*, 287.
- (114) Minetti, M.; Pietraforte, D.; Carbone, V.; Salzano, A. M.; Scorza, G.; Marino, G. *Biochemistry* **2000**, *39*, 6689.
- (115) Mark, G.; Korth, H. G.; Schuchmann, H. P.; von Sonntag, C. *J. Photochem. Photobiol., A* **1996**, *101*, 89.
- (116) Dierks, E. A.; Hu, S. Z.; Vogel, K. M.; Yu, A. E.; Spiro, T. G.; Burstyn, J. N. *J. Am. Chem. Soc.* **1997**, *119*, 7316.
- (117) Zhao, Y. D.; Hoganson, C.; Babcock, G. T.; Marletta, M. A. *Biochemistry* **1998**, *37*, 12458.
- (118) Migita, C. T.; Salerno, J. C.; Masters, B. S.; Martasek, P.; McMillan, K.; Ikeda-Saito, M. *Biochemistry* **1997**, *36*, 10987.
- (119) Abu-Soud, H. M.; Wu, C. Q.; Ghosh, D. K.; Stuehr, D. J. *Biochemistry* **1998**, *37*, 3777.
- (120) Endo, I.; Odaka, M.; Yohda, M. *Trends Biotechnol.* **1999**, *17*, 244.
- (121) Witting, P. K.; Douglas, D. J.; Mauk, A. G. *J. Biol. Chem.* **2001**, *276*, 3991.
- (122) Brown, G. C. *Biochim. Biophys. Acta* **1999**, *1411*, 351.
- (123) Brown, G. C. *Eur. J. Biochem.* **1995**, *232*, 188.

CR000078Y