Kinetics and Mechanism of Thermal Oxidation and Photooxidation of Nitrosylmyoglobin in Aqueous Solution

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In aqueous solution, the rate of oxidation of chromatographically purified nitrosylmyoglobin (MbNO), synthesized from equine metmyoglobin (MMb), is strongly dependent on oxygen partial pressure and temperature but shows little dependence on pH, ionic strength, and solution viscosity. At an oxygen partial pressure of 0.2 atm and above, the rate approaches a limiting value with an observed first-order constant at 25 °C of k_{obs} = (2.32 ± 0.09) × 10^{-4} s^{-1} ($\Delta H^{\ddagger}$ = 110.1 ± 0.4 kJ mol^{-1}, $\Delta S^{\ddagger}$ = 55 ± 3 J mol^{-1} K^{-1}). The oxidation products are MMb and nitrate, as identified spectrophotometrically and by means of a nitrate electrode, respectively, and the rate of MbNO oxidation and the rate of nitrate formation are very similar; a reaction mechanism is suggested in which dissolved oxygen forms a complex with MbNO (K_{ass} = 5 ± 3 atm^{-1}) prior to intermolecular rate-determining electron transfer with a high activation barrier. The rate of exchange of nitric oxide in MbNO with solution nitric oxide, as determined by ^{15}N isotopic labeling, is fast (greater than 3.4 × 10^{-7} mol L^{-1} s^{-1} in a NO-saturated aqueous 3.4 × 10^{-5} M MbNO solution at 0 °C), excluding a common rate-determining step for ligand substitution and oxidation of MbNO. The reaction quantum yield for photooxidation of MbNO to MMb in air-saturated aqueous solution at 5 °C (i) is only moderately dependent on the wavelength of irradiation and ranges from 6.9 × 10^{-3} (254 nm) to 4.3 × 10^{-4} mol $einstein^{-1}$ (546 nm), (ii) is proportional to the partial oxygen pressure, and (iii) increases with increasing solution viscosity (glycerol/water mixtures). A bimolecular reaction between an electronically excited MbNO and oxygen with a transition state with a partly dissociated nitric oxide molecule is suggested.

INTRODUCTION

Cured meat products attain their bright red color when muscle myoglobin reacts with nitrite under the reducing conditions established in the curing process (Fox and Thomson, 1963; Fox and Ackerman, 1968; Killday et al., 1988). Heat processing transforms the initially formed MbNO into denatured nitrosylmyochrome, which provides products such as ham with the characteristic pink color. The different nitrosylheme pigments formed improve the oxidative stability of the product and have been found to inhibit lipid oxidation (Kanner et al., 1980, 1984). The role of the nitrosyl pigments as antioxidants is not well understood, although nitrosylmyoglobin (MbNO) has been suggested to act as a scavenger of oxygen radicals (Kanner et al., 1987). However, nitrosylmyoglobin and nitrosylmyochrome are both rather susceptible to oxidation, and cured meat products should, accordingly, be stored under conditions where pigment degradation is prevented to protect against development of rancidity (Skibsted, 1992). The pigment oxidation is easily recognized, since it causes fading of the bright red or pink color which is normally associated with different types of cured meat products. As for sliced products, because of the large surface area and the better contact with oxygen, color stability becomes particularly important, and the conditions for storage and retail-trade display should be optimized accordingly to minimize discoloration. For sliced ham, color fading during display in chill cabinets has been found to be caused by the combined action of light and oxygen. Therefore, the photochemistry of nitrosylmyoglobin and nitrosylmyochrome is of immediate interest in connection with food packaging (Andersen et al., 1988, 1990a). On exposure to light, even when the product is packed with a high initial vacuum, the residual oxygen present may cause photooxidation with concomitant discoloration and reduced general oxidative stability of the product. The color fading has been demonstrated to be a partly reversible process, indicating a role of residual nitrite and excess of ascorbate (Skibsted, 1992).

Although oxidation of MbNO has been the subject of several kinetic studies (Urbain and Jensen, 1940; Kampschmidt, 1955; Walsh and Rose, 1956; Bailey et al., 1964), the understanding of the mechanism of thermal activation and activation by light in the oxidation of MbNO is rather incomplete. To contribute to a clarification, we have investigated the kinetics of oxidation of both nitrosylmyoglobin and nitrosylmyochrome in aqueous model systems under different conditions of oxygen pressure and temperature and under illumination with monochromatic light of different wavelengths. The results of these model experiments, which are reported here, provide part of the information required for optimizing packaging conditions for cured meat products. Moreover, recent results obtained from storage experiments with sliced ham (Andersen et al., 1988, 1990a), together with results of related investigations (Lin and Sebranek, 1979; Lin et al., 1980; Acton et al., 1986; Feldhusen et al., 1986), provide a critical test of the applicability of the results obtained in the present study with chemical models.

MATERIALS AND METHODS

Nitrosylmyoglobin. Equine metmyoglobin (MMb: Sigma type III, dissolved in 5 mM equimolar $H_2PO_4^-/HPO_4^{2-}$ buffer, 100 mg/L) was purified on a Sephadex G-50 column (1.6 × 30 cm) by elution with 5 mM equimolar phosphate buffer (pH 6.8). The effluent myoglobin was diluted with the phosphate buffer to 0.20 mM, as monitored by absorbance measurement (Cary 219 spectrophotometer, ϵ_{525} = 7700).

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Ten milliliters of the 0.20 mM MMb solution was completely deoxygenated by passing N_2 gas through the solution for 2 h. The deoxygenated MMb solution was mixed anaerobically in an array of Zwickel flasks (Kuehn and Taube, 1976) with a deoxygenated $NaNO_2$ solution and a deoxygenated solution of ascorbate dissolved in 0.1 M $H_2PO_4^-/HPO_4^{2-}$ buffer (pH 6.4), resulting in concentrations of 0.10 mM MMb, 10 mM $NaNO_2$, and 20 mM ascorbate, respectively. This solution was protected from light and kept for 2 h at ambient temperature, during which time MMb was converted to MbNO. The MbNO solution was cooled to 0 °C in an ice bath and passed through a Sephadex G-25 (medium) column (2.0 × 30 cm) by elution with a degassed aqueous solution of 0.16 M NaCl solution at 4 °C to remove excess $NaNO_2$ and ascorbate. MbNO in the eluate was identified by its visible absorption spectrum (Kamarei and Karel, 1982) (Figure 1) and was used for kinetic or photochemical experiments the same day it was synthesized.

Nitrosylmyochrome. A solution of MMb in 5 mM $H_2PO_4^-/HPO_4^{2-}$ buffer, purified as described above, was adjusted to pH 5.6 with phosphoric acid to correct for the smaller concentration of ascorbic acid used. The MMb solution was diluted to 0.20 mM and completely deoxygenated; 10 mL of this solution was mixed with degassed solutions of $NaNO_2$ and ascorbate to yield concentrations of 0.10 mM MMb, 0.10 mM $NaNO_2$, and 1.76 mM ascorbate, respectively. The solution was protected from light, and the synthesis was completed overnight.

The solution of MbNO (0.10 mM MbNO and 1.7 mM ascorbate) was used either directly or heated to 72 °C in a water bath prior to kinetic or photochemical experiments. The heating procedure was intended to imitate the cooking procedure used in the meat industry for cured cooked meat products, resulting in denatured MbNO, which in the following will be denoted nitrosylmyochrome (dMbNO).

Other Materials. Freeze-dried Cu_2/Zn_2-superoxide dismutase (SOD) from *Saccharomyces cerevisia* was a gift from Dr. Morten Bjerrum. Catalase (Sigma) was from bovine liver (twice crystallized) in aqueous suspension containing 0.1% thymol. Analytical grade chemicals and doubly deionized water were used throughout. Dioxygen, dinitrogen, and nitric oxide of analytical grade and certified gas mixtures (O_2 + N_2) from Dansk Ilt & Brint, Inc., were used for the exchange experiments and experiments with controlled atmospheres.

Buffers and pH Measurements. Phosphate and acetate buffers were used for both kinetic and photolysis experiments, and ionic strength was adjusted with sodium chloride or sodium sulfate. pH was in each case measured relative to concentration standards in the actual salt medium (titrated solutions of strong acid), and the definition pH = −log [H^+] was employed for all kinetic and photolysis measurements. A Metronom 605 pH-meter and a combination glass electrode (Ingold type 405) were used for pH measurement with 3 M KCl in the reference part.

Kinetic Experiments. The solutions of (i) MbNO in 0.16 M NaCl, (ii) MbNO in 0.16 M NaCl in the presence of ascorbate, and (iii) dMbNO in 0.16 M NaCl in the presence of ascorbate were each mixed with buffer solutions to give an appropriate absorbance and a total buffer concentration of 25 mM. The ionic strength was adjusted with sodium chloride or sodium sulfate, and the solution viscosity was increased with glycerol in certain experiments. The reaction mixture was transferred to 2-cm silica cells and placed in a thermostated cell compartment of a Cary 219 spectrophotometer, and after temperature equilibrium was obtained, spectra were recorded at known times (450 nm < λ < 650 nm). The recorded spectra were read at four wavelengths, 500, 544, 580, and 630 nm. A Hewlett-Packard 8452A diode array spectrophotometer with a thermostated cell compartment with magnetic stirring was used for the kinetic experiment with reduced oxygen partial pressure and for $P(O_2)$ = 1 atm. After saturation of the solution with oxygen or the actual oxygen–nitrogen mixture for 30–60 min, a gentle gas flow of the same gas was maintained in the headspace, together with stirring of the solution, to ensure a constant oxygen concentration throughout the experiment.

For electrochemically monitored oxidation reactions, the MbNO solution (dissolved in 0.053 M Na_2SO_4 with 5 mM phosphate buffer and synthesized in a procedure in which sulfate had replaced chloride in the purification step) was air-saturated and transferred to a thermostated beaker, and a Radiometer nitrate ion Selectrode (F2412NO_3) in conjunction with a calomel reference electrode (Radiometer K 401, saturated KCl) was used to follow the formation of nitrate. The nitrate electrode was standardized in the actual medium using $NaNO_3$ dissolved in 0.053 M Na_2SO_4 for calibration. The change to the sulfate medium was necessary to avoid interference from chloride ions on the nitrate-electrode response.

Exchange Experiments. ^{15}N-Labeled nitrosylmyoglobin ($Mb^{15}NO$) was synthesized from equine metmyoglobin and $Na^{15}NO_2$ (99% ^{15}N, CEA-Oris), using the same procedure as described for unlabeled nitrosylmyoglobin. For each exchange experiment, MbNO was identified by its visible absorption spectrum, and the labeling was validated by $^{15}N/^{14}N$ isotopic analysis on half of the effluent MbNO solution (approximately 10 mL, 0.08 mM) following oxidation with 5 mL of H_2O_2 (15%) for 20 min. During the oxidation, the color of the solution bleached, as the pigment was oxidized, and NO was converted to NO_3^-. pH was subsequently adjusted to 4.0 with 0.1 M H_2SO_4, and the solution was frozen at −30 °C and freeze-dried overnight. The lyophilized residue was mixed with copper and copper(II) oxide and subjected to a high-vacuum Dumas destruction at 400 °C in a sealed glass tube, and the $^{15}N/^{14}N$ isotopic distribution was subsequently determined by optical emission spectroscopy (Johansen and Middelboe, 1982). The other half of the labeled MbNO solution was diluted with phosphate buffer (50 mM, pH 6.2) to yield a MbNO concentration of 3.4×10^{-5} M as determined spectrophotometrically, and 20 mL of the resulting solution was transferred to a reaction vessel and thermostated at 0 °C. Residual oxygen was removed by flushing for 1 h with N_2 (purified by passage through a chromium(II)/zinc scrubber). The oxygen-free $Mb^{15}NO$ solution was subsequently saturated with NO (purified for NO_2 by passage through nitrogen-saturated water), and after 5 min of reaction, the NO was removed by flushing vigorously with N_2 for 1 h. The exchange experiments were conducted in a closed system of glass equipment (connected with Teflon tubings), and oxygen was rigorously excluded. After exchange and removal of NO, absorption spectra of the MbNO solution were recorded for each experiment to ensure that no oxidation had occurred during the exchange. Five milliliters of MbNO solution was subsequently mixed with 5 mL of H_2O_2 (15%), and the isotopic analysis was performed as described above.

Continuous Wave Photolysis. Photolysis solutions were prepared according to the same procedure as for the solutions used in the kinetic experiments. The photolysis solutions were irradiated with monochromatic light and the extent of photooxidation was monitored at regular intervals by spectrophotometric measurements (450–650 nm plus irradiation wavelength, Cary 219 spectrophotometer). The photolysis solutions were either air-saturated and in contact with air during the photolysis or saturated with 100% N_2 or 100% O_2, and a gentle flow of gas was maintained in the headspace during photolysis. Photolysis was carried out in an optical train consisting of a light source, an Osram HBO 100/2 high-pressure Hg lamp, a light condenser, a heat filter, an interference filter, a precision shutter connected to an electronic timer, and lenses focusing the light into a thermostated photolysis cell (5.0 ± 0.5 °C for MbNO and MbNO in the presence of ascorbate and 13.2 ± 0.5 °C for dMbNO in the presence of ascorbate). The photolysis solution was stirred by means of a Teflon-coated magnetic bar. All parts and cells were of quartz (Spindler und Hoyer, Göttingen, GFR). Light intensities were determined by ferrioxalate actinometry (Hatchard and Parker, 1956) or for 546- and 578-nm light by Reinecke salt actinometry (Wegner and Adamson, 1966).

Calculation of Rate Constants. Rate constants for the thermal oxidation were calculated by nonlinear regression analysis, using a least-squares criterion for minimization. Four different wavelengths were included in each calculation for the spectrophotometric determinations of rate constants, and the quantity to be minimized was

$$\text{var} = \sum_{\lambda}\sum_{t} \frac{[A_{\lambda,t}(\text{obs}) - A_{\lambda,t}(\text{calc})]^2}{\sigma^2_A} \quad (1)$$

Determination of Photooxidation Quantum Yields. Photooxidation quantum yields (in moles per einstein) defined as

$$\Phi_{irr} = \frac{\text{no. of products formed}}{\text{no. of photons absorbed by NO complex}} \quad (2)$$

were calculated from the degree of conversion of nitric oxide complex to product from the spectrophotometric data (20% photoconversion in a typical experiment with MbNO and 5% photoconversion in experiments with dMbNO) in combination with the light intensity and the molar absorptivities for the nitric oxide complex and photoproduct at the wavelength of irradiation according to the following procedure.

$Q_{MbNO}(t_i)$ and $Q_{MMb}(t_i)$, the number of photons (in einstein) absorbed by nitrosylmyoglobin and metmyoglobin, respectively, were evaluated numerically by adding the light absorbed in small but finite time intervals $(t_i - t_{i-1})$

$$Q_{MMb}(t_i) = \sum_i \frac{(\gamma/V)\epsilon_{irr}^{MMb} l}{A_{irr}} I_0(1 - 10^{-A(irr)})(t_i - t_{i-1}) \quad (3)$$

$$Q_{MbNO}(t_i) = \sum_i [I_0(1 - 10^{-A(irr)})(t_i - t_{i-1})] - Q_{MMb}(t_i) \quad (4)$$

where γ is the number of moles of MbNO reacted, A_{irr} is the absorbance at the wavelength of irradiation, each at time $^1/_2(t_i - t_{i-1})$, V is the volume of the solution irradiated, l is the cell path length, ϵ_{irr}^{MMb} is the molar absorptivity of MMb at the wavelength of irradiation, and I_0 is the light intensity as determined by chemical actinometry. For a reversible photoreaction, the net reaction $\gamma(t_i)$ is related to the absorbed light by

$$\gamma(t_i) = \Phi^{MbNO} Q_{MbNO}(t_i) - \Phi^{MMb} Q_{MMb}(t_i) \quad (5)$$

in which Φ^{MbNO} and Φ^{MMb} are the reaction quantum yields for the oxidation and the reverse reaction, respectively. This method corrects rigorously for inner-filter effects (light absorption by photoproducts), and a zero slope in the linear relationship

$$\frac{\gamma(t_i)}{Q_{MbNO}(t_i)} = \Phi^{MbNO} - \Phi^{MMb}\frac{Q_{MMb}(t_i)}{Q_{MbNO}(t_i)} \quad (6)$$

indicates that the reverse photoreaction is insignificant and that subsequent photoreactions are negligible (see Figure 6).

RESULTS

When allowed to react with nitrite in aqueous solution in the presence of excess ascorbate, metmyoglobin (MMb) is converted quantitatively to the nitric oxide–iron(II) complex nitrosylmyoglobin (MbNO) (Kamarei and Karel, 1982). When this compound, synthesized either according to the method with equivalent concentration of MMb and nitrite (Kamarei and Karel, 1982) or with excess nitrite (see Materials and Methods) and identified by its visible absorption spectrum (Figure 1), was heated anaerobically, only minor spectral changes were observed. The spectral changes resulting from 10 min of heating to 72 °C of a 0.1 mM MbNO solution with excess ascorbate are shown in Figure 1. The minor spectral changes noted show that the iron(II) chromophore is almost unaffected by the heating process. Similar heat treatment of the iron(III) compound MMb likewise caused only minor spectral changes (Figure 1).

When a solution of MbNO, prepared under anaerobic conditions, is saturated with air, the characteristic red color of MbNO slowly changes to brown. The products of the autoxidation process were identified by spectrophotometric and electrochemical methods. The spectral changes observed as a result of the autoxidation process are shown in Figure 2. On admission of air into the solution, the initial MbNO spectrum is converted to the spectrum of MMb, and 95% of the coordinated NO was detected as NO_3^- by a nitrate-selective electrode (Figure 4). However, other changes in the molecule, besides the change in the oxidation state of the iron center from Fe(II) to Fe(III), are evident, since the product could be only

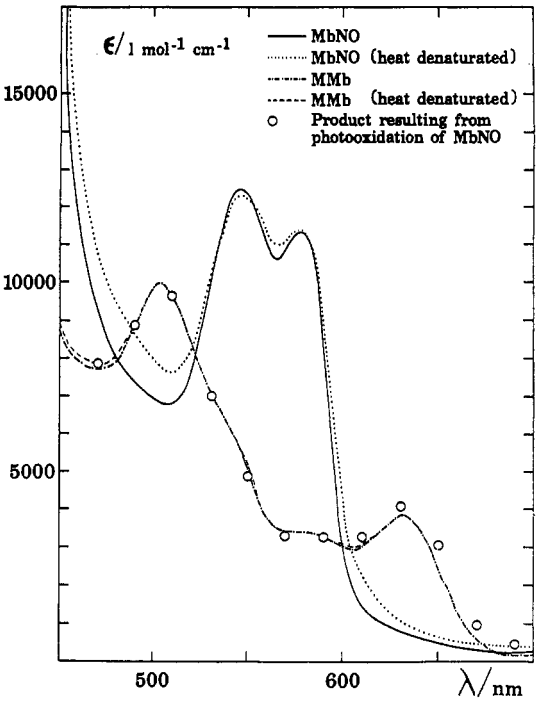

Figure 1. Absorption spectrum in the visible region of nitrosylmyoglobin and metmyoglobin in aqueous 0.16 M NaCl at ambient temperature. The spectrum of the heat-denaturated MbNO is recorded after MbNO is heated to 72 °C for 10 min under anaerobic conditions in the presence of 1.7 mM ascorbate. The spectrum of the heat-denaturated MMb is recorded after a similar heat treatment of a MMb solution. Also shown is the spectrum of the product resulting from photooxidation of MbNO, following excitation with monochromatic light (546 nm, see Table II), in air-saturated aqueous solution.

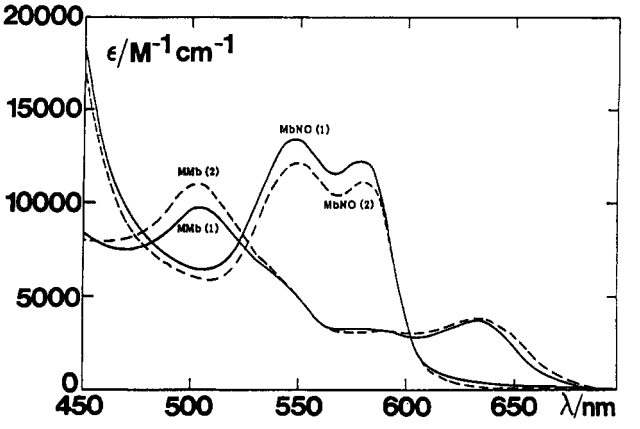

Figure 2. Spectral changes during formation of nitrosylmyoglobin [MbNO(1)] from metmyoglobin [MMb(1)] under anaerobic conditions in aqueous solution with pH 5.9, subsequent thermal oxidation in air-saturated solution to yield MMb(2), and partial regeneration of nitrosylmyoglobin [MbNO(2)] under reestablished anaerobic conditions. In the two steps involving synthesis of nitrosylmyoglobin, $NaNO_2$ and ascorbate were added to yield concentrations of 10 and 20 mM, respectively.

partly converted (approximately 85%) back to MbNO. Moreover, MMb formed as product in a second nitrosylation/oxidation cycle using the same solution and conditions (ascorbate, nitrite, pH) as in the first nitrosylation likewise showed further spectral deviation from the MMb formed in the first cycle. The spectral changes are most likely caused by concomitant oxidation or denaturation of the globin part of the pigment.

The rate of oxidation was determined spectrophotometrically in an aqueous sodium chloride solution under different conditions of temperature, pH, ionic strength,

Table I. Observed First-Order Rate Constants for Oxidation of Nitrosylmyoglobin in Aqueous Solution

	medium					
t, °C	I[a]	supporting electrolyte	pH	$P(O_2)$, atm	k_{obs}[b], s^{-1}	remarks
9.7	0.16	NaCl	5.9	0.21	$2.0(4) \times 10^{-5}$	only followed 1 half-life
19.2	0.16	NaCl	5.5	0.21	$9.0(2) \times 10^{-5}$	
19.2	0.16	NaCl	6.2	0.21	$8.4(2) \times 10^{-5}$	
19.2	0.05	NaCl	6.2	0.21	$8.7(2) \times 10^{-5}$	
19.3	0.16	NaCl	5.6	0.21	$9.5(5) \times 10^{-5}$	
19.3	0.16	NaCl	5.6	0.21	$9.2(2) \times 10^{-5}$	catalase[c]
19.3	0.16	NaCl	5.6	0.21	$9.3(3) \times 10^{-5}$	SOD[d]
19.3	0.16	NaCl	5.6	0.21	$9.3(2) \times 10^{-5}$	catalase[c] + SOD[d]
25.0	0.16	NaCl	5.8	0.21	$2.27(5) \times 10^{-4}$	
25.0	0.16	Na_2SO_4	5.2	0.21	$2.22(2) \times 10^{-4}$	
25.0	0.16	Na_2SO_4	5.2	0.21	$3.5(5) \times 10^{-4}$	see Figure 4; mean of three electrochemical determinations
25.0	0.16	Na_2SO_4	5.1	0.21	$3.4(3) \times 10^{-4}$ [e]	catalase,[c] determined electrochemically
30.0	0.16	NaCl	5.8	1.00	$5.10(2) \times 10^{-4}$	
30.0	0.16	NaCl	5.8	0.21	$5.05(2) \times 10^{-4}$	
30.0	0.16	NaCl	5.8	0.030	$4.8(3) \times 10^{-4}$	
30.0	0.16	NaCl	5.8	0.010	$3.0(2) \times 10^{-5}$ [e]	
30.0	0.16	NaCl	5.8	0.0020	$5.6(2) \times 10^{-6}$ [e]	only followed 1 half-life
35.5	0.16	NaCl	5.9	0.21	$1.08(2) \times 10^{-3}$	

[a] Ionic strength. [b] Determined spectrophotometrically (mean of at least two determinations, standard deviation in parentheses), except where otherwise noted. [c] 8800 units mL^{-1} in reaction mixture, as determined according to Sigma's procedure (Sigma, 1989); similar result was obtained with 17.000 units mL^{-1}. [d] Superoxide dismutase: 500 units mL^{-1}, as determined by the pyrogallol assay (Bjerrum, 1988). [e] Only a single determination.

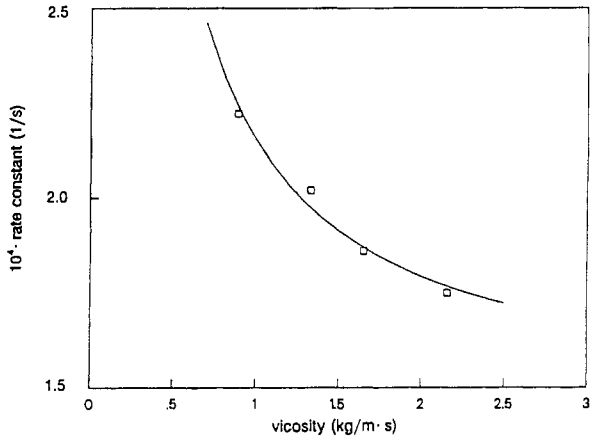

Figure 3. First-order rate constants determined spectrophotometrically for oxidation of MbNO in air-saturated water/glycerol mixtures (0, 2, 3, and 4 M glycerol in 0.16 M NaCl) at 25 °C as function of η, the dynamic viscosity (Miner and Dalton, 1953), according to the empirical equation $k = a + b/\eta$.

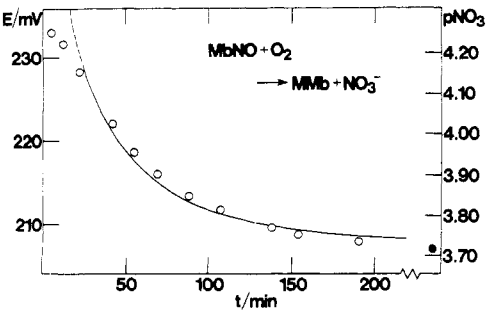

Figure 4. Oxidation of nitrosylmyoglobin in air-saturated 0.053 M Na_2SO_4 with pH 5.1 at 25 °C. The increasing nitrate concentration in a 2.08×10^{-4} M MbNO solution was monitored with a nitrate-selective electrode in conjugation with a calomel electrode. The full line is based on the equation $E(t) = c - 21.10 \ln(1 - \exp(-kt))$, from which the pseudo-first-order rate constant $k_{obs} = (2.9 \pm 0.2) \times 10^{-4}$ s^{-1} was calculated by nonlinear regression (χ^2 goodness-of-fit test). The pNO_3 axis is based on a non-Nernstian slope determined from a standard curve in the actual medium. The dashed line indicates the nitrate concentration region in which the electrode shows an almost constant potential (below detection limit). ● marks the overnight reading, from which $[NO_3^-]_\infty = (2.0 \pm 0.1) \times 10^{-4}$ M is calculated, corresponding to approximately 95% conversion of coordinated NO to NO_3^-.

solution viscosity, and oxygen partial pressure. The oxidation of chromatographically purified MbNO was found to be strictly first order in MbNO, and values for the observed first-order rate constants may be found in Table I. Solution pH and ionic strength had little, if any, effect on the rate of oxidation of MbNO used as a model for the different colored meat pigments in the range relevant for cured meat products. The presence of glycerol in a concentration up to 4 M only decreased the rate by 25% (Figure 3). The spectrophotometrically determined rate constant had the value $k_{obs} = (2.27 \pm 0.05) \times 10^{-4}$ s^{-1} at 25.0 °C for an air-saturated solution. Under similar experimental conditions, except for the substitution of chloride with sulfate, a nitrate-selective electrode showed that nitrate was formed during the oxidation process. The observation that 0.95 mol of NO_3^- was formed when 1 mol of pigment was oxidized (Figure 4) established the reaction stoichiometry as

$$\mathrm{MbNO} + \mathrm{O_2} \xrightarrow{k_{obs}} \mathrm{MMb} + \mathrm{NO_3^-} \qquad (7)$$

and the nitrate concentration is expected to increase according to a first-order rate expression:

$$[NO_3^-] = C^\circ_{MbNO}(1 - \exp(-k_{obs}t)) \qquad (8)$$

The Nernstian relation between the measured potential and the increasing nitrate concentration (in which f is an empirical correction factor) is

$$E = E^\circ - f(RT/F) \ln [NO_3^-] \qquad (9)$$

This expression yields, when combined with the first-order rate expression

$$E = c - f(RT/F) \ln (1 - \exp(-k_{obs}t)) \qquad (10)$$

The rate constant, calculated by nonlinear regression analysis from the variation of the electrode potential with time, as shown in Figure 4, had the value $k_{obs} = (2.9 \pm 0.2) \times 10^{-4}$ s^{-1} (experiment of Figure 4). Given the different salt media used, the agreement in results obtained by the two methods, notably monitoring reactant and product, respectively, is satisfactory, and since the nitrate formation, if different in rate, tends to be more rapid than conversion of MbNO, no significant concentrations of reaction in-

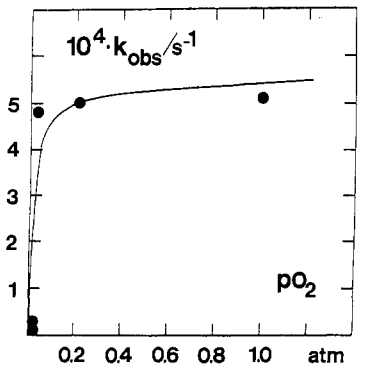

Figure 5. Observed first-order rate constant for thermal oxidation of nitrosylmyoglobin in aqueous solutions at different partial pressures of oxygen. Conditions: temperature, 30.0 °C; pH 5.8; ionic strength, 0.16, adjusted with sodium chloride. Full line calculated by nonlinear regression analysis according to the equation $k_{obs} = [P(O_2)K_{ass}/(1 + P(O_2)K_{ass})]k_1$ with $K_{ass} = 5 \pm 3$ atm^{-1} and $k_1 = (5.5 \pm 0.9) \times 10^{-4}$ L mol^{-1} s^{-1} (see text).

termediates accumulate during the course of the reaction (Table I). The presence of the enzymes catalase and superoxide dismutase did not affect the rate of oxidation, as monitored spectrophotometrically and electrochemically, showing that hydrogen peroxide and superoxide are not reaction intermediates as has been found for the oxidation of oxymyoglobin (Gotoh and Shikama, 1976). The presence of sulfamate ($H_2NSO_3^-$, 10 mM) had likewise no effect on the rate of formation of nitrate, excluding nitrite as an reaction intermediate.

The dependence of the first-order rate constant on the partial pressure of oxygen is shown in Figure 5 for 30.0 °C. As seen in Table I, the rate does not increase significantly between an air-saturated solution and a solution saturated with 1 atm of oxygen. At lower oxygen pressure, the rate decreases rapidly with decreasing oxygen partial pressure. This result is in agreement with the semiquantitative results reported for nitrosylhemoglobin (Urbain and Jensen, 1940). The dependence of rate on oxygen pressure can be accounted for by invoking an initial binding of oxygen to the pigment prior to monomolecular reaction:

$$MbNO + O_2 \rightleftarrows MbNO\cdots O_2 \qquad K_{ass} \qquad (11)$$

$$MbNO\cdots O_2 \rightarrow MMb + NO_3^- \qquad k_1 \qquad (12)$$

According to this mechanism, at least in the pressure region where Henry's law is valid, the rate of reaction of MbNO and the rate of formation of nitrate are related to the concentration of oxygen and MbNO by

$$-\frac{d[MbNO]}{dt} = \frac{d[NO_3^-]}{dt} = k_1\left[\frac{P(O_2)K_{ass}}{1 + P(O_2)K_{ass}}\right]C_{MbNO} \qquad (13)$$

in which

$$K_{ass} = [MbNO\cdots O_2]/[MbNO]P(O_2) \qquad (14)$$

The observed first-order rate constant

$$k_{obs} = P(O_2)K_{ass}k_1/(1 + P(O_2)K_{ass}) \qquad (15)$$

approaches the limiting value of k_1 at higher oxygen partial pressures.

A crude estimate of $K_{ass} = 5 \pm 3$ atm^{-1} was obtained by nonlinear regression as shown in Figure 5, and the limiting rate is almost reached in air-saturated solution. The rate constant determined in air-saturated solution is accordingly a good approximation of k_1. Accordingly, the temperature dependence of k_{obs} determined in air-saturated solution permitted deduction of the parameters of activation for the oxidation step by use of the Arrhenius equation (Figure 6) and transition-state theory. The enthalpy and entropy of activation given in Table II were not affected significantly by the presence of 1.7 mM ascorbate during the oxidation (Figure 6 and Table II). For oxidation in the presence of ascorbate, the rate constant was calculated for only 50% conversion to avoid influence from the subsequent slow reduction yielding oxymyoglobin (MbO_2).

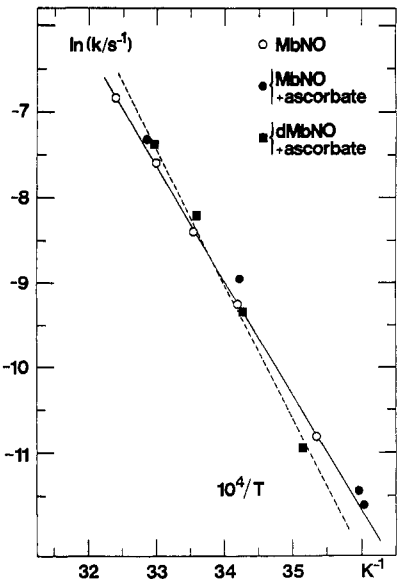

Figure 6. Temperature dependence of thermal oxidation of nitrosylmyoglobin in air-saturated 0.16 M NaCl plotted according to the Arrhenius equation. No difference between the regression line for nitrosylmyoglobin (MbNO: $\ln k = 37.06 - 1.354 \times 10^4/T$) and for the nitrosylmyoglobin in the presence of 1.7 mM ascorbate (MbNO + ascorbate: $\ln k = 37.28 - 1.355 \times 10^4/T$) was found (full line). However, the common regression line is different from the regression line for the heat-denatured nitrosylmyoglobin (dMbNO + ascorbate: $\ln k = 44.22 - 1.566 \times 10^4/T$), marked with a broken line.

$$2MMb + \text{ascorbate} + 2O_2 \rightarrow 2MbO_2 + \text{dehydroascorbate} \qquad (16)$$

The heat-denatured MbNO had, in contrast, a significantly higher enthalpy of activation (Figure 6 and Table II) for the oxidation. The presence of ascorbate was found to be essential to avoid precipitation of the heat-denatured nitrosylmyoglobin, nitrosylmyochrome, during the heat treatment. The role of ascorbate is not clear, although ascorbate may prevent oxidation during the heat treatment. However, the noted effect on the activation barrier is caused by the denaturation rather than by the presence of ascorbate as may be inferred from the result in Table II.

Isotopic labeling and optical emission spectroscopy were used to follow the exchange process

$$Mb^{15}NO + NO \rightarrow MbNO + {}^{15}NO \qquad (17)$$

to get a measure of the kinetic inertness of MbNO. In NO-saturated solution at 0 °C ($C_{NO} = 3.3 \times 10^{-4}$ M; Linke and Seidell, 1965), the enrichment of MbNO ($C_{MbNO} = 3.4 \times 10^{-5}$) was found, after 5 min of reaction, to decrease by $0.47 \pm 0.03\%$ from an initial value of $0.90 \pm 0.02\%$ ^{15}N (mean of two independent experiments). The initial enrichment is close to the theoretical value of 0.83% for nitrosylmyoglobin with one ^{15}NO label (Andersen et al., 1990b), and the decrease corresponds to a fraction of

Table II. Rate and Activation Parameters at 25 °C for Thermal Oxidation[a] and Quantum Yields[b] at 5 °C for Photooxidation of Nitrosylmyoglobin and Nitrosylmyochrome[c] in Aqueous Air-Saturated Solution[d]

	nitrosylmyoglobin	nitrosylmyoglobin + ascorbate[e]	nitrosylmyochrome + ascorbate[e]
k_{298}, s^{-1}	$(2.32 \pm 0.09) \times 10^{-4}$	$(2.8 \pm 0.2) \times 10^{-4}$	$(2.5 \pm 0.2) \times 10^{-4}$
$\Delta H^{\ddagger}$, kJ·mol^{-1}	110.1 ± 0.8	110 ± 4	128 ± 4
$\Delta S^{\ddagger}$, J·mol^{-1}·K^{-1}	55 ± 3	57 ± 15	114 ± 13
Φ_{366}, mol·einstein^{-1}	$(1.47 \pm 0.06) \times 10^{-3}$ [f]	$(1.5 \pm 0.1) \times 10^{-3}$	$(1.5 \pm 0.1) \times 10^{-3}$ [c]
Φ_{405}, mol·einstein^{-1}	$(1.11 \pm 0.04) \times 10^{-3}$ [f]	$(1.0 \pm 0.1) \times 10^{-3}$	$(1.2 \pm 0.2) \times 10^{-3}$ [c]
Φ_{436}, mol·einstein^{-1}	$(8.6 \pm 0.2) \times 10^{-4}$ [f]	$(1.0 \pm 0.1) \times 10^{-3}$	$(1.9 \pm 0.6) \times 10^{-3}$ [c]
Φ_{578}, mol·einstein^{-1}	$(5.7 \pm 0.3) \times 10^{-4}$ [f]	$(7.3 \pm 0.6) \times 10^{-4}$	$(1.0 \pm 0.4) \times 10^{-3}$ [c]

[a] Determined as shown in Figure 6. [b] Determined as shown in Figure 7, cf. Table III. For photolysis in the presence of ascorbate (1.7 mM), quantum yields were determined in experiment with approximately 5% photoconversion of the nitrosyl pigment to metmyoglobin. [c] Reaction quantum yields for nitrosylmyochrome were determined at 13 °C to avoid precipitation. [d] pH 5.8 ± 0.1, 0.020 M phosphate buffer, ionic strength adjusted to 0.16 with NaCl. [e] 1.7 mM ascorbate. [f] From Table III.

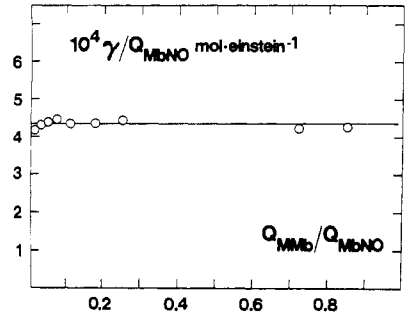

Figure 7. Determination of photooxidation quantum yield for MbNO in air-saturated aqueous 0.16 M NaCl with pH 5.8 at 5 °C. Wavelength of irradiation was 546 nm ($\epsilon_{546}^{MbNO} = 11\,500$ and $\epsilon_{546}^{MMb} = 5700$ L mol^{-1} cm^{-1}; see Figure 1), with $I_0 = 1.34 \times 10^{-7}$ einstein s^{-1} as determined by Reinecke-salt actinometry. The zero slope demonstrates that the photooxidation is nonreversible (see text). The last point corresponds to 98% conversion of MbNO to MMb.

exchange of $F = 1.03 \pm 0.07$ (number of exchanged ^{15}NO in each MbNO). Since the exchange is complete after 5 min of reaction and quenching of the exchange after shorter reaction time was not possible with the present experimental technique, only a lower limit for the rate constant for the exchange process can be estimated according to the McKay equation (Espenson, 1981):

$$\ln(1 - F) = -k_{obs}t = -R_{ex}[(C_{MbNO} + C_{NO})/(C_{MbNO}C_{NO})]t \quad (18)$$

Using the lower limit for the exchange ($F = 0.94$), limiting values of $k_{obs} \geq 1.0 \times 10^{-2}$ s^{-1} and $R_{ex} \geq 3.4 \times 10^{-7}$ mol L^{-1} s^{-1} for the rate constant and the rate of exchange are calculated, respectively.

In the presence of oxygen, light was found to increase the oxidation rate significantly. The product resulting from photooxidation of MbNO was identified spectrophotometrically as MMb, at least when visible light was used for excitation (Figure 1). The photooxidation appeared as a single reaction not complicated by subsequent thermal or photochemical reaction. This is demonstrated in Figure 7 for up to 98% photooxidation. The reaction quantum yield was determined at 5 °C to separate the thermal oxidation from the photooxidation, and the results for eight different wavelengths throughout the UV–visible range are given in Table III for air-saturated solutions. The reaction quantum yield provides an objective measure of the light sensitivity of MbNO, and from Table III it is seen that the wavelength dependence is only moderate. The photooxidation quantum yield was found to depend linearly on the oxygen pressure (Figure 8) and to increase with increasing viscosity (Table IV). Again, severe experimental difficulties were encountered at low oxygen pressures, and the results obtained at higher solution viscosity were difficult to reproduce. However, the qualitatively different oxygen pressure dependence and qualitatively different dependence on solution viscosity indicate a different reaction mechanism for the thermal oxidation and the photooxidation.

Table III. Quantum Yields for Photooxidation of Nitrosylmyoglobin in Air-Saturated Aqueous Solution at 5 °C[a]

λ_{irr},[b] nm	ϵ_{irr}^{MbNO},[c] L mol^{-1} cm^{-1}	Φ_{in},[d] mol einstein^{-1}	rel rate of photooxidation[e]
254	29 500	$(6.9 \pm 0.4) \times 10^{-3}$	41
313	14 700	$(2.25 \pm 0.10) \times 10^{-3}$	6.6
334	19 600	$(1.63 \pm 0.03) \times 10^{-3}$	6.4
366	24 000	$(1.47 \pm 0.06) \times 10^{-3}$	7.1
405	64 400	$(1.11 \pm 0.04) \times 10^{-3}$	14
436	40 800	$(8.6 \pm 0.2) \times 10^{-4}$	7.0
546	11 500	$(4.33 \pm 0.03) \times 10^{-4}$ [f]	1
578	10 600	$(5.7 \pm 0.3) \times 10^{-4}$	1.3

[a] pH 5.8 ± 0.1, 0.020 M phosphate buffer, ionic strength adjusted to 0.16 with NaCl. [b] Wavelength of irradiation. [c] Molar absorptivity of MbNO at wavelength of irradiation. [d] Photooxidation quantum yield. [e] Photooxidation rate (monochromatic illumination) relative to that for 546-nm illumination (yellow light) as calculated from rate(λ_{irr})/rate(546 nm) = $(\Phi_{irr}/\Phi_{546}) \times (\epsilon_{irr}^{MbNO}/\epsilon_{546}^{MbNO})$ [cf. Bertelsen and Skibsted (1987)]. [f] Determined as shown in Figure 7.

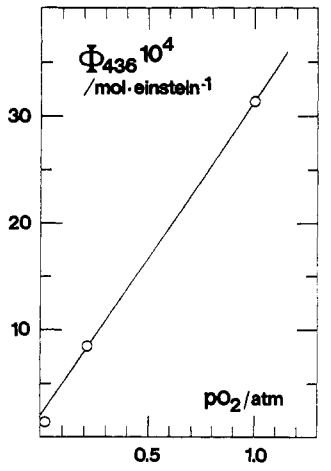

Figure 8. Quantum yield for photooxidation of nitrosylmyoglobin resulting from excitation with 436-nm monochromatic light as a function of oxygen pressure. The linear relationship has a slope of $(2.9 \pm 0.3) \times 10^{-4}$ mol einstein^{-1} atm^{-1} and an intercept of $(2 \pm 2) \times 10^{-4}$ mol einstein^{-1}.

The proportionality between the reaction quantum yield and the partial oxygen pressure is consistent with a mechanism in which an electronically excited state of MbNO produced by absorption of a photon

$$MbNO + h\nu \rightarrow MbNO^* \quad (19)$$

reacts in a bimolecular process with a ground-state oxygen (triplet state: $^3O_2$)

Table IV. Estimations of Quantum Yields for Photooxidation of Nitrosylmyoglobin in Air-Saturated Water/Glycerol Mixtures (0.16 M NaCl, pH 5.9) Excited with 578-nm Monochromatic Light[a]

glycerol, M	Φ, mol einstein^{-1}	glycerol, M	Φ, mol einstein^{-1}
0	5.7×10^{-4}[b]	3	12×10^{-4}
2	9.0×10^{-4}	4	10×10^{-4}

[a] Quantum yields determined relative to quantum yield in water. [b] See Table II.

$$MbNO^* + {}^3O_2 \rightarrow MMb + \text{other products} \quad (20)$$

in competition with deactivation to the ground state

$$MbNO^* \rightarrow MbNO + \text{heat} \quad (21)$$

The reaction quantum yield for reaction out of the excited state is the ratio between the rate of reaction and the sum of all rates deactivating the reactive state:

$$\Phi = k_{react}[O_2]/(k_{react}[O_2] + \sum k_{deact}) \quad (22)$$

The rate of oxidation is small as compared to the rate of deactivation ($k_{react}[O_2] \ll \sum k_{deact}$) for all of the experimental conditions employed, yielding small reaction quantum yields which are proportional to the oxygen pressure:

$$\Phi \approx k_{react}[O_2]/\sum k_{deact} = k'P(O_2) \quad (23)$$

The reaction quantum yield was not influenced by the presence of 1.7 mM ascorbate (Table II), and heat denaturation had no significant effect on the photooxidation either. The photolysis experiment with MbNO in the presence of ascorbate was not extended to wavelengths below 366 nm, where light absorption and photoreaction of ascorbate represent further complications, and photolysis was only followed up to 5% photoconversion to avoid complications from subsequent reaction of products with ascorbate. The heat-denatured pigment, dMbNO, precipitated slowly at 5 °C, and the photooxidation yield was accordingly determined at a higher temperature (13 °C).

DISCUSSION

Besides affording information relevant for mechanistic assignments for the oxidation of nitrosylmyoglobin, MbNO, and the heat denatured pigment of cured meat products, the results obtained in the present study are also of immediate interest for practical food packaging. The ensuing discussion will focus on both aspects. The oxidation reaction

$$MbNO + O_2(g) \rightarrow MMb + NO_3^- \quad (24)$$

is strongly energetically favored and has a net reaction free energy of $\Delta G° = -145$ kJ mol^{-1} at 25 °C, as may be calculated from available thermodynamic data (Linke and Seidell, 1965; Moore and Gibson, 1976; Brunori et al., 1971; Maloy, 1985; Hoare, 1985).

The rate of oxidation (Table II) is governed by an enthalpy barrier of $\Delta H^‡ = 110$ kJ mol^{-1} at 25 °C, which is partly counteracted by the favorable entropy of activation, $\Delta S^‡ = 55$ J mol^{-1} K^{-1}. Walsh and Rose (1956) have previously given an estimate of 92 kJ mol^{-1} for the energy of activation. To identify the mechanistic step in the overall reaction, which corresponds to this high activation barrier, a comparison with kinetic data for the closely related reaction might prove valuable:

$$MbO_2 + NO \xrightarrow{k'_2} MMb + NO_3^- \quad (25)$$

The rate of reaction for this "reverse" oxidation has been estimated at 25 °C (Doyle and Hoekstra, 1981) to have a second-order rate constant of 3.7×10^7 mol^{-1} L s^{-1}, corresponding closely to the rate obtained for the association of oxygen or nitric oxide with myoglobin. This observation of a very fast reaction, in which the rate-determining step apparently is the entering of NO into the heme pocket, could suggest that the establishment of an equilibrium between nitrosylmyoglobin and oxymyoglobin preceded a fast oxidation (the reverse reaction):

$$MbNO + O_2 \rightarrow MbO_2 + NO \qquad K_{O_2,NO} \quad (26)$$

The value of the equilibrium constant can be estimated to have the value $K_{O_2,NO} = 10^{-5}$ (Moore and Gibson, 1976; Armstrong and Sykes, 1986), and the equilibrium is thus strongly in favor of nitrosylmyoglobin. According to this mechanism, the rate of oxidation will, for moderate oxygen pressures, be approximated by

$$-d[MbNO]/dt = k'_2K_{O_2,NO}[O_2][MbNO] \quad (27)$$

and the observed first-order rate constant is, in the pressure range in which Henry's law is valid, expected to be proportional to the oxygen pressure:

$$k_{obs} = k'_2K_{O_2,NO}K_{Henry}P(O_2) \quad (28)$$

At $P(O_2) = 0.21$ atm and 25 °C, and with $K_{Henry} = 1.3 \times 10^{-3}$ mol L^{-1} atm^{-1} (Linke and Seidell, 1965), the observed rate constant is estimated to have the value $k_{obs} = 0.10$ s^{-1}. The observed value is, however, only 2.3×10^{-4} s^{-1} (Table II), leading to the following two conclusions: (i) The oxidation of nitrosylmyoglobin is not initiated by the substitution of nitric oxide by oxygen; (ii) the equilibrium constant for the substitution of nitric oxide by oxygen in myoglobin must have a value less than $K_{O_2,NO} = 2 \times 10^{-8}$, corresponding to a stronger binding of nitric oxide. Oxygen is thus not capable of replacing nitric oxide in a simple substitution step prior to the redox reaction. Dissociation of nitric oxide from nitrosylmyoglobin followed by oxidation of nitric oxide by oxygen

$$MbNO \rightarrow Mb + NO \quad (29)$$

$$NO + O_2 \rightarrow \text{oxidation products} \quad (30)$$

will show a limiting rate for increasing oxygen pressure equal to the rate of dissociation of nitric oxide. The rate constant for dissociation has indirectly been estimated to have the value $k_d = 10^{-4}$ s^{-1} (Moore and Gibson, 1976), very similar to the limiting value obtained in the present study, $k_1 = 2.3 \times 10^{-4}$ s^{-1}. However, the primary oxidation product of the reaction following dissociation is nitrite:

$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (31)$$

This reaction has been reported to be third-order and very fast, with a rate constant larger than 10^{10} mol^{-2} L^2 s^{-1} (Springborg et al., 1989).

$$-d[NO]/dt > 10^{10}[NO]^2[O_2] \text{ mol L}^{-1} \text{ s}^{-1} \quad (32)$$

Our findings indicate that nitrate is formed at a rate very similar to the rate of formation of MMb and in a 1:1 ratio. This result is not easily understood on the basis of this dissociative reaction mechanism, since it would involve formation of nitrate from nitrite and oxidation of Mb to MMb in processes that are both fast as compared to the initial dissociation, at least under the limiting conditions.

Moreover, the reaction, competing for nitrite

$$H_2NSO_2O^- + NO_2^- \rightarrow N_2 + SO_4^{2-} + H_2O \quad (33)$$

was not found to affect the rate of formation of nitrate. The Mb formed by dissociation of nitric oxide is furthermore expected to combine with oxygen.

$$Mb + O_2 \rightarrow MbO_2 \quad (34)$$

This reaction has a rate constant $k_f = 2 \times 10^7$ mol^{-1} L s^{-1} and is very rapid as compared to the dissociation of nitric oxide from MbNO. The rate of autoxidation of MbO_2 to MMb has a rate constant of $k_{obs} = 2 \times 10^{-5}$ s^{-1} at pH 5.8 and would become rate determining for the production of MMb. Oxidation of Mb or MbO_2 to MMb is also expected to involve the superoxide ion or hydrogen peroxide as reaction intermediate and thus to be sensitive to the presence of superoxide dismutase or catalase, in contrast to our findings.

The rate of exchange of NO in MbNO ($R_{ex} \geq 3.4 \times 10^{-7}$ mol L^{-1} s^{-1} at 0 °C) found by isotopic labeling

$$Mb^{15}NO + NO \rightleftarrows MbNO + {}^{15}NO \quad (35)$$

is likewise not understandable on the basis of a limiting dissociative mechanism. The rate-determining step for a dissociative exchange reaction

$$MbNO \rightarrow Mb + NO \quad (36)$$

corresponds to the rate law

$$R_{ex} = k_1^{ex} C_{MbNO} \quad (37)$$

from which a lower limit for the first-order rate constant of $k_1^{ex} \geq 1.0 \times 10^{-2}$ s^{-1} at 0 °C is calculated. The fact that the value of this first-order rate constant is several orders of magnitude greater than the observed first-order rate constant for oxidation of MbNO in air-saturated solution ($k_{obs} = 2.3 \times 10^{-4}$ s^{-1} at 25 °C) excludes NO dissociation from MbNO as a common rate-determining step for the two reactions. For both the exchange process in MbNO and the oxidation of MbNO, a certain degree of association has accordingly to be assumed for the transition state of the rate-determining step. As for exchange, a bimolecular process corresponds to the rate expression

$$R_{ex} = k_2^{ex} C_{MbNO} C_{NO} \quad (38)$$

from which a lower limit for the second-order rate constant of $k_2^{ex} \geq 3.0$ L mol^{-1} s^{-1} at 0 °C is calculated. For the exchange process, $\Delta H^{\ddagger} = 47$ kJ mol^{-1} has been determined from temperature dependence of NO exchange in MbNO dissolved in nitrite/ascorbate solution (Andersen et al., 1990b), and a combination of this value for the enthalpy of activation with the value for the rate constant yields a moderately negative entropy of activation ($\Delta S^{\ddagger} \geq -70$ J mol^{-1} K^{-1}), which is consistent with an associative transition state for the exchange process.

For the oxidation of MbNO, we then arrive at a reaction mechanism in which oxygen does not replace nitric oxide nor does nitric oxide dissociate prior to oxidation. For complexes like MbNO in which the FeNO group is bent, it has been argued that a substantial transfer of electron density from the metal center to the nitrosyl group (π-back bonding) is responsible for the strong binding of nitric oxide (Nocek et al., 1988).

$$\{Fe(II)\cdots NO\} \leftrightarrow \{Fe(III)\cdots NO^-\} \quad (39)$$

Binding of oxygen in the heme pocket prior to a rate-determining rearrangement

$$\{Fe(III)\cdots NO^- + O_2\} \rightleftarrows \{Fe(III)\cdots NO^-\cdots O_2\} \quad (40)$$

$$\{Fe(III)\cdots NO^-\cdots O_2\} \rightarrow Fe(III) + NO_3^- \quad (41)$$

accommodates the kinetic observations, including the fact that the rate of transformation of MbNO to MMb is similar to the rate of nitrate formation. The binding of oxygen is rather weak, and the rate-determining step is an addition of oxygen to the negatively charged nitrosyl ligand. The activation barrier for the final rearrangement is substantial and sensitive to heat denaturation of the polypeptide chain and may involve assistance from amino acid residues in the heme pocket. A unimolecular elementary reaction as rate-determining step for the oxidation of MbNO also accounts for the lack of dependence on rate of ionic strength and pH and for a moderate dependence on solution viscosity.

The most notable observation for the photochemical oxidation is the modest dependence of the quantum yield on the wavelength of excitation, which is in contrast to the strong wavelength dependence reported for the photooxidation of MbO_2 (Bertelsen and Skibsted, 1987). The dramatic wavelength dependence for the photooxidation of MbO_2 ($\Phi_{254}/\Phi_{546} = 2300$) indicates that excited states of different parentage (ligand field states and charge-transfer states) are likely to be involved in the photooxidation of MbO_2. For MbNO, the ratio Φ_{254}/Φ_{546} is only 16 in favor of a common reaction excited state, populated by efficient intersystem crossing from the excited state initially populated by light absorption. MbNO is a low-spin d^6 electronic system, and although the exact nature of the reactive state is unknown, it is likely to involve a substantial lengthening of the Fe–NO bond, e.g., a triplet ligand field state with a σ-antibonding e_g orbital populated. The lowest energy for this excited state involves pentacoordination of the iron(II) center, with NO (or NO$^-$) trapped in the heme cavity. Under anaerobic conditions, NO reenters the coordination sphere of iron upon deactivation of the excited state. However, with the presence of oxygen in or near the heme cavity, chemical reaction is competing with binding of NO to Fe(II):

$$Fe\text{–}NO \overset{h\nu}{\rightleftarrows} Fe\text{- - -}NO \quad (42)$$

$$Fe\text{- - -}NO + O_2 \rightarrow \text{primary reaction products} \quad (43)$$

Although the non-heme primary reaction product(s) could not be identified, the first-order dependence of oxygen partial pressure can be accounted for by this mechanism. The increase in reaction quantum yield with solution viscosity reflects the decrease in mobility of O_2 in escaping from the heme cavity.

The thermal oxidation has a large temperature dependence, and in Figure 9 the relative rate of photooxidation resulting from monochromatic light of four different wavelengths of particular abundance in fluorescence light is compared to the rate of thermal oxidation at different temperature. Photooxidation is seen to be dominant at temperatures below room temperature even for visible light, and it may be concluded that cured meat products should be protected against light in the presence of oxygen to avoid discoloration resulting from oxidation of MbNO. This prediction, which can be made from Figure 9, has been confirmed in storage experiments with sliced ham (Andersen et al., 1988, 1990a). The wavelength dependence for photooxidation is small compared to that for MbO_2, and in contrast to what has been found for frozen

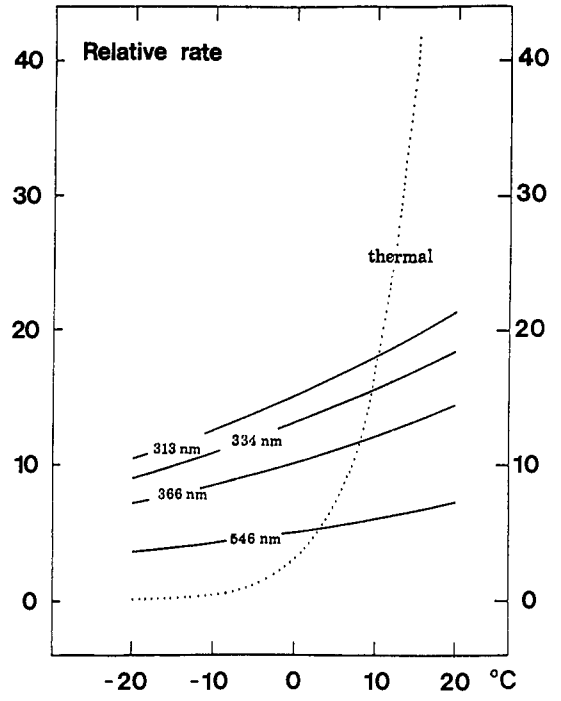

Figure 9. Rate of competitive thermal and light-induced oxidation of nitrosylmyoglobin (relative to thermal autoxidation at 1 °C) calculated for a model air-saturated aqueous MbNO solution illuminated by monochromatic light, with indicated wavelengths of particular intensity in fluorescent light (an approximate intensity of 5 mW mL^{-1} was used for the calculation). Photooxidation is dominant at temperatures below room temperature even for visible light.

beef (Andersen et al., 1989) no improvement is to be expected in the color stability of cured meat by application of UV-light barriers in the packaging material. For vacuum-packaged cured meat, reappearance of the cured meat color after fading depends on the presence of residual nitrite and excess of ascorbate (Skibsted, 1992). The result of Figure 2 provides an illustration of this important phenomenon and shows that a complete reappearance is not to be expected. However, the data provided in the present study are only part of the information required for a dynamic description of the processes in real meat systems, and further work is in progress.

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