

STABILITY OF THE NITRITE-CURED PIGMENT NITROSYLHMYOGLOBIN TOWARDS ELECTRON ACCEPTORS. MODELLING THE OPTIMAL PACKAGING GAS COMPOSITION FOR CURED MEAT

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Background

The oxidative stability of nitrite-cured meat products towards rancidity was recognized many years ago (1). However, the mechanism by which nitrosylmyoglobin (MbFe^{II}NO) may exert a radical inactivating action is still not well known. This bright red coloured heme ferrous iron complex of NO was first recognized as the pigment formed in meat following addition of nitrite curing process (2), and has been studied in relation to meat colour stability (3). More recently, MbFe^{II}NO has also been described as a potential radical scavenger preventing pathophysiological conditions such as arteriosclerosis or apoptosis [(4,5)], and MbFe^{II}NO is known to act as a chain-breaking antioxidant in model systems (6,7).

The oxidation of deoxymyoglobin (MbFe^{II}) or oxymyoglobin (MbFe^{II}O₂) by various transition metal complexes has been the subject of various studies related to formation of the physiological inactive metmyoglobin, MbFe^{III}, under conditions of oxidative stress (8,9). The dissociation of oxygen from MbFe^{II}O₂ ($k_{\text{diss}} \approx 10 \text{ s}^{-1}$) was found to be the rate-determining step in the oxidation by other transition metal complexes. For the oxidation by ferricyanide, Fe(CN)₆³⁻, under anaerobic conditions of various mutants of MbFe^{II} a marked difference in the second order rate constant, k_2 , was observed for wild-type ($10^6 \text{ M}^{-1} \text{ s}^{-1}$) in comparison to mutants, where His64 is substituted for other amino acids (Phe, Gly, Val or Leu; k_2 ranging from 8.7 to $13.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) (9). This difference was ascribed to an alteration in the interaction between water and distal amino acid residues leading to changes in the accessibility to the central iron atom. Comparison of the reaction rates of Fe(CN)₆³⁻ with MbFe^{II}/MbFe^{II}O₂ and MbFe^{II}NO, respectively, may elucidate further how the nitrosylated complex acts in radical scavenging processes and serves as an antioxidant.

Objectives

Two individual experimental sections have been carried out:

- Investigations of the reaction rate between nitrite-cured pigment, MbFe^{II}NO, and the electron acceptor, Fe(CN)₆³⁻
- Studies of the effect of varying gas composition, especially O₂/CO₂-levels, on thermal oxidation of MbFe^{II}NO

Methods

Kinetic experiments: MbFe^{II}NO was synthesised as described previously by Andersen & Skibsted (3). Stock solutions of potassium hexacyanoferrate(III) (K₃Fe(CN)₆) were prepared and used for stopped-flow spectrophotometric measurements after dilution to required concentration. Solutions of MbFe^{II}NO (either 100 or 50 mM) and the electron acceptor both in 10 mM phosphate buffer (pH = 7.0, I = 0.16 M) were placed in separate syringes of the DX-17MV stopped-flow spectrofluorometer equipped with a diode array detector for recording spectra for global analysis and a grating monochromator coupled to a photo multiplier for recording of changes at single wavelengths. For all measurements sample light path was 10 mm. Reactions were followed by recording spectra in the wavelength region 480 < λ < 670 nm with 2 nm intervals or by monitoring changes in absorbance maximum for MbFe^{II}NO at 548 nm. For all measurements pseudo first order kinetics were established by having the electron acceptor in at least 10 times excess relative to MbFe^{II}NO. Reaction kinetics between MbFe^{II}NO and Fe(CN)₆³⁻ was investigated for different concentrations of electron acceptor at 15°C in order to estimate the second order reaction rate constant, k_2 .

The reaction of MbFe^{II}NO with molecular O₂ at varying gas composition (O₂: 1.5% and CO₂: 0, 20 and 90% balanced with N₂) was investigated on a HP8452A UV-vis diode array spectrophotometer with a thermostatted cuvette holder and magnetic stirring. Solutions was saturated with gas mixture by purging for 30 min, before spectral changes were recorded, while a gentle gas flow was maintained in the headspace of the cuvette.

Numerical analysis: Spectral changes during reaction were analysed using singular value decomposition (SVD) followed by non-linear regression utilizing the Levenberg-Marquardt algorithm of the programme SPECFIT Global Analysis System Version 3.0.30. Global analysis was either carried out using a kinetic model with a single exponential decay or a model with two consecutive decay steps corresponding to a single first order reaction or a bi-exponential reaction with two consecutive first order reactions, respectively. Absorbance values recorded at a single wavelength were fitted to either single or bi-exponential decay models build-in the software of the Applied Photophysics workstation DX-17MV, which apply the Marquardt-Levenberg algorithm for non-linear fitting.

Results and Discussion

The presence of Fe(CN)₆³⁻ increased the rate of oxidation of MbFe^{II}NO as compared to the rate of simple autoxidation, and the heme containing reaction product was shown by visible spectroscopy to be MbFe^{III}. For excess of Fe(CN)₆³⁻ the reaction shows simple first order kinetics and observed pseudo first order rate constant depends linearly on the concentration of excess of Fe(CN)₆³⁻. The global analysis with singular value decomposition (SVD) showed that only two species MbFe^{II}NO and MbFe^{III} were present during the reaction (Figure 1a). In order to obtain rate constants, the reaction was monitored at λ = 548 nm and a single exponential decay expression was used in curve fitting, as shown in Figure 2 from which it also can be seen that residuals tend to increase as the excess Fe(CN)₆³⁻ is increased.

A plot of the observed pseudo first order rate constant, k_{obs} , for oxidation of MbFe^{II}NO as a function of [Fe(CN)₆³⁻] was linear under the conditions studied (inset, Figure 2), indicating that the reaction is first order in both MbFe^{II}NO and Fe(CN)₆³⁻. The general expression in Eq. 1 can be used to derive the second order rate constant, k_2 .

$$k_{\text{obs}} = k_{\text{auto}} + k_2[\text{Fe(CN)}_6^{3-}] \quad [1]$$

The intercept was found to be $4 \pm 7 \times 10^{-4} \text{ s}^{-1}$ and should be equal to k_{auto} , the rate of autoxidation, of MbFe^{II}NO ($2.0 \times 10^{-5} \text{ s}^{-1}$ at 10°C and pH 5.9 [(3)]). The value at 15°C for k_{auto} from Eq. 1 does not differ significantly from this value (or from zero), while the second order rate constant for MbFe^{II}NO oxidation by Fe(CN)₆³⁻ has the value $1.67 \pm 0.1 \text{ M}^{-1} \text{ s}^{-1}$. The parameters observed for reaction between MbFe^{II}NO and Fe(CN)₆³⁻ indicate an alternative reaction mechanism in comparison to oxidation of MbFe^{II}O₂ or MbFe^{II} by Fe(CN)₆³⁻.

For the autoxidation of MbFe^{II}NO at varying CO₂ partial pressure (pCO₂) a model with two consecutive reactions had to be employed in order to accommodate the experimental data. This means that an intermediate species is present during the autoxidation of MbFe^{II}NO, and the exact nature of this myoglobin species still needs to be identified. It is possible that the initial reaction step between MbFe^{II}NO and O₂ as electron acceptor, or possibly the unknown intermediate are affected by varying pCO₂, thus resulting in either an overall stabilizing or increased rate of discoloration of the nitrite-cured pigment in modified atmosphere packages.

Conclusion

It was shown that the one-electron acceptor, Fe(CN)₆³⁻, reacted with a moderate rate constant towards MbFe^{II}NO, and the identified second order rate constant indicates that NO dissociation is not the rate determining step. However, it is likely that an initial oxidation of the central iron from ferrous to ferric form will labilize the binding of NO to the complex, thus resulting in degradation into the product MbFe^{III} yielding a dull grey colour. Furthermore, investigation are undertaken to study the possible effect of varying CO₂ concentration on the stability of MbFe^{II}NO towards reaction with molecular O₂ as electron acceptor in relation to modified packaging employing high levels of CO₂.

References

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Data

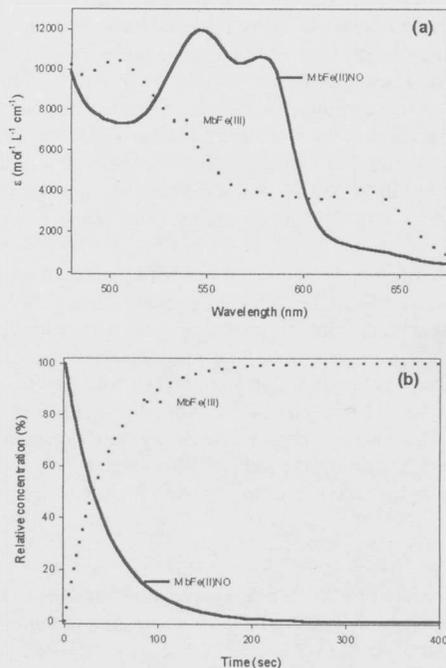


Figure 1. Global analysis of MbFe^{II}NO and Fe(CN)₆³⁻ reaction showing resolved spectral profiles (a) and time profiles for myoglobin species (b).

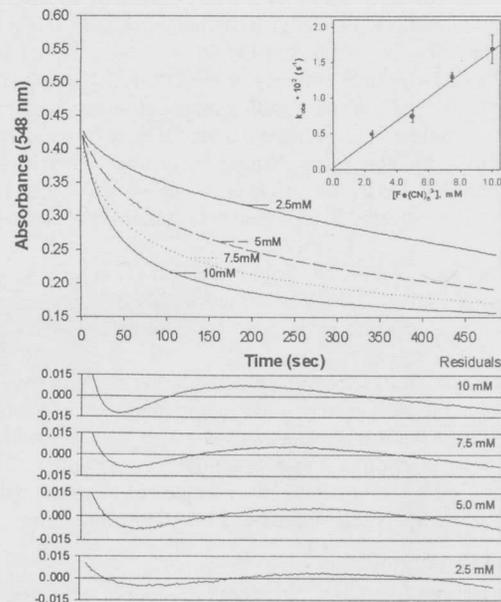


Figure 2. Spectral changes of MbFe^{II}NO for varying concentration of Fe(CN)₆³⁻ with corresponding residuals and plot of pseudo first order rate constants (inset).