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Deactivation of hypervalent meat pigments. Kinetics of reduction of ferrylmyoglobin by nitrite and iodide

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Abstract

The hypervalent heme pigment ferrylmyoglobin, MbFe(IV)=O, a potential pro-oxidant in meat products, is deactivated efficiently by the antioxidant nitrite to a nitrite complex of metmyoglobin, MbFe(III). The second-order rate constant for direct reduction in aqueous 0.16 M NaCl with pH = 7.4 was found to have the value $k_2 = 13.9 \pm 0.4$ M^{-1} s^{-1} at 25°C with the activation parameters $\Delta H^{\#} = 30 \pm 2$ kJ mol^{-1} and $\Delta S^{\#} = -123 \pm 7$ J $mol^{-1} \times K^{-1}$. Iodide, an example of a simple spheric electron donor, is less efficient with $k_2 = 0.34 \pm 0.01$ M^{-1} s^{-1} as the result of a higher enthalpy of activation ($\Delta H^{\#} = 43 \pm 3$ kJ mol^{-1} and $\Delta S^{\#} = -109 \pm 8$ J mol^{-1} K^{-1}). Although nitrite, like iodide, thus belongs to a class of deactivators, for which intra-molecular electron transfer to form a cation radical $^{+\cdot}$MbFe(III)–O^- is rate-determining and characterized by a negative entropy of activation, it differs from the spheric iodide, by inducing conformation changes in the protein to lower the barrier of activation. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

The hypervalent myoglobin species ferrylmyoglobin (MbFe(IV)=O) and perferrylmyoglobin ($^{+\cdot}$Mb Fe(IV)=O) are powerful pro-oxidants formed by reaction of MbFe(II)O_2 and MbFe(III) with hydrogen peroxide (George & Irvine, 1954, 1956; Whitburn, 1987; Yusa & Shikama, 1987). They are known to initiate peroxidation of lipids (Kanner & Harel, 1985a,b) and to induce oxidative damage to proteins (Hanan & Shaklai, 1995; Irwin, Østdal & Davies, 1999; Østdal, Daneshvar & Skibsted, 1996). Ferrylmyoglobin and perferrylmyoglobin are deactivated by reaction with antioxidants such as flavonoids and ascorbate (Jørgensen & Skibsted, 1998; Kröger-Ohlsen & Skibsted, 1997; Laranjinha, Almeida & Madeira, 1995; Laranjinha, Vieira, Almeida & Madeira, 1996) or, in the absence of external electron donors, by the so-called "autoreduction" reaction, where ferrylmyoglobin is slowly transformed to metmyoglobin by pathways leading to oxidation of the globin protein and to heme-protein crosslinking (King & Winfield, 1963; Tajima & Shikama, 1993).

The mechanism for deactivation of hypervalent myoglobin is largely unknown, although detailed studies have been undertaken of the reduction of ferrylmyoglobin by NADH (Mikkelsen & Skibsted, 1995), β-lactoglobulin (Østdal, et al., 1996), ascorbate (Kröger-Ohlsen & Skibsted, 1997) and chlorogenate (Carlsen, Kröger-Ohlsen, Bellio & Skibsted, 2000), consistently confirming that protonated ferrylmyoglobin (pK_a~5) reacts faster than the unprotonated form. The activation parameters are rather similar for the reduction of the unprotonated form of ferrylmyoglobin by the different reductants, suggesting that an intra-molecular electron transfer in ferrylmyoglobin might be a common rate-determining step for reduction of the unprotonated form of ferrylmyoglobin (Kröger-Ohlsen & Skibsted, 1997). For ascorbate and chlorogenate, complex-binding of the reducing compound with ferrylmyoglobin prior to reduction by a second molecule, has been found to occur in parallel with direct reaction, and recently it was found that reduction of ferrylmyoglobin by thiocyanate proceeds via a reaction intermediate that may be a protein radical stabilized by binding of a thiocyanate (Kröger-Ohlsen & Skibsted, 1999). The present study of reduction of ferrylmyoglobin by the antioxidant nitrite was undertaken to provide further insight into the mechanisms by which ferrylmyoglobin is

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deactivated. Nitrite is of special interest for meat systems, since the mechanism by which nitrite prevents lipid oxidation in cured meats is still highly speculative (Arendt, Skibsted & Andersen, 1997). However, one possible mechanism is deactivation of pro-oxidants such as hypervalent heme proteins. Iodide was included in the study as an example of a simple spheric electron donor, from which further mechanistic insight for the more complex reductants like nitrite and ascorbate is expected by a comparison of the activation parameters. Iodide is also of practical relevance, since fortification of salt to be used also for curing of meat is becoming mandatory in some countries.

2. Materials and methods

Metmyoglobin (MbFe(III), horse heart, type III) was purchased from Sigma Chemical Co. (St. Louis, MO). 0.00200 M HCl was from Bie & Berntsen Laboratory (Bie & Berntsen A/S, Rødovre, Denmark), and sodium nitrite, potassium iodide, and other chemicals (all of analytical grade) were from Merck (Darmstadt, Germany). The concentration of H_2O_2 (8 mM stock solution) was determined spectrophotometrically ($\varepsilon_{240} = 39.4$ M^{-1} cm^{-1} (Nelson & Kiesow, 1972). Water was purified through a Millipore Q-Plus purification train (Millipore Corp., Bedford, MA). Metmyoglobin purification and preparation of ferrylmyoglobin were carried out as previously described and a Sephadex G25-column (Pharmacia Biotech AB, Uppsala, Sweden) was used for purification of MbFe(III) instead of Sephadex G-50 (Kröger-Ohlsen & Skibsted, 1999); ferrylmyoglobin concentration was determined according to Rice-Evans & Miller (1994). pH was measured relative to concentration standards (0.0100 and 0.00100 M HCl, ionic strength 0.16 adjusted with NaCl), employing the definition pH = −log [H^+] throughout.

2.1. Product identification

The heme product of the reaction between MbFe(IV)=O and iodide or nitrite was identified spectrophotometrically (HP8452 and HP8453 UV-vis diode array spectrophotometers, Hewlett–Packard, Palo Alto, CA). MbFe(IV)=O was prepared by mixing solutions to yield (final concentrations) 95 μM MbFe(III) and 95 μM hydrogen peroxide (pH = 7.4, 25°C, $I = 0.16$). For nitrite as reductant, NO_2^- (13 mM in final solution) was added after 10 min and, after a further 2 min, a 2.5 ml aliquot of the myoglobin-nitrite-solution was passed through a PD-10 Sephadex G-25 column (Pharmacia Biotech AB, Uppsala, Sweden). Another aliquot of the solution was diluted to match the myoglobin concentration resulting from the chromatographic procedure assuming an isosbestic point at 525 nm. The experiment was repeated with MbFe(III) (98 μM) instead of MbFe(IV) = O. The reaction between NO_2^- and MbFe(III) was also studied using the stopped-flow kinetic technique and global analysis in order to identify the product resulting from the reaction between NO_2^- and MbFe(IV)=O (Fig. 5).

2.2. Kinetic experiments

Solutions of I^- or NO_2^- were freshly prepared each day to yield the final conditions, pH = 7.40 ± 0.05 (20 mM phosphate buffer), $I = 0.16 \pm 0.01$ (NaCl), in mixture with ferrylmyoglobin. The concentration of I^- or NO_2^- was in excess relative to MbFe(IV)=O by at least a factor of 10 in all experiments. For reactions with I^-, cuvettes containing 1.50 ml I^- solution were thermostatted in the cell holder of an HP 8453 UV-vis diode array spectrophotometer (Hewlett–Packard, Palo Alto, CA) before addition of 1.50 ml prethermostatted MbFe(IV)=O solution, and recordings of spectra of stirred solutions were started after mixing. The reaction temperature was measured in the cuvettes after completion of the reaction, and pH was subsequently measured in thermostatted samples of the reaction mixtures. For reactions with NO_2^-, MbFe(IV)=O solution and a buffer solution containing NO_2^- were placed in each syringe of a DX17MV stopped-flow spectrofluorometer (Applied Photophysics, London, UK), and the reaction was followed by absorbance measurements at 580 nm, except for a product identification experiment, where the stopped-flow spectrofluorometer was equipped with a diode array detector. For this particular experiment (spectra shown in Fig. 2 see below) a global analysis was made in order to ensure that only one reaction is contributing to the observed kinetics. pH was measured in thermostatted 1:1 mixtures of the MbFe(IV)=O and NO_2^- solutions for each combination of NO_2^- concentration and temperature.

2.3. Data analysis

Pseudo-first order rate constants k_{obs} for reduction of MbFe(IV)=O by I^- were calculated using the PC Pro-K Global Analysis software obtained from Applied Photophysics Ltd. (Applied Photophysics, London, UK). For reduction by NO_2^-, k_{obs} were calculated from the absorbance change at 580 nm; k_{obs} and concentration data for reduction of MbFe(IV) = O by NO_2^- were transformed logarithmically prior to linear regression analysis to yield the second-order rate constant to stabilize the variance.

3. Results

Reduction of MbFe(IV) = O by I^- could for excess of iodide, be described by (pseudo) first-order kinetics

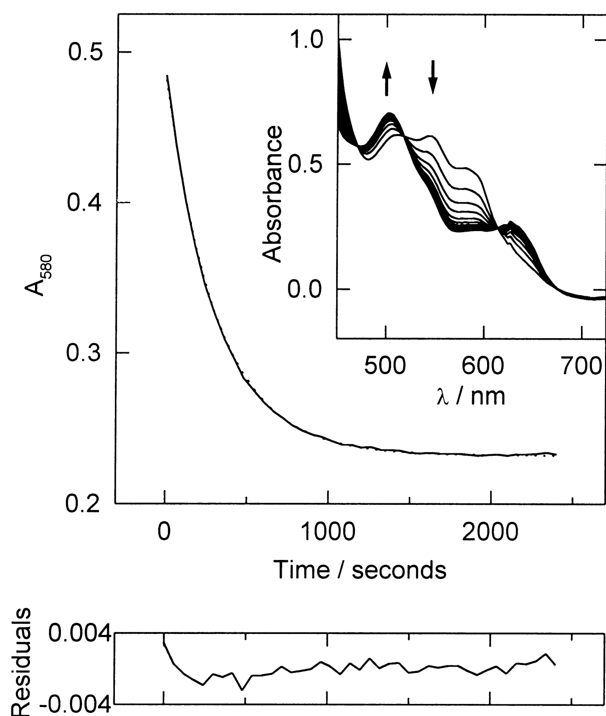


Fig. 1. Reaction between 3.7×10^{-5} M MbFe(IV)=O and 0.013 M I^- at pH 7.4 (0.020 M phosphate buffer) and 21.0°C at ionic strength 0.16 (NaCl) as followed by absorption spectroscopy at 580 nm. Dotted line: First-order decay calculated according to the expression $A_\lambda(t)=a_\lambda+b_\lambda$ exp($-k_{obs}t$), with the rate constant $k_{obs}=3.214\times10^{-3}\pm3\times10^{-6}$ s^{-1} as calculated by global analysis for 251 wavelengths in the spectral range 450–700 nm (residuals at 580 nm shown in lower panel). Insert: absorption spectra from the same experiment recorded with 120 s interval. Forty minutes elapsed from initiation of the reaction to recording of the final spectrum. Arrows indicate direction of spectral changes during the reaction.

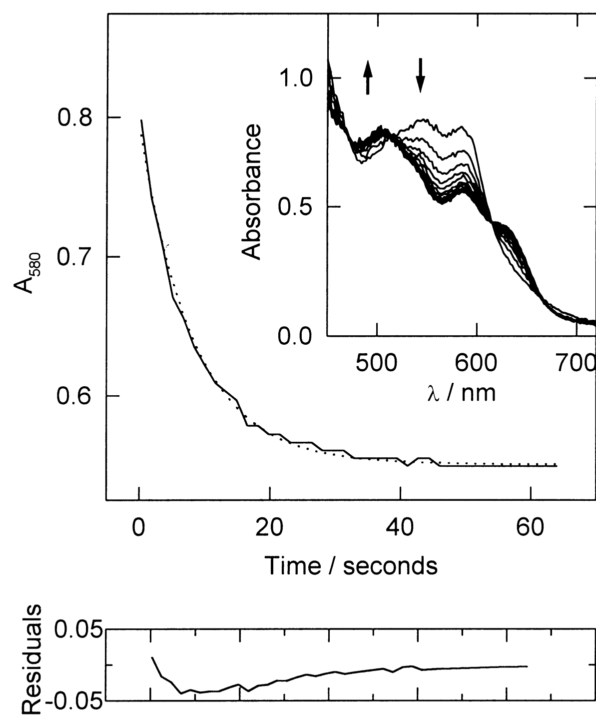


Fig. 2. Reaction between 4.9×10^{-5} M MbFe(IV)=O and 0.010 M NO_2^- at pH 7.4 (0.020 M phosphate buffer) and 24.8°C at ionic strength 0.16 (NaCl) followed by stopped-flow absorption spectroscopy at 580 nm. Dotted line: first-order decay calculated according to the expression $A_\lambda(t)=a_\lambda+b_\lambda$ exp($-k_{obs}t$), with the rate constant $k_{obs}=0.1198\pm0.0001$ s^{-1} as calculated by global analysis for 135 wavelengths in the spectral range 450–733 nm (residuals at 580 nm shown in lower panel). Insert: absorption spectra from the same experiment recorded with 1.8 s interval. Sixty-four seconds elapsed from initiation of the reaction to recording of the final spectrum. Arrows indicate direction of spectral changes during the reaction.

(Fig. 1), and the reaction product can be identified spectrally as MbFe(III) (Andersen, Bertelsen & Skibsted, 1988). Reduction of MbFe(IV)=O by NO_2^- also followed (pseudo) first order kinetics for conditions of excess NO_2^-, and the final absorption spectrum from the reaction between MbFe(IV)=O and NO_2^- showed strong resemblance to the spectrum of MbFe(IV)=O reduced by NO· (Gorbunov, Osipov, Day, Zayas-Rivera, Kagan & Elsayed, 1995), in which case a one-electron reduction of MbFe(IV)=O to MbFe(III) is suggested (Fig. 2). However, the product spectrum is clearly different from that of MbFe(III) but similar to the spectrum of MbFe(III) reacted with NO_2^-. When MbFe(III) is allowed to react with excess of NO_2^-, MbFe(III) is converted (at least partly) into a nitrite complex:

$$\mathrm{MbFe(III)}^{z+} + \mathrm{NO_2^-} \rightleftarrows \mathrm{MbFe(III)NO_2}^{(z-1)+} \qquad (1)$$

for which a dissociation constant $K_d=3.3$ mM is reported (Sono & Dawson, 1982). This complex formation was studied spectrophotometrically, and, as may be seen in Fig. 3, the equilibration can be described by a (pseudo) first-order reaction. The product spectra for reaction between MbFe(III) and NO_2^- and between MbFe(IV)=O and NO_2^- are seen (compare Figs. 2 and 3) to be very similar, but to depend on the concentration of excess NO_2^- in agreement with an equilibrium distribution between MbFe(III) and its nitrite complex. For the experimental conditions of Fig. 3, it is calculated from $K_d=3.3$ mM that approximately 30% of MbFe(III) is present as the nitrite complex. The complex formation caused very small changes in the absorption in the Soret band (results not shown). The reversibility of the reaction of nitrite with MbFe(III) was further demonstrated by size exclusion chromatography. When a product solution from reduction of MbFe(IV)=O with NO_2^- or from reaction of MbFe(III) with NO_2^- was passed through a Sephadex G-25 column on which NO_2^- is effectively separated from the heme pigments, the spectra corresponding to a mixture of MbFe(III) and its nitrite complex were converted into a MbFe(III) spectrum (Fig. 4).

For both I^- and NO_2^-, the observed first-order rate constant k_{obs} depended linearly on the concentration of

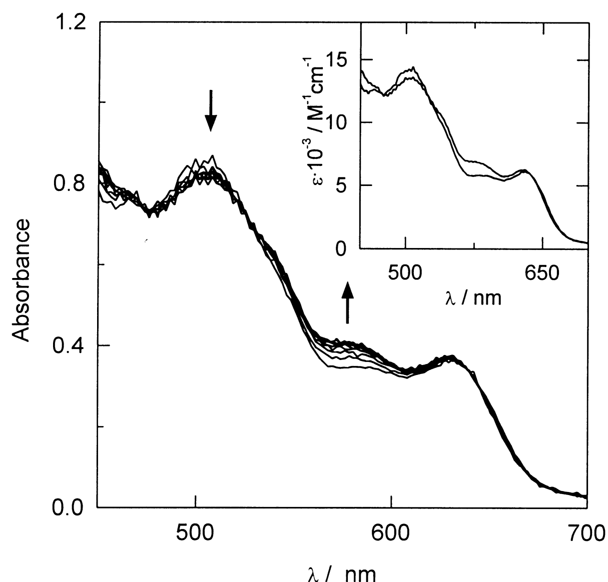


Fig. 3. Rection between 7.5×10^{-5} M MbFe(III) and 10 mM NO_2^- as followed spectrophotometrically using the stopped-flow technique with photodiode array detection. First spectrum was recorded 3.8 ms after mixing, the subsequent spectra with 3.8 ms interval and the final spectrum after 0.46 s. The kinetics could (by global analysis) be described by one first order reaction with $k_{obs}=8.35\pm0.54$ s^{-1}. The initial spectrum and the spectrum of the product solution are shown in the insert. Arrows indicate direction of spectral changes.

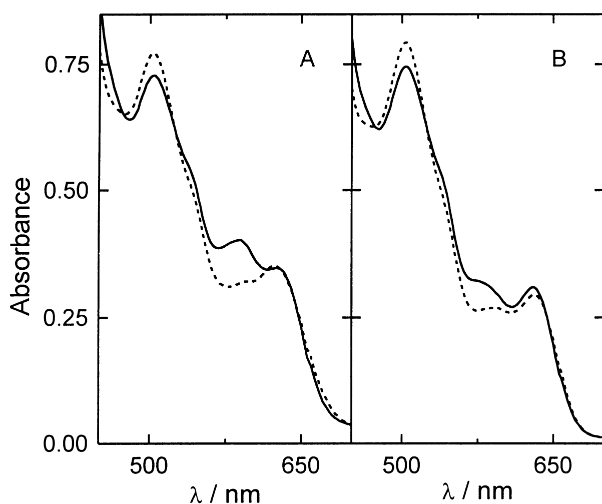


Fig. 4. Absorption spectra of products resulting from reaction of nitrite with heme proteins ($\sim1\times10^{-4}$ M). A: MbFe(IV)=O + NO_2^- (13 mM) before (full line) and after (dashed line) passing through a Sephadex-G25 column (pH = 7.4, 25°C, I = 0.16). B: As in A, but MbFe(III) substituted for MbFe(IV)=O.

the reducing compound (Fig. 5) in agreement with the expression

$$k_{obs} = k_{auto} + k_2 \text{ [reducing compound]} \quad (2)$$

where k_2 is the second-order rate constant for reduction of ferrylmyoglobin by the reducing compound, and

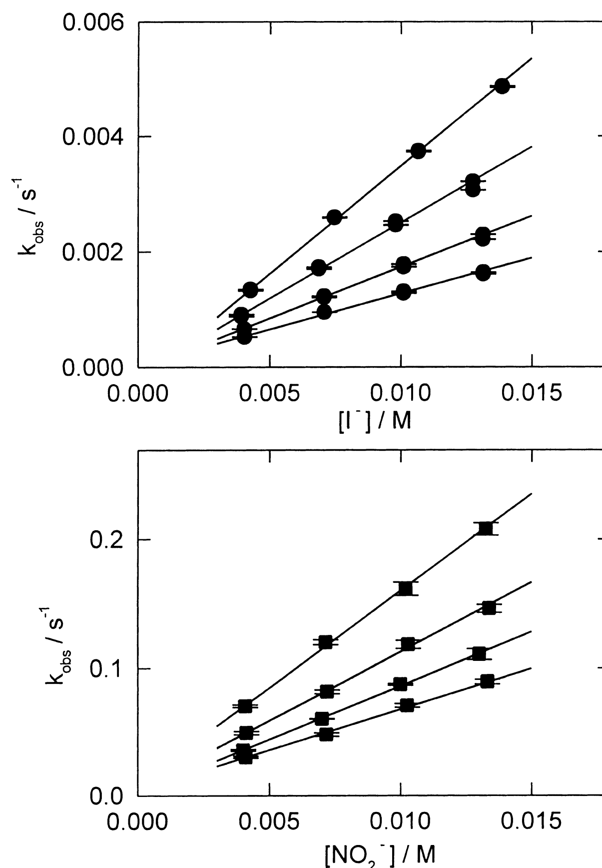


Fig. 5. Observed pseudo-first order rate constants k_{obs} for reduction of MbFe(IV)=O by I^- and NO_2^- at different temperatures (26.8, 20.3, 14.4, and 8.1°C for I^-, shown from top to bottom, and 26.3, 20.0, 14.0, and 8.0°C for NO_2^-, shown from top to bottom), pH = 7.4, I = 0.16. Second-order rate constants k_2 were obtained by linear regression analysis according to the expression $k_{obs}=k_{auto}+k_2$ [reducing compound], where k_{auto} is the rate constant for autoreduction of MbFe(IV)=O. For I^-, k_{obs} measured in individual exeriments are plotted together with the line fitted by linear regression analysis; NO_2^- data are shown as mean values together with the line fitted by linear regression analysis after logarithmic transformation of data.

k_{auto} is the rate constant for parallel autoreduction of ferrylmyoglobin (approximately 2×10^{-4} s^{-1} at pH 7.4 and 25°C (Mikkelsen & Skibsted, 1995). Nitrite is seen to be a more efficient reductant than iodide. The temperature dependence for both reactions could be described by the Arrhenius equation in the temperature interval 8–27°C, and linear regression analysis of the second-order rate constants for four temperatures as a function of temperature (Fig. 6) resulted in the activation parameters shown in Table 1.

4. Discussion

Nitrite reduces MbFe(IV)=O to MbFe(III), which partly reacts with excess of nitrite to form a nitrite complex. The reduction is slower than complex formation

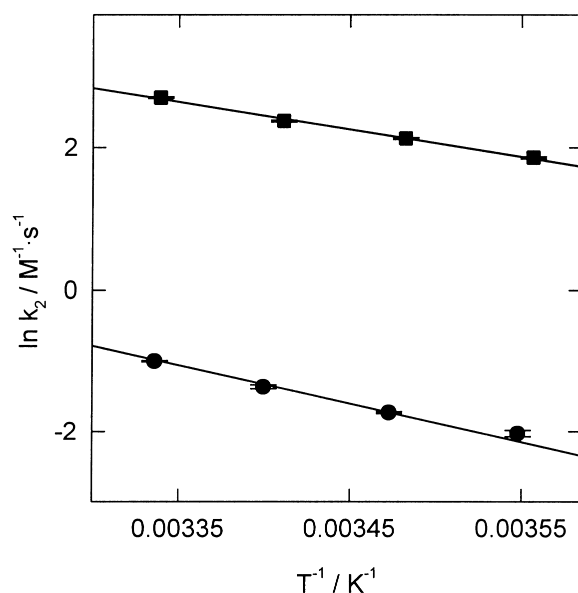
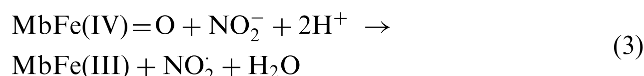


Fig. 6. Arrhenius plots for reduction of MbFe(IV)=O by I^- (●) and NO_2^- (■), from which the activation parameters $\Delta H^{\#}=43\pm3$ kJ mol^{-1} and $\Delta S^{\#}=-109\pm8$ J (mol^{-1} K^{-1}) for I^- and $\Delta H^{\#}=30\pm2$ kJ mol^{-1} and $\Delta S^{\#}=-123\pm7$ J (mol^{-1} K^{-1}) for NO_2^- were calculated.

by a factor of approximately 40, as seen from a comparison of Fig. 3 and Fig. 5, and complex formation has consequently no effect on the observed kinetics of the reduction. The reduction of MbFe(IV)=O is a one-electron transfer and the following stoichiometry is suggested for the primary reaction step:



$$\text{MbFe(IV)}{=}\text{O} + NO_2^- + 2H^+ \rightarrow \text{MbFe(III)} + NO_2^{\cdot} + H_2O \quad (3)$$

Table 1
Rate constants and activation parameters for deactivation of MbFe(IV)=O by different reducing compounds in aqueous solution at neutral pH and 25.0°C

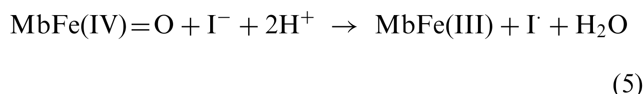
Reducing compound	Rate constant (M^{-1} S^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})
Iodide[a]	0.34 ± 0.01	43 ± 3	−109 ± 8
Nitrite[a]	13.9 ± 0.4	30 ± 2	−123 ± 7
Ascorbate[b,c]	2.95 ± 0.7	–	–
β-Lactogolbulin[d]	1.2 ± 0.1	45 ± 6	−93 ± 20
Thiocyanate[e]	0.119 ± 0.006	45 ± 6	−113 ± 19
Chlorogenate[c]	216 ± 50	73 ± 8	41 ± 30
Apigenin[f]	125 ± 6	69 ± 1	23 ± 4
Rutin[f]	105 ± 1	65 ± 3	13 ± 9

[a] Present work.
[b] Kröger-Ohlsen & Skibsted, 1997.
[c] Carlsen et al., 2000.
[d] Østdal et al., 1996.
[e] Kröger-Ohlsen & Skibsted, 1999.
[f] Jørgensen & Skibsted, 1998.

most likely followed by a disproportionation of the primary oxidation product, eventually to yield nitrate:

$$2NO_2^{\cdot} + H_2O \rightleftarrows NO_2^- + NO_3^- + 2H^+ \quad (4)$$

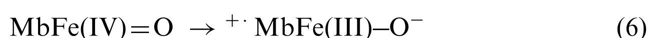
and by complex formation [reaction of Eq. (1)]. Iodide was not found to bind to MbFe(III) like NO_2^- (see Fig. 1), and the reaction stoichiometry is accordingly



$$\text{MbFe(IV)}{=}\text{O} + I^- + 2H^+ \rightarrow \text{MbFe(III)} + I^{\cdot} + H_2O \quad (5)$$

followed by a combination of iodine radicals to yield an equilibrium mixture of I_2 and I_3^- in the medium of excess I^-.

NO_2^- is clearly the most efficient reductant at ambient temperature despite the fact that, thermodynamically, NO_2^- is a poorer reductant ($E^o = +0.88$ V for N_2O_4 to yield NO_2^-) than I^- ($E^o = +0.54$ V for I_2 to yield I^-) (Lide, 1995). The difference in rate can be assigned to differences in activation enthalpy rather than differences in activation entropy (Table 1). Previous studies of deactivation of the unprotonated form of MbFe(IV)=O, dominating at neutral pH, have shown that certain substrates are oxidized by MbFe(IV)=O at remarkably similar rates, resulting from very similar activation parameters ($\Delta H^{\#}\sim45$ kJ mol^{-1}, $\Delta S^{\#}\sim-100$ J mol^{-1} K^{-1}), despite the very different natures of these substrates (Table 1). It seems likely that a common activation step is rate-determining for these reactions, possibly an intramolecular electron transfer in MbFe(IV)=O yielding a ferric protein radical as the reactive species (Kröger-Ohlsen & Skibsted, 1997), as has been shown for microperoxidase-8 (Low, Winkler & Gray, 1996):



$$\text{MbFe(IV)}{=}\text{O} \rightarrow {}^{+\cdot}\,\text{MbFe(III)–}O^- \quad (6)$$

The activation parameters found in the present study, for reduction of MbFe(IV)=O by I^-, further substantiate the hypothesis of a common activation step, as they lie in the previously established range (Table 1). However, reduction of MbFe(IV)=O by NO_2^- is about an order of magnitude faster at 25°C (favoured by a lower activation enthalpy). It is noteworthy that the entropy of activation is consistently negative and around −100 J mol^{-1} K^{-1} as also for NO_2^-. This common value could be accounted for by an increased solvation of the protein radical cation, independent of the actual reductant. NO_2^- belongs to this class of reductants, but has a lower enthalpy of activation, and it is suggested that NO_2^-, maybe through binding near the heme cleft (as in MbFe(III), see Fig. 4), lowers the energy barrier for intramolecular electron transfer, which, in the (common) rate determining step, precedes fast reduction of the protein radical cation by (another) nitrite.

Notably, iodide does not induce such specific effects in the protein, and it seems simply to act as an electron donor to the protein radical, not affecting the rate of intramolecular electron transfer.

However, iodide may catalyze other redox reactions in meat, which should be further explored in view of the increased use of salt fortified with iodide in meat curing.

Other important antioxidants clearly deactivate MbFe(IV)=O by other mechanisms. Parameters of activation for reduction are now available for three plant phenols (Table 1). Although they all have large enthalpies of activation, the positive entropy of activation in each case makes the plant phenols very efficient as reductants. This could be understood through (hydrophobic) binding of the phenols to the heme protein, modulating the reactivity of the heme protein towards external reductants. This important aspect of antioxidant interaction and the role of plant phenols is in focus in our recent research.

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