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ARTICLE

Reduction of ferrylmyoglobin by cysteine as affected by pH

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Reduction of the hypervalent meat pigment ferrylmyoglobin, MbFe(IV)=O, by cysteine is enhanced by acid due to protonation of ferrylmyoglobin to yield sulfmyoglobin as the main product, while at alkaline conditions, the rate decreases with the cysteine dianion as reactant forming oxymyoglobin, MbFe(II)O₂. The second-order rate constant for cysteine reacting with protonated ferrylmyoglobin is $5.1 \pm 0.4 \text{ l mol}^{-1} \text{ s}^{-1}$ at 25°C in 0.16 M aqueous sodium chloride and for the cysteine dianion reacting with ferrylmyoglobin $0.31 \pm 0.15 \text{ l mol}^{-1} \text{ s}^{-1}$. For pH = 7.4 the activation parameters for sulfmyoglobin formation is $\Delta H^\ddagger = 75 \pm 2 \text{ kJ mol}^{-1}$ and $\Delta S^\ddagger = -250 \pm 7 \text{ J mol}^{-1} \text{ K}^{-1}$ with similar values for homocysteine and glutathione. The difference in product is indicative of a shift from an electron-transfer/radical addition mechanism at low pH as in the stomach to two-step electron-transfer mechanism at higher pH as in the intestine, and is discussed in relation to protection against formation of radicals by sulphurous compounds during digestion of red meat.

Introduction

Hypervalent iron is formed in the digestive tract during digestion of meat by activation of metmyoglobin (MbFe(III)) by reactive oxygen species in the gut e.g. fatty acid hydroperoxide (2) and *in vitro* also by hydrogen peroxide (3) forming the very short lived perferryl compound ($\cdot\text{MbFe(IV)=O}$) and subsequently the more long-lived ferrylmyoglobin (MbFe(IV)=O) (1,2,3,4). Both $\cdot\text{MbFe(IV)=O}$ and MbFe(IV)=O are known to be radical initiators of lipid oxidation and protein oxidation in meat and meat products during meat processing and storage affecting product quality negatively (4,2,3). Such radical mechanisms may also operate during digestion of red meat, and besides being radical initiators these hypervalent heme pigment may also liberate free iron forming reactive oxygen species in the gut. Red meat has been suggested to be one of a main causes in the development of colon cancer (3,7), and research are actively carried out to understand the toxic effects of reactive oxygen species (ROS) generated by hypervalent iron and how this can be counteracted by combining the intake of red meat with foods rich in natural antioxidants (8).

Some thiols and hydrogen sulfide are known to react with MbFe(IV)=O and to form sulfmyoglobin (SulfMb) which is a protein with a modified chlorine type tetrapyrrole structure with a sulphur atom added to the pyrrole B ring of the porphyrin (10,11). SulfMb can, like myoglobin, exist as SulfMbFe(II) and SulfMbFe(III) with iron in different oxidation states (12, 13). Cysteine is among the most reducing amino acids and may play a role in the protection of epithelial cells against radical attack, both as a free amino acid and incorporated into peptides like

glutathione and larger proteins. Cysteine and glutathione are thus known to have cytoprotective effects against acute renal failure due to myoglobin toxicity, a condition that depends on iron catalyzed peroxidation (14). As previously reported, the products formed by reduction of MbFe(IV)=O by cysteine depends on pH (9,15). However, the complex structure of the data obtained by UV-Vis spectroscopy invited for the use of multivariate curve resolution (MCR) in order to identify intermediate and reaction products for different conditions of pH.

Based on the extracted component spectra and the time concentration profiles of the absorbing components in the reactions mixture several reactions mechanisms were suggested (9). Accordingly, a more focused kinetic study has been initiated in order to identify reaction pathways under different pH-conditions and to determine rate constants for the dominating reactions for discussion of reaction mechanisms behind protection against hypervalent heme pigments by cysteine and cysteine derivatives and homologues.

Materials and Methods

Chemicals

L-cysteine was obtained from Merck (Darmstadt, Germany). Glutathione, L-homocysteine, and hydrogen peroxide (30% v/v) were obtained from Sigma-Aldrich (Steinheim, Germany) and used without further treatment. Catalase from bovine liver and myoglobin from horse heart (purity > 90 %) was obtained from Sigma-Aldrich (Steinheim, Germany). Myoglobin was purified according to procedures

previously described (9,11). Acetic acid was from AppliChem GmbH (Darmstadt, Germany). K_2HPO_4 and KH_2PO_4 were of analytical grade and supplied by J. T. Baker (Phillipsburg, NJ, US). Deionized water was obtained using a Milli-Q system Millipore Co. (Billerica, MA, US).

Reaction kinetics

Aqueous L-cysteine stock solutions ($1.5 \times 10^{-2} \text{ mol L}^{-1}$) were prepared in appropriate buffers. Ferrylmyoglobin was prepared from reaction of metmyoglobin ($1 \times 10^{-4} \text{ mol L}^{-1}$) with H_2O_2 in a proportion of 1:2. Hydrogen peroxide excess was consumed by addition of catalase as previous described (9,11). Kinetic measurements at pH 6.0, 6.5, 7.0, 7.4, and 8.2 were performed using appropriate aqueous buffer using a two-compartment 1 cm x 1 cm quartz cell for fast mixture (Hellma Analytics, Müllheim, Germany) and UV-Vis spectra recorded from 200 nm to 800 nm at different time intervals in an Multiskan GO Microplate Spectrophotometer (ThermoScientific, Bremen, Germany) thermostatted at 20.0, 25.0, or 30.0 °C. Kinetic measurements at pH 4.5, 5.0, 5.6, and 6.2 were performed using appropriate aqueous buffer using a SX 20 sequential mixing stopped-flow spectrophotometer (Applied Photophysics Ltd, Leatherhead, UK). Ferrylmyoglobin is unstable under acid conditions (pH 4.5-6.2), and kinetic data were obtained using a pH-jump technique. In acidic medium, the reaction was initiated by mixing a ferrylmyoglobin solution in a pH 7.4 phosphate buffer with low ionic strength with an acidic cysteine solution in acetate buffer at pH 4.5, 5.0, and 5.6 or phosphate buffer at 6.2 and 6.5 with an ionic strength of 0.32 M yielding a final ionic strength of 0.16 M at the desired pH. pH was measured in the reaction mixture using a combined glass electrode pH-meter model PHM240 (Radiometer Analytical, Copenhagen, Denmark). The formation of sulfmyoglobin was monitored at 625 nm and the decay of ferrylmyoglobin was monitored at 580 nm under pseudo-first order conditions for increasing concentration of cysteine. The auto-decay rate constant for ferrylmyoglobin was determined by mixing equal volumes of ferrylmyoglobin in low ionic strength buffer solution at pH 7.4 and an acid buffer at ionic strength 0.32 M resulting in a final 0.16 M ionic strength at the desired pH. The auto-decay followed first-order kinetics. The first-order rate constants were obtained by non-linear regression (Matlab R2012a, Mathworks, Natick MA, US). Concentration/time profiles were modelled as previously reported (9) in agreement with different reactions scheme using Levenberg-Marquardt algorithm in an optimized toolbox at Matlab R2012a.

Results and Discussion

Ferrylmyoglobin, MbFe(IV)=O, is known to be reduced by cysteine (15), and both metmyoglobin, metMbFe(III), and sulfmyoglobin, sulfMbFe(II), have been detected as the heme reaction product using UV-vis spectroscopy to monitor the reactions, see Figure 1. Using multivariate curve resolution (MCR) of UV-vis spectra for reaction mixture of MbFe(IV)=O and cysteine for conditions of excess of cysteine in varying concentrations, it was previously detected that the product formed for low pH conditions was sulfMbFe(II) with an increasing contribution from metMbFe(III) for higher pH (9). The spectral profile and product assignment obtained by MCR analysis provide the proposed pathways for the reduction of MbFe(IV)=O by cysteine under different pH conditions (pH 6, 7, 8 and 9) as illustrated in Scheme 1. For pH 6, 7, and 8,

MbFe(IV)=O reacted with cysteine forming mainly sulfMbFe(II) accordingly to the following rate equations:

$$-d[MbFe(IV)=O]/dt = -k_1[MbFe(IV)=O][cys] - k_2[MbFe(IV)=O][cys] \quad (1)$$

$$d[metMbFe(III)]/dt = k_2[MbFe(IV)=O][cys] - k_3[metMbFe(III)][cys] \quad (2)$$

$$d[sulfMbFe(II)]/dt = k_1[MbFe(IV)=O][cys] + k_3[metMbFe(III)][cys] \quad (3)$$

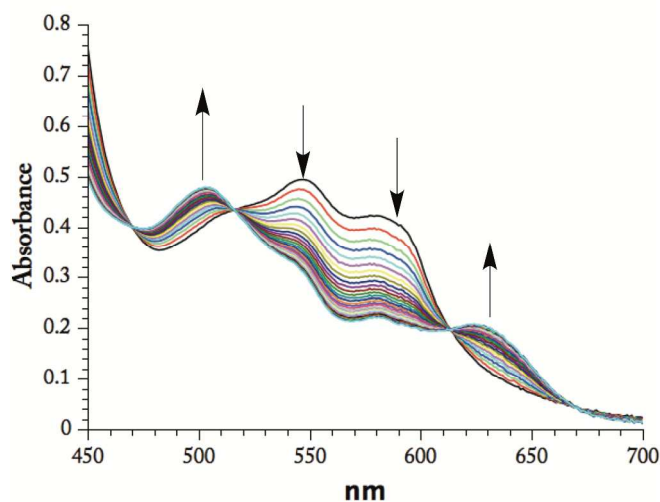


Figure 1. Spectral changes for the reaction between MbFe(IV)=O ($5 \times 10^{-5} \text{ mol L}^{-1}$) and cysteine ($1 \times 10^{-2} \text{ mol L}^{-1}$) in aqueous solution with pH 7.4 at 25.0 °C. Absorption spectra were recorded with intervals of 10 seconds.

The rate constants as obtained from the MCR analysis and subsequent fitting of concentrations of eq. (1) to (3) for experiments at pH 6, 7, and 8 separately showed that only the reaction of MbFe(IV)=O with cysteine to yield sulfMbFe(II) significantly contribute to the reduction of MbFe(IV)=O, since $k_1 \gg k_2 > k_3$, see Figure 2 (A, B, and C), which show the observed pseudo-first order rate constants k_1' , k_2' , and k_3' as a function of increasing cysteine concentration. For pH 6, 7 and 8 only the reaction corresponding to the first term in eq.(1) contributes to the over-all reduction with k_1' (s^{-1}) equal to:

$$k_1' = k_{\text{autoreduction}} + k_1[cys] \quad (4)$$

where $k_{\text{autoreduction}}$ is the natural decay of ferrylmyoglobin yielding metmyoglobin with the polypeptide chain slightly modified by oxidation (16).

Any formation of metMbFe(III) under these conditions seems to result from auto-reduction of MbFe(IV)=O and since k_3 may be considered insignificant compared to k_1 , metMbFe(III) could be considered a spectator in the reaction. Notably, the second-order rate constant k_1 ($l \text{ mol}^{-1} \text{ s}^{-1}$) determined as the slope of k_1' as a linear function of $[cys]$, depends on pH in a complex way which was analysed in separate experiments. As an example of concentration/time profile, concentrations of sulfMbFe(II) and metMbFe(III) as function of time in the reaction of MbFe(IV)=O with cysteine at pH 7 for 9.43 mM of cysteine is seen in Figure 3. The concentration of metMbFe(III) present in the reaction mixture prior to reaction with cysteine was not changing during the progress of reaction in agreement with the insignificance of

other reactions than the reaction corresponding to the first term in eq.(1) under these conditions.

Scheme 1. Possible reaction pathways for the reduction of MbFe(IV)=O by cysteine (RSH) under acidic, neutral, and alkaline yielding SulfMbFe(II), MbFe(III), and MbFe(II)O₂. The proposed pathways are the basis for writing the differential rate laws 1-6. $k_{\text{autoreduction}}$ is related to the autoreduction of ferrylmyoglobin yielding metmyoglobin in which the protein backbone becomes modified (16). $k_{\text{autooxidation}}$ is related to the auto decay of oxymyoglobin producing superoxide and the oxidized metal center (17).

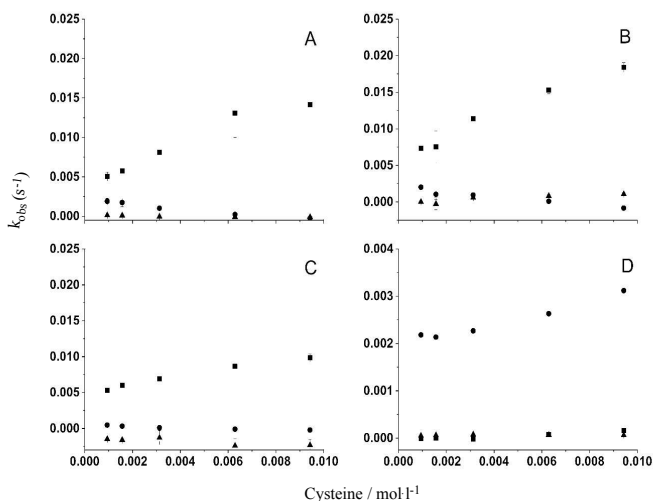
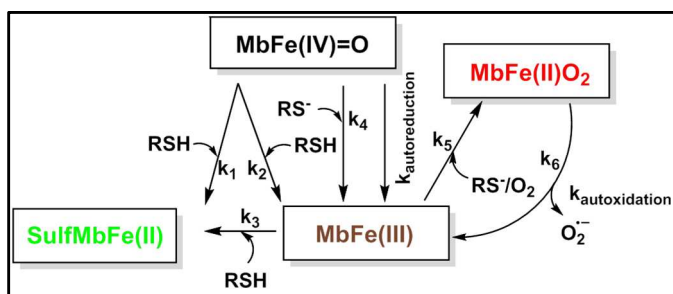


Figure 2. Derived first-order rate constants in s^{-1} for the reaction of MbFe(IV)=O and metMbFe(III) with cysteine at 25.0 °C as function of excess [cysteine] for pH = 6 (A: $k_1' = \blacksquare$, $k_2' = \bullet$, $k_3' = \blacktriangle$), for pH = 7 (B: $k_1' = \blacksquare$, $k_2' = \bullet$, $k_3' = \blacktriangle$), for pH = 8 (C: $k_1' = \blacksquare$, $k_2' = \bullet$, $k_3' = \blacktriangle$), for pH = 9 (D: $k_4' = \blacksquare$, $k_5' = \bullet$, $k_6' = \blacktriangle$). The quality of the non-linear fitting was evaluated by the residuals; see Table 1 of supplementary information.

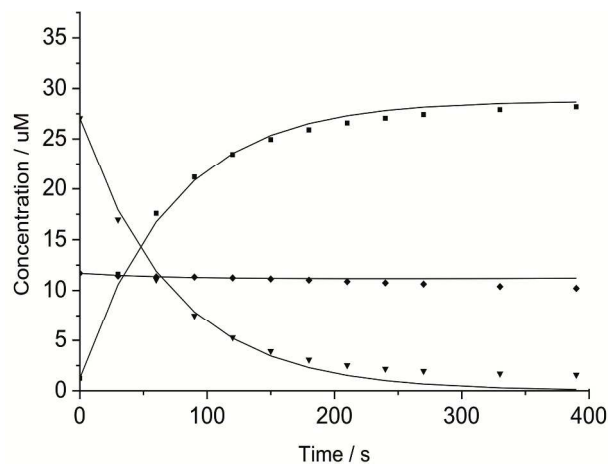


Figure 3. Non-linear fitting of rate equations of eq.(1) to eq.(3) to concentration profiles of MbFe(IV)=O (\blacktriangledown) as reactant and sulfMbFe(II) (\blacksquare) and metMbFe(III) (\blacklozenge) as products in the reaction of MbFe(IV)=O with cysteine at pH = 7 and 25 °C.

For pH 9, MbFe(IV)=O reacts with cysteine forming metMbFe(III) and subsequent MbFe(II)O₂ rather than sulfMbFe(II) according to the spectral analysis (9). The concentration/time profile of MbFe(IV)=O, metMbFe(III), MbFe(II)O₂ (and sulfMbFe(II)) corresponds to contributions from the following rate equations:

$$-d[\text{MbFe(IV)=O}]/dt = k_4[\text{MbFe(IV)=O}][\text{cys}^-] \quad (4)$$

$$\begin{aligned} d[\text{metMbFe(III)}]/dt = \\ k_4[\text{MbFe(IV)=O}][\text{cys}^-] - k_5[\text{metMbFe(III)}][\text{cys}^-] + k_6[\text{MbFe(II)O}_2] \end{aligned} \quad (5)$$

$$d[\text{MbFe(II)O}_2]/dt = k_5[\text{metMbFe(III)}][\text{cys}^-] - k_6[\text{MbFe(II)O}_2][\text{cys}^-] \quad (6)$$

The rate equations as obtained from the MCR analysis and subsequently fitting of concentrations to the eq.(4) to (6) for experiments at pH 9 showed that the reaction corresponding to the first term in eq.(6) determine the rate of formation of MbFe(II)O₂ under the present conditions with an excess of cysteine, which at this high pH is deprotonated to the thiolate anion, since pK_a for the thiol of cysteine is 8.3 and 10.7 for the amine group (18).

In order to analyse the pH-dependence of cysteine reduction of MbFe(IV)=O at conditions of relevance for meat products and for digestion of meats, formation of sulfmyoglobin(II) was followed for $4.5 < \text{pH} < 8.2$ using absorption at 625 nm, see Figure 4A, at which wavelength sulfMbFe(II) has an absorption maximum, and metMbFe(III) and MbFe(IV)=O are in the isobestic point (9). The observed pseudo-first order rate constant corresponds to k_1' in eq.(1) and eq.(2) and in Figure 2 (A, B, and C) and k_1' was found to increase linearly on the concentration of excess cysteine as shown for pH 7.4 and 25 °C in Figure 4B. The intercept ($1.2 \cdot 10^{-2} \text{ s}^{-1}$) in Figure 4B is in agreement with the reported auto-reduction of ferrylmyoglobin, $k_{\text{autoreduction}} = 1.8 \cdot 10^{-2} \text{ s}^{-1}$, at pH 7.5 and 25 °C (16).

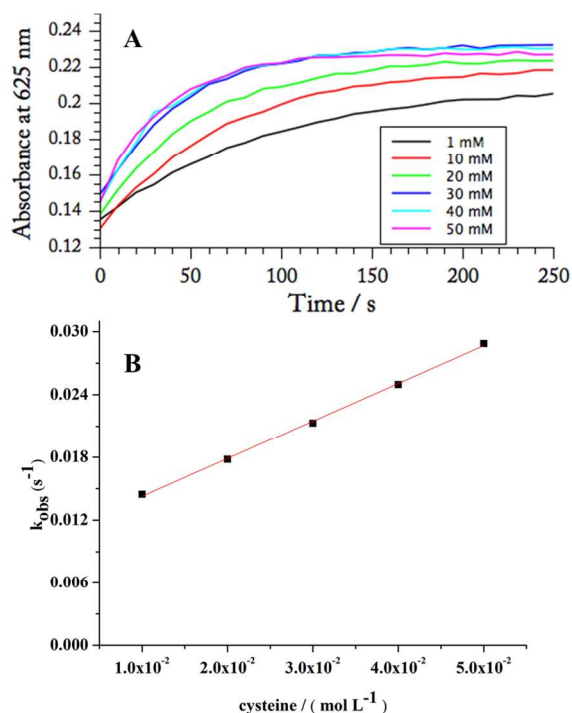
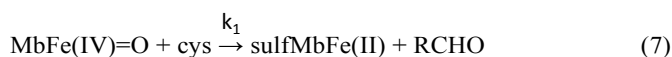
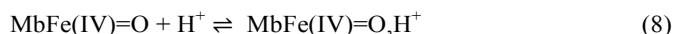


Figure 4. A: Absorbance time profile at 625 nm for the formation of sulfMbFe(II) during the reduction of 5×10^{-5} mol L⁻¹ of MbFe(IV)=O at pH = 7.4 and 25 °C with increasing concentrations of cysteine as indicated. B: Observed first-order rate constant for formation of sulfMbFe(II) as dependent on excess of cysteine concentration.

SulfMbFe(II) is the main product for reduction of MbFe(IV)=O by cysteine for acidic and neutral conditions, eq.(7).



The second-order rate constant, k_1 , for reduction of MbFe(IV)=O by cysteine to yield sulfMbFe(II) and an aldehyde form of oxidized cysteine (19) depends on pH similarly to the dependence seen for ascorbate and chlorogenate (20), Figure 5. The increase in rate for decreasing pH depends on protonation of MbFe(IV)=O



with a $pK_a = 4.9$ (20) rather than on specific acid catalysis as seen for autoreduction of MbFe(IV)=O and for auto-oxidation of MbFe(II)O₂ (16,21). For neutral conditions, protonization of His₄₈ and His₁₁₉ in globin seems to enhance or hamper electron-transfer, respectively, as has been found for electron-transfer between cytochrome-C and oxymyoglobin (22). Considering all three protolytic active groups, as in Scheme 2, the observed pseudo-first order rate constant, k_{obs} (s⁻¹) for the formation of sulfmyoglobin as function of pH can be described as follow:

$$k_{obs}^{\text{sulfMbFe(II)}} = \frac{(k_1^{3H^+}[H^+]^3 + k_1^{2H^+}[H^+]^2 K_{a1} + k_1^{H^+}[H^+] K_{a1}K_{a2} + k_1^N K_{a1}K_{a2}K_{a3})}{([H^+]^3 + [H^+]^2 K_{a1} + [H^+] K_{a1}K_{a2} + K_{a1}K_{a2}K_{a3})} \quad (9)$$

where K_{a1} is the acid dissociation constant of MbFe(IV)=O,H⁺, K_{a2} is the acid dissociation constant of His₄₈ is ferrylmyoglobin and K_{a3} is the acid dissociation constant of His₁₁₉ in ferrylmyoglobin, see Scheme 2. The pH-profile as seen in Figure 5 is based on a non-linear fitting procedure including the values $pK_{a1} = 4.9$, $pK_{a2} \approx 5.6$ (23), $pK_{a3} \approx 6.6$ (23), to yield $k_1^{3H^+} = 5.1 \pm 0.4$ l mol⁻¹ s⁻¹, $k_1^{H^+} = 2.1 \pm 0.4$ l mol⁻¹ s⁻¹, and $k_1^N = 0.31 \pm 0.15$ l mol⁻¹ s⁻¹. The value for $k_1^{2H^+}$ could not be satisfactory determined based on the non-linear fitting of the experimental data in Figure 5 to eq.(9), from the non-linear fitting $k_1^{2H^+} = 0.29 \pm 0.5$ l mol⁻¹ s⁻¹.

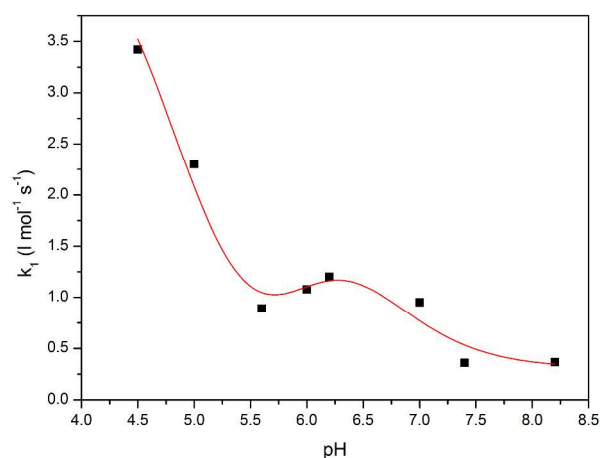
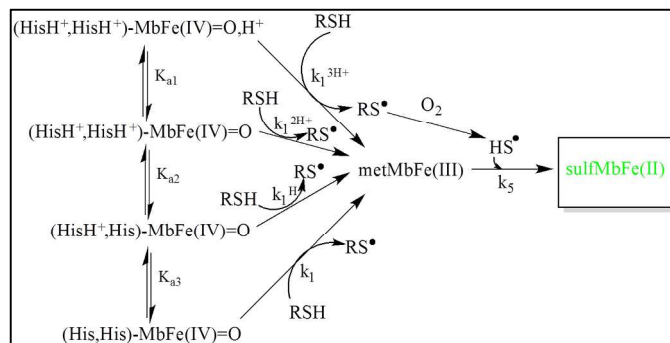


Figure 5. pH dependence at 25 °C for second-order rate constant for reduction of ferrylmyoglobin by cysteine in appropriate buffer. Solid line is obtained by non-linear regression to eq.(9), goodness-of-fit $r^2 = 0.9658$.

Scheme 2. Suggested reaction scheme for reduction of MbFe(IV)=O by cysteine under acidic and neutral conditions. From the reaction pH dependence (Figure 5) the involvement of two histidine (His₄₈/His₁₁₉) residues in an acid-base equilibrium is proposed.



For higher pH, where the reductant is the thiolate dianion of cysteine with pKa of 8.3 for the thiol and 10.4 for the amino group, the product is oxymyoglobin (MbFe(II)O₂):



for which a value of $k_5 = 0.12 \pm 0.01 \text{ l mol}^{-1} \text{ s}^{-1}$ was found at pH 9.0 and 25 °C (see Figure 2D).

Homocysteine together with glutathione were compared with cysteine at an intermediate pH of 7.4, where sulfMbFe(II) was the product of reduction. The reaction rates were very similar as were the activation parameters derived from the temperature dependence for the three reductants, see Table 1.

Table 1. Second-order rate constant for reduction of MbFe(IV)=O by cysteine, homocysteine, and glutathione in 0.16 M NaCl at pH 7.4 and calculated enthalpy and entropy of activation.

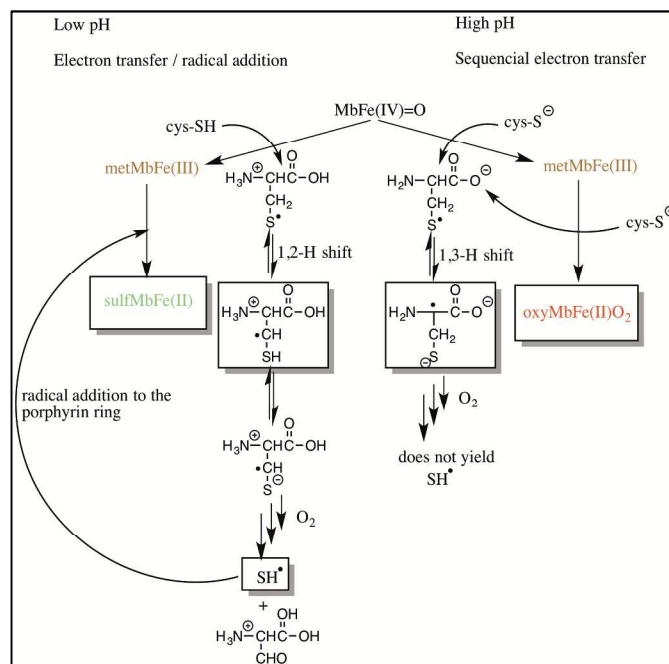
	$k_1 \text{ (l mol}^{-1} \text{ s}^{-1}\text{)}$			$\Delta H^\ddagger \text{ (kJ mol}^{-1}\text{)}$	$\Delta S^\ddagger \text{ (J mol}^{-1} \text{ K}^{-1}\text{)}$
	20.0°C	25.0°C	35.0°C		
Cysteine	0.30	0.72	1.49	75 ± 2	-250 ± 7
Homocysteine	0.23	0.70	1.39	89 ± 4	-251 ± 13
Glutathione	0.32	0.79	1.56	77 ± 3	-248 ± 9

Discussion

Cysteine is concluded to reduce MbFe(IV)=O less efficiently than hydrogen sulfide as studied previously (11). For hydrogen sulfide the reaction with protonated ferrylmyoglobin has a rate constant of $2.5 \times 10^6 \text{ l mol}^{-1} \text{ s}^{-1}$ at 25°C to be compared with the rate constant for cysteine $k_1^{H^+} = 5.1 \pm 0.4 \text{ l mol}^{-1} \text{ s}^{-1}$. For ferrylmyoglobin at higher pH, the reaction of HS⁻ has a rate constant of $1.0 \times 10^4 \text{ l mol}^{-1} \text{ s}^{-1}$ at 25°C while the thiolate dianion reduction of ferrylmyoglobin has a rate constant of $0.31 \pm 0.15 \text{ l mol}^{-1} \text{ s}^{-1}$ at 25°C. Notably, the product of the reduction of ferrylmyoglobin by hydrogen sulfide is sulfMbFe(II) independent of the pH, while sulfMbFe(II) is formed by cysteine reduction only at pH lower than the pK_a for the thiol and amino group in cysteine, and MbFe(II)O₂ is formed at higher pH.

One-electron oxidation of H₂S results in the sulfur radicals HS[•] and S^{•-}, which will add to the porphyrin in metMbFe(III) forming sulfMbFe(II). For cysteine only the protonated form is supposed to form HS[•] following a 1,2-hydrogen shift as shown in Scheme 3, while the dianion form of cysteine will favor a 1,3-hydrogen shift yielding a less reactive tertiary carbon-centered radical (19). For higher pH conditions, it is accordingly suggested reduction of the initially formed metMbFe(III) yields oxymyoglobin. The two different reaction pathways for MbFe(IV)=O (and the protonated form MbFe(IV)=O, H⁺) reacting with cysteine and the cysteine dianion are outlined in Scheme 3.

Scheme 3. Proposed reaction mechanisms for the reduction of MbFe(IV)=O by cysteine under acidic and alkaline conditions (19).



Cysteine is known to protect kidneys against myoglobin toxicity under myoglobinuric tubular injury (14). The mechanism is not obvious, since glutathione and homocysteine apparently worsen this pathology condition, in which hydrogen peroxide seems to be involved. Hypervalent myoglobin may be the damaging reactant, and cysteine may under the slightly acidic conditions reduce the hypervalent iron pigment preventing damaging lipid and especially protein oxidation otherwise harming sensitive membranes.

Conclusions

It is often recommended to add vegetables and fruits to meals rich in red meat in order to protect the digestive tract from radicals formed by the meat pigments during digestion. Fruits are rich in ascorbate and ascorbate is known to reduce protonated ferrylmyoglobin with a second-order rate constant of $2.0 \times 10^3 \text{ l mol}^{-1} \text{ s}^{-1}$, while chlorogenate, a common antioxidant in vegetables, is even faster with $3.0 \times 10^6 \text{ l mol}^{-1} \text{ s}^{-1}$ at 25°C (20). Cysteine is far less efficient, but may still be of relevance, since meat proteins like myosin are rich in cysteine (24). Structural meat proteins like myosin are closely associated with myoglobin during meat processing and during digestion and may thus for steric reasons contribute to reduction of any hypervalent meat pigment formed during digestion.

From a mechanistic point of view, the difference between hydrogen sulfide and cysteine is interesting, since hydrogen sulfide react so much faster and since hydrogen sulfide, which may be formed in the intestine, form sulfMbFe(II) also under alkaline conditions. SulfMbFe(II) may serve as a dead-end for myoglobin reactions, while MbFe(II)O₂ formed by cysteine reduction in alkaline conditions or by ascorbate and

chlorogenate reduction may still be catalytic for radical formation. The formation of sulfmyoglobin may also impair iron absorption since heme iron account to approximately 40 % of total iron bioavailability and inorganic iron absorption in the gut is very inefficient (25).

Meat proteins like myosin may accordingly serve as an internal protection against hypervalent iron formed by peroxide oxidation of myoglobin and hemoglobin, since sulfmyoglobin as the product of reduction by cysteine during digestion prevents catalytic activity of the heme, in effect serving as a dead-end. This is in contrast to plant-derived antioxidants like ascorbate and chlorogenate, which although reacting faster form reduced heme pigment still with catalytic activity.

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Notes and references

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Electronic Supplementary Information (ESI) available: [Quality of the non-linear fitting evaluated by residual values and total residual and individual electronic absorption spectra for ferrylmyoglobin, metmyoglobin, sulfmyoglobin, and cysteine]. See DOI: 10.1039/b000000x/

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Herein we report the kinetics and mechanism by which hypervalent heme-iron species formed in the gut may be deactivated by thiols like cysteine and glutathione.

