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Reduction of Ferrylmyoglobin by Hydrogen Sulfide. Kinetics in Relation to Meat Greening

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ABSTRACT: The hypervalent meat pigment ferrylmyoglobin, MbFe(IV)=O, characteristic for oxidatively stressed meat and known to initiate protein cross-linking, was found to be reduced by hydrogen sulfide to yield sulfmyoglobin. Horse heart myoglobin, void of cysteine, was used to avoid possible interference from protein thiols. For aqueous solution, the reactions were found to be second-order, and an apparent acid catalysis could be quantitatively accounted for in terms of a fast reaction between protonated ferrylmyoglobin, MbFe(IV)=O,H$^+$, and hydrogen sulfide, H_2S ($k_2 = (2.5 \pm 0.1) \times 10^6$ L mol^{-1} s^{-1} for 25.0 °C, ionic strengh 0.067, dominating for pH < 4), and a slow reaction between MbFe(IV)=O and HS^- ($k_2 = (1.0 \pm 0.7) \times 10^4$ L mol^{-1} s^{-1} for 25.0 °C, ionic strengh 0.067, dominating for pH > 7). For meat pH, a reaction via the transition state {MbFe(IV)=O···H···HS}‡ contributed significantly, and this reaction appeared almost independent of temperature with an apparent energy of activation of 2.1 ± 0.7 kJ mol^{-1} at pH 7.4, as a result of compensation among activation energies and temperature influence on pK_a values explaining low temperature greening of meat.

KEYWORDS: *ferrylmyoglobin, meat greening, kinetics, hydrogen sulfide*

INTRODUCTION

Hypervalent heme pigments are formed during oxidative stress in muscles and blood by reaction of myoglobin and hemoglobin with peroxides.[1,2] Initially formed perferryl compounds, often denoted as Compound I, are as iron(IV) protein radical cations short-lived and are in muscle and meat converted to longer-lived ferryl compounds, also known as Compound II, like ferrylmyoglobin, MbFe(IV)=O.[3,4] MbFe(IV)=O reacts with proteins like myosin forming protein radicals initiating protein polymerization mainly via sulfur−sulfur bridges between cysteine residues.[5]

Endogenous metabolites or added compounds may protect muscle or meat through reactions as antioxidants by competing in deactivation of MbFe(IV)=O, in effect protecting protein against cross-linking and meat lipids against development of rancidity.[6] Small molecules important in cellular regulation like nitric oxide, carbon monoxide, and hydrogen sulfide may also be of importance in protection against hypervalent heme pigment in meat products as in muscles.[7] While the reaction of nitric oxide with hypervalent iron has been studied in some details and found important in controlling lipid and protein oxidation in meat systems, the reaction of MbFe(IV)=O with hydrogen sulfide is less understood.[7,8] Accordingly, we have studied the reaction between H_2S and MbFe(IV)=O at physiological pH as in muscles and for decreasing pH as in meat and during food digestion using fast kinetic techniques to contribute to an understanding of the role of hydrogen sulfide in mediating the metabolic processes during transformation of muscle to meat and further during processing and storage of meats.

The reaction of hydrogen sulfide with meat pigments like ferrylmyoglobin is further of interest for meat packaging, because meats despite chilled storage under some conditions develop a green color.[9−11] This so-called greening of meat has been assigned to formation of sulfmyoglobin as a compound or group of compounds originating from reaction of myoglobins with various sulfur-containing compounds present in or formed in meat.[12]

MATERIALS AND METHODS

Chemicals. Iron(II) sulfide, iron(III) chloride, zinc acetate, *N*,*N*-dimethyl-*p*-phenylenediamine (purity 97%), sodium sulfide, 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%) were obtained from Sigma-Aldrich (Steinheim, Germany). Myoglobin was purified according to procedures previously described.[13] K_2HPO_4 and KH_2PO_4 were of analytical grade and supplied by J. T. Baker (Phillipsburg, NJ), and boric acid, borax, and phosphoric acid were from Merck (Darmstadt Germany). Acetic acid was from AppliChem GmbH (Darmstadt, Germany), and hydrochloric acid 37% was from VWR international (Leuven, Belgium). Deionized water was obtained using a Milli-Q system Millipore Co. (Billerica, MA).

Reaction Kinetics. Hydrogen sulfide was generated by adding 3 mL of hydrochloric acid (2.5 mol L^{-1}) to 0.4 g of iron(II) sulfide using a separation funnel for slow addition of HCl to FeS followed by a standard washing procedure. Hydrogen sulfide solutions were obtained by bubbling the generated gas through 200 μL of buffer solution for 12 min. Next, 3.8 mL of fresh buffer solution was added to obtain hydrogen sulfide stock solutions. Aliquots of hydrogen sulfide stock solution (200−800 μL) were diluted to a final volume of 5.00 mL. The total sulfur concentration was determined by the so-called methylene blue method.[14,15] A stock solution of metmyoglobin (45 μmol L^{-1})

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was freshly prepared in the appropriate aqueous buffer. For kinetic measurements at pH 6.2, 6.9, 8.0, a phosphate buffer was used, while for pH 9.0 a borax buffer was used. The ionic strength 0.067 was kept constant for all pH investigated. For acidic conditions (pH 5.5, 5.0, 4.2, and 3.1), pH-jump experiments in the stopped-flow spectrophotometer were carried out using a mixture of phosphate and acetate buffer to yield the final ionic strength of 0.067 and desired pH. For all experiments, pH was measured in the reaction mixture at the actual temperature. Ferrylmyoglobin at a concentration of 30 μmol L^{-1} was freshly prepared from purified metmyoglobin and hydrogen peroxide as previously described.[13] This procedure involves addition of catalase to remove excess hydrogen peroxide and any hydrogen peroxide possibly formed during reaction.

Formation of sulfmyoglobin was followed spectrophotometrically using an Applied Photophysics SX20 sequential stopped flow system coupled to a PDA photodiode array detector. Spectra were recorded from 200 to 800 nm at different time intervals depending on reaction rate.

RESULTS AND DISCUSSION

Ferrylmyoglobin, MbFe(IV)═O, as synthesized from purified equine metmyoglobin by reaction with hydrogen peroxide, was found to react readily with hydrogen sulfide in aqueous solutions with a rate strongly dependent on pH, but less on temperature. The reaction was followed spectrophotometrically in the visible absorption range where absorption around 525 nm as characteristic for MbFe(IV)═O, and used to confirm the identity of the reactant,[13] disappeared, while a new absorption band with a maximum at 616 nm appeared. Absorption at 616 nm has been reported as characteristic for the green color of sulfmyoglobin.[9] The reaction

$$\text{MbFe(IV)═O} + \text{H}_2\text{S} \rightarrow \text{sulfmyoglobin} \quad (1)$$

was found to be characterized by well-defined isosbestic points, the position of which, however, depended on pH; see Figure 1. The nature of the product, sulfmyoglobin, is still a matter of some discussion and may depend on from which myoglobin form it is formed.[10−12] For the reaction of hydrogen sulfide with hypervalent heme compounds, evidence of porphyrin-ring modification by addition of sulfur across *N*-heterocycles has been reported.[11,12] The product spectrum as found in the present study is in agreement with the spectral characteristic of the iron(II) sulfmyoglobin.[11] The dependence on pH of the intermediate absorption spectra is further in agreement with a iron(III) myoglobin as an reaction intermediate with a water coordinated to iron(III) with a pK_a value around 8, as it is known for metmyoglobin, MbFe(III).[16] On the basis of the spectral characteristic, it is accordingly concluded that the final product of the reaction of eq 1 is a sulfmyoglobin with iron(II) as the metal center and sulfur incorporated in the porphyrin ring in agreement with a structural assignment based on Raman spectroscopy.[10−12] The rate determining step is, however, an initial reaction in which iron(IV) is reduced to iron(III), followed by subsequent faster reactions as discussed below.

For all pH and temperature conditions, the reaction was found to follow first-order kinetics for excess of hydrogen sulfide ($10^{-4} < C_{H2S} < 10^{-3}$ mol L^{-1}, as compared to $C_{MbFe(IV)=O}$ around 10^{-5} mol L^{-1}); see Figure 2. The reaction was completed in less than a few seconds when slowest, and a rapid stopped-flow spectroscopic technique was used in all experiments. In most experiments, a freshly prepared MbFe(IV)═O solution and a hydrogen sulfide solution buffered to the appropriate pH were mixed, and absorption was recorded with time. For the most acidic reaction conditions, a mixing technique was used, in which the MbFe(IV)═O solution was adjusted to the low pH by acid in the hydrogen sulfide solution in the other syringe prior to recording of time-dependent absorption. pH was in all experiments recorded in the final reaction mixture. The pH-jump technique was used to avoid longer time exposure of MbFe(IV)═O to strongly acidic conditions with the risk of protein denaturation, which is expected to occur within a few minutes.[17] The pseudofirst-order rate constants as determined by exponential fitting, see Figure 2A−C:

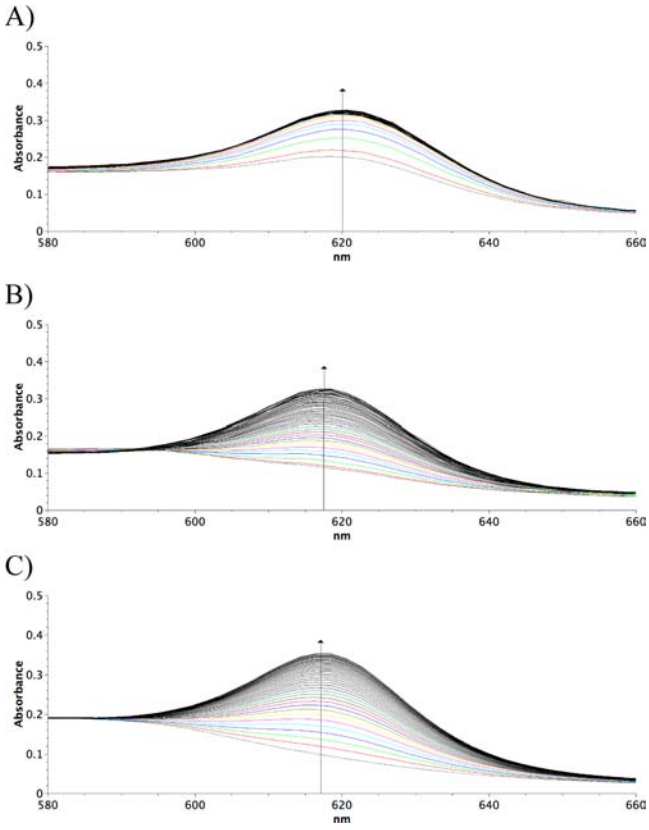


Figure 1. Spectral changes corresponding to formation of a "green band" for reaction of ferrylmyoglobin (1.5×10^{-5} mol L^{-1}) with hydrogen sulfide ($\sim 3 \times 10^{-4}$ mol L^{-1}) in aqueous solution of ionic strength 0.067 at 25.0 °C for pH 3.1 (A), pH 6.9 (B), and pH 9.0 (C). Spectra recorded every 1 ms for pH 3.1 and 6.9, and every 50 ms for pH 9.0. Light path 10 mm.

$$A_{(t)} = A_o + (\text{plateau} - A_o)\cdot(1 - e^{(-k\cdot t)}) \quad (2)$$

were for constant values of pH and temperature found to depend linearly on the total concentration of hydrogen sulfide, see Figure 2A−C, in agreement with a bimolecular rate-determining step for reaction of MbFe(IV)═O with hydrogen sulfide. From the plot of k_{obs} versus total hydrogen sulfide, the second-order rate constant, k_2, was extracted as the slope. This rate constant depends on pH as two reactants each in acid/base equilibrium are involved in the reactions; see Scheme 1. For hydrogen sulfide:

$$\text{H}_2\text{S} \rightleftharpoons \text{HS}^- + \text{H}^+ \quad (3)$$

a pK_a value of 6.8 at 25.0 °C is reported with a temperature dependence corresponding to ΔH^o = 22 kJ mol^{-1} and ΔS^o = −58 J mol^{-1} K^{-1}.[18] For ferrylmyoglobin, increasing evidence is

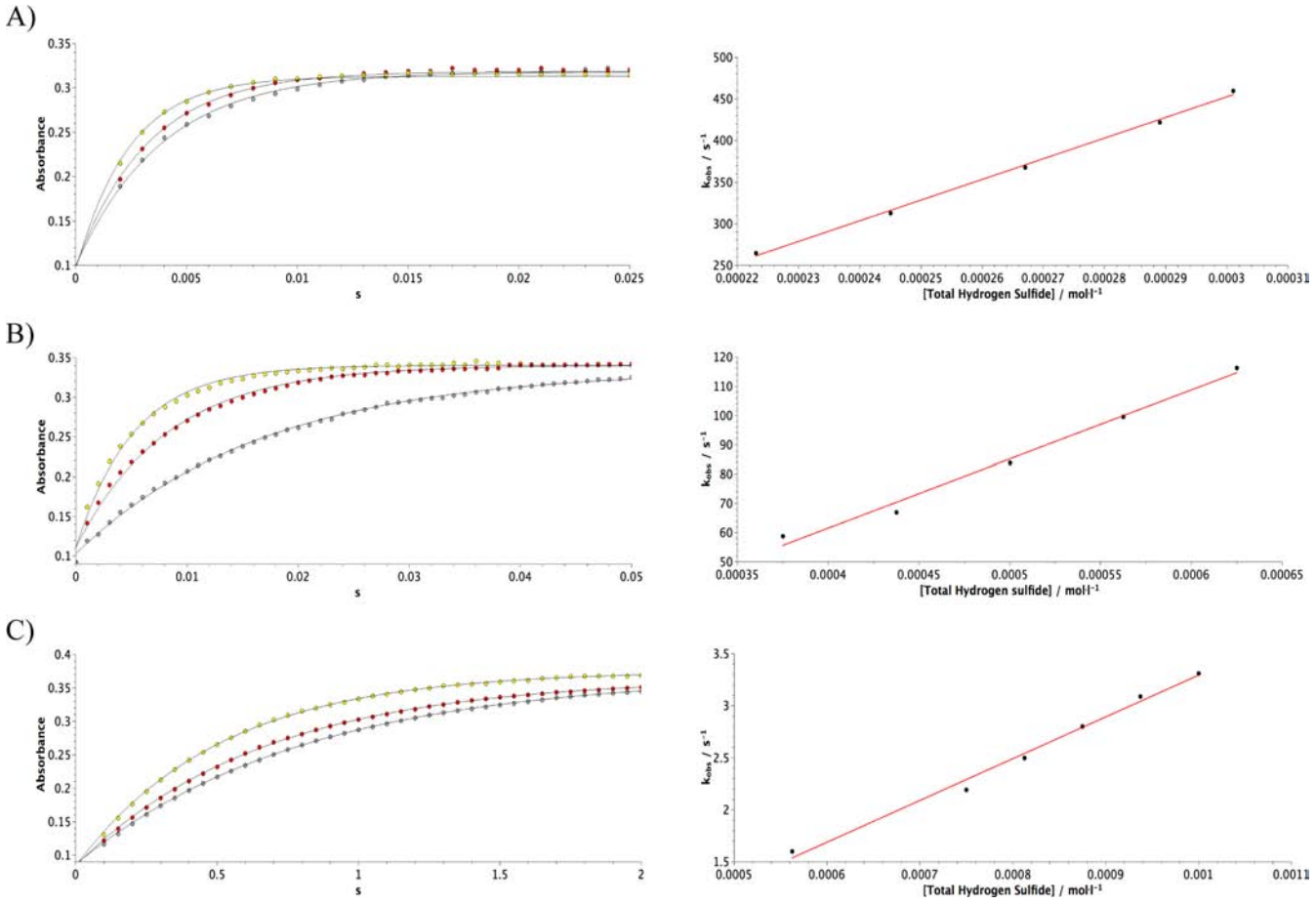
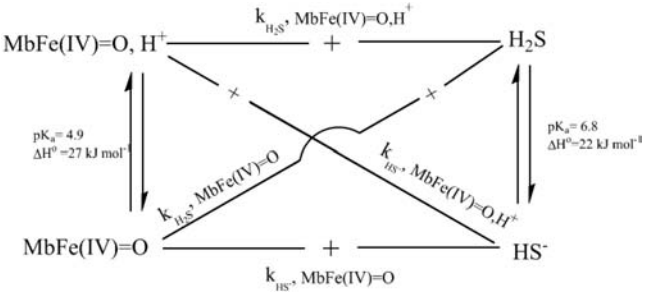


Figure 2. Time trace of absorption at 616 nm for reaction of ferrylmyoglobin (1.5×10^{-5} mol L^{-1}) with hydrogen sulfide (~3×10^{-4} mol L^{-1}) in aqueous solution of ionic strength 0.067 at 25.0 °C for pH 3.1 (A), pH 6.9 (B), and pH 9.0 (C) with curves from exponential fit to obtain pseudofirst-order rate constants, k_{obs}. To the right: k_{obs} for increasing concentration of hydrogen sulfide at respective values of pH to obtain second-order rate constants as the slope.

Scheme 1. Four Bimolecular Reactions Are Possible for Formation of Sulfmyoglobin from Ferrylmyoglobin and Hydrogen Sulfide at Meat pH



being presented for protonization being important for reduction to iron(III):

$$\text{MbFe(IV)=O, H}^+ \rightleftharpoons \text{MbFe(IV)=O} + \text{H}^+ \quad (4)$$

corresponding to a pK_a = 4.9 at 25.0 °C.[6] On the basis of the four possible reactions of the two-acid/base forms of the two reactants, the following pH-dependence of k_2 is deduced for constant temperature:[19]

$$k_2 = \frac{1}{([\text{H}^+] + K_a^{\text{H2S}}) \times ([\text{H}^+] + K_a^{\text{MbFe(IV)=O,H+}})} \times \left[k_{\text{H2S,MbFe(IV)=O,H+}} \times [\text{H}^+]^2 + (k_{\text{H2S,MbFe(IV)=O}} \times K_a^{\text{MbFe(IV)=O,H+}} + k_{\text{HS-,MbFe(IV)=O,H+}} \times K_a^{\text{H2S}}) \times [\text{H}^+] + k_{\text{HS-,MbFe(IV)=O}} \times K_a^{\text{H2S}} \times K_a^{\text{HbFe(IV)=O,H+}} \right] \quad (5)$$

For 25.0 °C and using pK_a = 6.8 and 4.9 for hydrogen sulfide and ferrylmyoglobin, respectively, nonlinear fitting of k_2 as a function of pH according to eq 5 leads to the value $k_{\text{H2S,MbFe(IV)=O,H+}} = (2.50 \pm 0.14) \times 10^6$ L mol^{-1} s^{-1} for the reaction dominating under acidic conditions:

$$\text{MbFe(IV)=O, H}^+ + \text{H}_2\text{S} \rightarrow \text{sulfmyoglobin} \quad (6)$$

and to the value of $k_{\text{HS-,MbFe(IV)=O}} = (1.0 \pm 0.7) \times 10^4$ L mol^{-1} s^{-1} for the reaction dominating under alkaline conditions:

$$\text{MbFe(IV)=O} + \text{HS}^- \rightarrow \text{sulfmyoglobin} \quad (7)$$

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For intermediate pH as relevant for meat, only the quantity $(k_{H2S,MbFe(IV)=O}\cdot K_a^{MbFe(IV)=O} + k_{HS^-,MbFe(IV)=O,H^+}\cdot K_a^{H2S}) = 5.0 \pm 0.8$ is obtained, because the following reactions:

$$MbFe(IV)=O,\ H^+ + HS^- \rightarrow \text{sulfmyoglobin} \quad (8)$$

$$MbFe(IV)=O + H_2S \rightarrow \text{sulfmyoglobin} \quad (9)$$

will have a transition state with the same composition, $\{MbFe(IV)=O\cdots H\cdots SH\}^{\ddagger}$, which due to the so-called "proton ambiguity" cannot be resolved into the individual reactions of eqs 8 and 9 solely based on kinetic data. However, a limiting value for $k_{HS^-,MbFe(IV)=O,H^+} = (3.2 \pm 0.5) \times 10^7$ L mol^{-1} s^{-1} may be calculated under the assumption that the reaction of eq 8 is dominating, while a limiting value of $k_{H2S,MbFe(IV)=O} = (4.0 \pm 0.7) \times 10^5$ L mol^{-1} s^{-1} may be calculated under the assumption that the rate of eq 9 is dominating.

Reduction of MbFe(IV)=O by H_2S seems accordingly to occur by three different reaction pathways. Under acidic conditions, the reaction of eq 6 dominates, while under basic conditions, the reaction accordingly to eq 7 is dominating, although slow and less well-defined. Considering the numerical values obtained at 25.0 °C and the low ionic strength of 0.067, the ordering of the rate constants $k_{H2S,MbFe(IV)=O,H+} = (2.5 \pm 0.1) \times 10^6$ L mol^{-1} s^{-1} > $k_{H2S,MbFe(IV)=O} = (4.0 \pm 0.7) \times 10^5$ L mol^{-1} s^{-1} > $k_{HS^-,MbFe(IV)=O} = (1.0 \pm 0.7) \times 10^4$ L mol^{-1} s^{-1}, however, suggests that the reaction of eq 9 is dominating for meat pH rather than the reaction of eq 8 (Figure 3). Acid

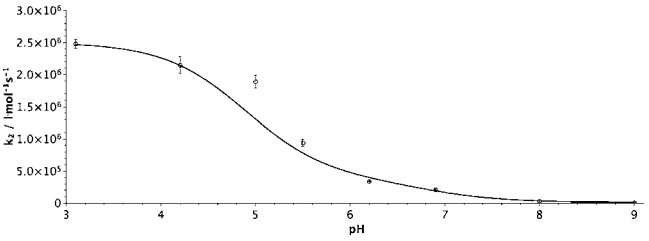


Figure 3. pH dependence at 25.0 °C for second-order rate constants for reaction of ferrylmyoglobin with hydrogen sulfide in aqueous solution of ionic strength 0.067. Line calculated by nonlinear regression analysis according to eq 5. Point at pH 5 not included in final fitting.

catalysis is important for MbFe(IV)=O as an oxidant,[6,20] and the reaction of eq 9 follows a mechanism also seen for other reductants, where H_2S as reactant transfers a proton to the reactive Fe(IV)=O center prior to electron transfer. A mechanism with the reaction of eq 9 being dominating over the reaction of eq 8 is moreover in accordance with general acid catalysis rather than with specific acid catalysis, where protonization of MbFe(IV)=O as in the reaction of eq 8 solely depends on pH providing circumstantial evidence for our suggestion of the reaction of eq 9 as dominating.

The temperature dependence of the reduction of MbFe(IV)=O by H_2S depends on pH. For the slow reaction at higher pH, the reduction has a significant temperature dependence, which, however, becomes small for decreasing pH as may be seen in Figure 4. The derived activation parameters at pH 9.0 ($\Delta H^{\circ} = 19 \pm 2$ kJ mol^{-1} and $\Delta S^{\circ} = -113 \pm 7$ J mol^{-1} K^{-1}) apply to the reaction of eq 7, while the values at lower pH are composite quantities depending both on the reaction of eq 6 and on the reaction of eq 9. Notably, for conditions of meat pH, the temperature dependence of the

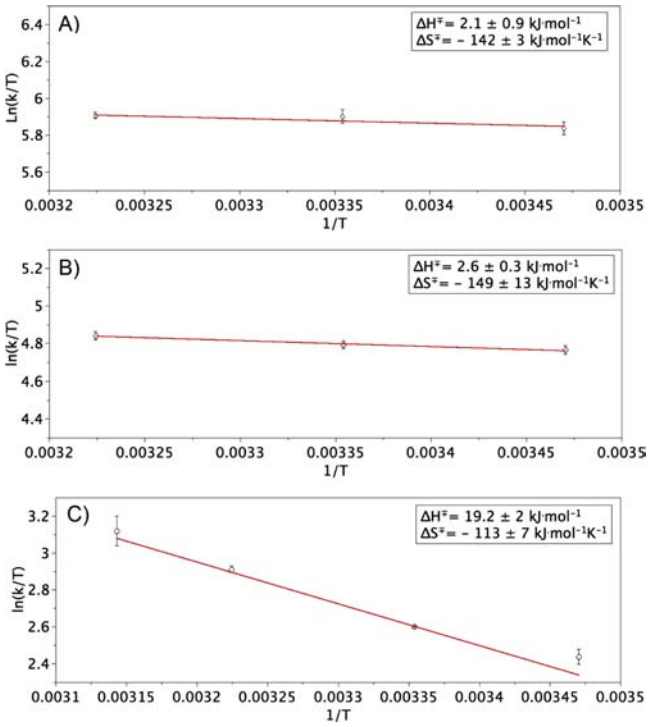


Figure 4. Eyring plot, ln k_2/T versus $1/T$, for second-order rate constant for reaction of ferrylmyoglobin with hydrogen sulfide in aqueous solution of ionic strength 0.067 at 25 °C for pH 7.4 (A), pH 8.0 (B), and pH 9.0 (C).

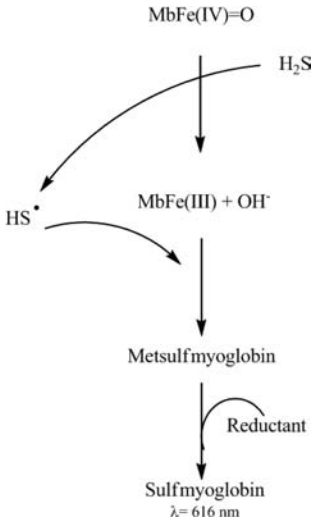
reaction between MbFe(IV)=O and H_2S almost disappears. Such temperature-compensated reactions are important in biological systems,[21] and may for the reactions under consideration be explained by the decrease in pK_a of H_2S (ΔH° = 22 kJ mol^{-1} for acidic dissociation) increasing the concentration of the less reactive HS^- at the expense of the more reactive H_2S with temperature. The temperature dependence is unknown for pK_a of MbFe(IV)=O,H$^+$, but a reasonable assumption is that the acid dissociation also is endothermic like for H_2S, and assuming that $\Delta H^{\circ} \approx \Delta G^{\circ}$ for the reaction of eq 4, increasing temperature will similarly increase the concentration of the less reactive MbFe(IV)=O at the expense of the more reactive MbFe(IV)=O,H$^+$ even more significantly than for H_2S, because ΔH° may be estimated to have the value of 27 kJ mol^{-1} for MbFe(IV)=O,H$^+$ as calculated from temperature dependence of pK_a.[6] The increase in the relative concentration of the less reactive form of both reactants for increasing temperature for constant pH apparently fully compensates for the increase in rate for a reaction with an expected modest energy of activation around 60 kJ mol^{-1} as was determined for acid catalyzed autoreduction of MbFe(IV)=O.[6] This temperature compensation clearly could seem of importance for greening of meat at low temperature, which accordingly will occur without decrease in rate as compared to physiological temperature.

On the basis of this kinetic description of a reaction, which may be important both in muscle under physiological conditions and in oxidatively stress meat, an outline of a reaction mechanism seems to appear for reduction of ferrylmyoglobin by hydrogen sulfide. For the study, horse heart myoglobin was preferred due to the absence of cysteine in the amino acid sequence. Competing reactions like:

$$RS^{\cdot} + HS{-}R + O_2 \rightarrow R{-}S{-}S{-}R + HO_2^{\cdot} \quad (10)$$

possibly depending on oxygen concentration and subsequent dismutation of superoxide to form hydrogen peroxide are accordingly not interfering with the kinetics of the reactions of Scheme 2. In addition, catalase was present in the reaction mixture to deplete hydrogen peroxide following ferrylmyoglobin synthesis or formed in subsequent reactions. For conditions of meat pH, hydrogen sulfide enters the globin cavity of MbFe(IV)=O initially transferring a proton followed by transfer of an electron from sulfur to the protonated moiety, MbFe(IV)=O,H$^+$, to yield iron(III). The thiyl radical formed in the cavity adds to the porphyrin ring to yield a metsulfmyoglobin with iron initially in the +3 oxidation state, see Scheme 2, but which subsequently is reduced in a faster reaction to yield the green iron(II) form of sulfmyoglobin. The exact nature of the reactants and the mechanism responsible for the fast transformation of the iron(III) form of sulfmyoglobin to the green sulfmyoglobin has not been indentified, but protein radicals may be involved and further studies are underway. The source of hydrogen sulfide may, however, be different in the living muscle where it is formed from cysteine as a signaling compound, and in meat where microbial activity under some conditions form hydrogen sulfide. Reactions of cysteine to form sulfmyoglobin are a subject of our current research.[22]

Scheme 2. The Dominating Reaction at Meat pH for Formation of Sulfmyoglobin from Ferrylmyoglobin and Hydrogen Sulfide



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Notes

The authors declare no competing financial interest.

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