Kinetics and Mechanism of Reduction of Ferrylmyoglobin by Ascorbate and D-Isoascorbate

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Reduction of iron(IV) in ferrylmyoglobin by ascorbate (and D-isoascorbate) is fast; at neutral or moderately acidic pH there are three parallel reactions: (i) a bimolecular reaction with 2.7 ± 0.8 $M^{-1}\cdot s^{-1}$, $\Delta H^{\#} = 45 \pm 13$ kJ·mol^{-1} , and $\Delta S^{\#} = -85 \pm 46$ J·mol$^{-1}\cdot$K^{-1}; (ii) a bimolecular reaction between ascorbate and protonated ferrylmyoglobin (28 ± 5 $M^{-1}\cdot s^{-1}$, $\Delta H^{\#} = 31 \pm 8$ kJ·mol^{-1}, and $\Delta S^{\#} = 113 \pm 28$ J·mol$^{-1}\cdot$K^{-1}); and (iii) an intramolecular electron transfer from ascorbate bound to protonated ferrylmyoglobin (7.5 ± 0.1 s^{-1}, $\Delta H^{\#} = 89.6 \pm 0.9$ kJ·mol^{-1}, $\Delta S^{\#} = 72 \pm 3$ J·mol$^{-1}\cdot$K^{-1}); rate constants and activation parameters are for aqueous 0.16 M NaCl solutions at 25.0 °C. Binding of ascorbate has $\Delta H^{\ominus} = -43 \pm 3$ kJ·mol^{-1} and $\Delta S^{\ominus} = -100 \pm 9$ J·mol$^{-1}\cdot$K^{-1}, and dissociation of protonated ferrylmyoglobin p$K_a = 5.34 \pm 0.02$ ($\Delta H^{\ominus} = 11 \pm 2$ kJ·mol^{-1}, $\Delta S^{\ominus} = -66 \pm 7$ J·mol$^{-1}\cdot$K^{-1}). One-electron transfer dominates to yield iron(III) metmyoglobin, and ascorbyl radical was detected by ESR spectroscopy. D-Isoascorbic acid was found to be slightly more acidic (p$K_a = 3.706 \pm 0.003$, $\Delta H^{\ominus} = 5.7 \pm 0.5$ kJ·mol^{-1}, $\Delta S^{\ominus} = -52 \pm 2$ J·mol$^{-1}\cdot$K^{-1}) than ascorbic acid (p$K_a = 3.772 \pm 0.003$, $\Delta H^{\ominus} = 5.8 \pm 0.4$ kJ·mol^{-1}, $\Delta S^{\ominus} = -53 \pm 1$ J·mol$^{-1}\cdot$K^{-1}), while D-isoascorbate had reducing properties similar to those of ascorbate (cyclic voltammetry and stopped-flow spectroscopy).

Keywords: *Ferrylmyoglobin; ascorbic acid; D-isoascorbic acid; protein binding; electron transfer*

INTRODUCTION

The oxidative stability of meat and meat products depends on the balance between prooxidants and antioxidants. Prooxidants may be endogenous metalloproteins or ingredients added to the product such as salt (Andersen and Skibsted, 1991) or processing aids such as the anticaking agent potassium ferrocyanide (Hansen et al., 1996). Among the endogenous prooxidants, myoglobins activated by hydrogen peroxides may play a key role, as both the iron(IV) protein radical perferrylmyoglobin, •MbFe(IV)═O, and ferrylmyoglobin, MbFe(IV)═O, may act as initiator of lipid peroxidation (Kelman et al., 1994; Rao et al., 1994; Kanner and Harel, 1985a,b; Harel and Kanner, 1985). Hypervalent myoglobins have further been found to induce oxidative cross-linking of proteins (Østdal et al., 1996; Hanan and Shaklai, 1995) and to oxidize NADH, the reducing cofactor important for enzymatic reduction of metmyoglobin, MbFe(III), to oxymyoglobin, MbFe(II)O_2 (Mikkelsen and Skibsted, 1995).

The formation of hypervalent myoglobin depends on reaction of MbFe(II)O_2 or MbFe(III) with peroxides (King and Winfield, 1963). In chilled meat, especially ground meat, hydrogen peroxide is formed by lactic acid bacteria and by autoxidation of MbFe(II)O_2, cf. Figure 1. The autoxidation is acid catalyzed (Andersen et al., 1988) and, as such, promoted by the pH decrease caused by growth of microaerophilic lactic acid bacteria, the growth of which, on the other hand, is accelerated by the oxygen depletion resulting from the pigment autoxidation. Autoxidation of MbFe(II)O_2 and growth of lactic acid bacteria thus act synergetically in the production of hydrogen peroxide (Skibsted et al., 1994).

Water-soluble reductants react readily with hypervalent myoglobin (Giulivi et al., 1992; Romero et al., 1992), and the reaction of MbFe(III) with H_2O_2 followed by the reduction of MbFe(IV)═O by ascorbate has been proposed to protect the living cell from more harmful reactions of H_2O_2; that is, myoglobin possibly functions as a pseudo ascorbic acid peroxidase (Galaris et al., 1989). For meat and meat products, endogenous as well as added ascorbate may likewise play an important role in deactivating the hydrogen peroxide through reaction with ferrylmyoglobin. The reaction mechanism of this and similar electron-transfer reactions is clearly of relevance for a better understanding of deactivation of hydrogen peroxide in biological systems and in meats, and we have undertaken a detailed kinetic study of the reduction of MbFe(IV)═O by ascorbate and by the epimeric D-isoascorbate in an aqueous model system.

MATERIALS AND METHODS

Materials. Metmyoglobin [MbFe(III), horse heart, type III] and bovine liver catalase were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium ascorbate (>99%) was from Fluka (Buchs, Switzerland), D-isoascorbic acid (D-*erythro*-hex-2-enoic acid γ-lactone) (99.8%) from Sigma, and H_2O_2 (35%) from Riedel-de Haën (Seelze, Germany). All other chemicals (analytical grade) were from Merck (Darmstadt, Germany). Water was purified through a Millipore Q-Plus (Millipore Corp., Bedford, MA) purification train.

Synthesis of Ferrylmyoglobin. MbFe(III) dissolved in 5.0 mM phosphate buffer/0.15 M NaCl (pH 6.7) was purified by elution on a Sephadex G50 column (40 × 2.5 cm) (Pharmacia Biotech AB, Uppsala, Sweden). The purified MbFe(III) was diluted with the phosphate buffer to a concentration within the range of 130−210 μM as determined spectrophotometrically ($\epsilon_{525} = 7700$ $M^{-1}\cdot cm^{-1}$ (Andersen et al., 1988)). A 10.0 mL aliquot of the diluted MbFe(III) was prewarmed to 25.0 °C and mixed with a 1.0 mL aliquot of 8.0 mM H_2O_2 (standardized iodometrically) and allowed to react for 2 min. A 25 μL aliquot of catalase [7000 units, activity measured as described by Sigma (1995)] was added to cleave excess H_2O_2, and the solution was allowed to react for at least 5 min. The formation of MbFe(IV)═O was confirmed spectrophotometrically (Whitburn et al., 1984).

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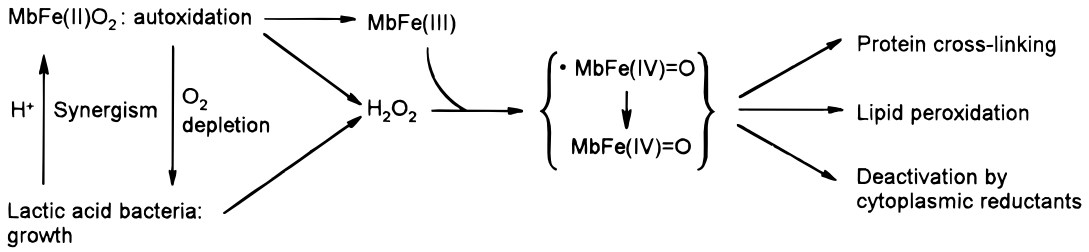

Figure 1. Ferrylmyoglobin as initiator of oxidative damage to proteins and lipids as the result of synergism between lactic acid bacteria growth and oxymyoglobin autoxidation. The initiation of lipid peroxidation and cross-linking of proteins by hypervalent iron is in living cell controlled by the presence of effective cytoplasmic reductants such as endogenous ascorbate.

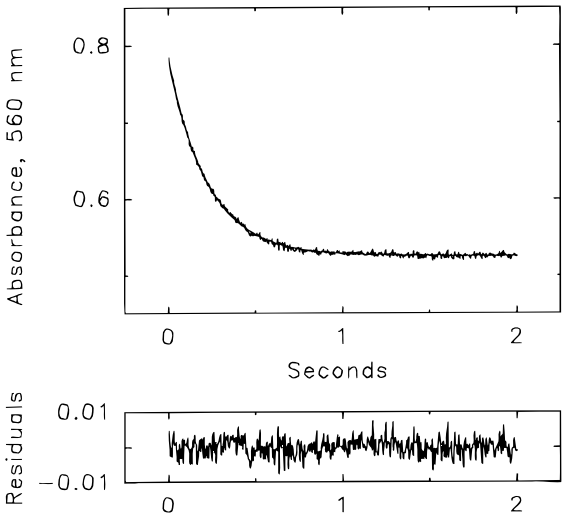

Figure 2. Relative absorbance at 560 nm during reaction between 7.5×10^{-5} M MbFe(IV)=O and 0.028 M ascorbate (total concentration) at pH 5.10 (0.020 M phthalate buffer) and 25.0 °C at ionic strength 0.16 (NaCl) using the stopped-flow technique. Lower panel shows residuals from a nonlinear regression analysis: $A(t) = a + b \exp(-k_{obs}t)$, from which a pseudo-first-order rate constant $k_{obs} = 4.43 \pm 0.02$ s^{-1} is obtained.

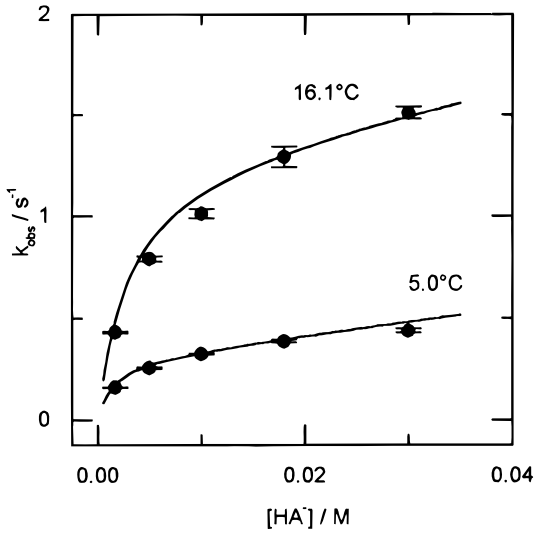

Figure 3. Pseudo-first-order rate constants, obtained as shown in Figure 2, as a function of ascorbate concentration at pH 5.34 at ionic strength 0.16 (NaCl). Experimental points are compared with full lines as calculated from the final kinetic parameters for the reaction mechanism depicted in Figure 4.

pH Measurements. In all experiments, 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$^+$]. pH was measured with a Metrohm 6.0234.100 combination glass electrode (Herisau, Switzerland), except for the determination of acid dissociation constants for ascorbic acid and D-isoascorbic acid.

Determination of Acid Dissociation Constants. The first acid dissociation constant (concentration basis) was determined for ascorbic acid and for D-isoascorbic acid at ionic strength 0.16 (±0.01) and four temperatures in the range 5.8−35.0 °C (±0.1 °C). The titration was carried out with a TitraLab 90 autotitrator (Radiometer, Copenhagen, Denmark), and the cell potential was measured with a Radiometer pHC2401 combination glass electrode.

Kinetic Experiments. Solutions of ascorbate were prepared by dissolving sodium ascorbate in NaCl-containing phosphate buffer and diluting with water shortly before use. For the solutions of D-isoascorbate, a solution of D-isoascorbic acid was made and a pH value in the range 7−9 achieved by titration with 1.0 M NaOH. The required volume of this solution was transferred to NaCl-containing phosphate buffer and diluted with water shortly before use. The stock solution was kept at 5 °C and used within the same day to limit autoxidation of D-isoascorbic acid.

MbFe(IV)=O solution and a buffer solution containing NaCl and either ascorbate or D-isoascorbate were placed in each syringe of a DX-17MV stopped-flow spectrofluorometer (Applied Photophysics, London, U.K.), and the reactions were followed by absorbance measurements as seen in Figure 2 at 560 nm, which is in the wavelength region with maximum difference between MbFe(IV)=O and MbFe(III). Other wavelengths were included in addition to 560 nm in some experiments, but no differences in rate constants were noted. In the reaction mixture, the buffer concentration was 0.020 M (phthalate or phosphate) and the ionic strength was 0.16 ± 0.01 adjusted with NaCl for all experiments. In all experiments, the ascorbate or D-isoascorbate concentration was in excess relative to MbFe(IV)=O by at least a factor of 30. For each combination, pH was measured in thermostated 1:1 mixtures of the MbFe(IV)=O and ascorbate/D-isoascorbate solutions.

Results of experiments, as those shown in Figure 3 for pH 5.34 and various temperatures and ascorbate concentrations, formed the basis of the design of the main investigation, which, for ascorbate, included a total of 476 experiments with 72 different combinations of pH, temperature, and ascorbate concentration. The comparison between ascorbate and D-isoascorbate was based on 159 further experiments with the latter isomer. Pseudo-first-order rate constants for the reactions were calculated by nonlinear regression analysis (the Marquardt−Levenberg algorithm) (Figure 2).

Product Identification, Myoglobin. Solutions of MbFe(IV)=O and ascorbate were mixed in the thermostated cell of an HP 8452 UV−vis diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA), yielding reaction mixtures with 3.6 $\times 10^{-3}$ M ascorbate and 1.0×10^{-4} M MbFe(IV)=O in 0.020 M buffer (phosphate or phtalate) at an ionic strength of 0.16, adjusted with NaCl. Spectra were recorded each 0.5−5 s (depending on reaction rate at the actual conditions), beginning 2 s after mixing and continuing to completion of the reaction. pH was subsequently measured in the cell. For each experiment the relative concentrations of MbFe(II), MbFe(II)O_2, and MbFe(III) were calculated according to the method of Krzywicki (1982).

Product Identification, Ascorbate. Solutions of MbFe(IV)=O and ascorbate were mixed at room temperature

Table 1. Acid Dissociation Constants[a] and Thermodynamic Parameters for Acid Dissociation of Ascorbic Acid and D-Isoascorbic Acid in Aqueous 0.16 M NaCl at 25.0 °C

	ascorbic acid[b]	D-isoascorbic acid[b]
pK_{a1}	3.772 ± 0.003	3.706 ± 0.003
$\Delta H^{\ominus}$ (kJ·mol^{-1})	5.8 ± 0.4	5.7 ± 0.5
$\Delta S^{\ominus}$ (J·mol^{-1}·K^{-1})	−53 ± 1	−52 ± 2

[a] Presented as $pK_a = -\log K_a$; K_a in M. [b] Parameter calculated from data at 5.8, 16.5, 25.2, and 35.0 °C using van't Hoff plots (not shown) and given with standard error of estimate.

(reaction mixture: 0.020 M phosphate buffer, 3.0×10^{-3} M ascorbate, 1.06×10^{-4} M MbFe(IV)═O, pH 7.3, ionic strength 0.16 adjusted with NaCl) , and the mixture was transferred by means of a flow system to a flat ESR cell mounted in a Bruker 4103 TM/9216 rectangular cavity (Bruker, Karlsruhe, Germany). The magnetic field was modulated with a frequency of 100.0000 kHz using 0.142 Gauss field modulation amplitude, 10 mW microwave power, and receiver gain of 5×10^5.

The experiment was repeated with MbFe(III) (5.2×10^{-5} M in the reaction mixture) instead of MbFe(IV)═O, with MbFe(IV)═O alone (1.06×10^{-4} M), with ascorbate alone (3.0×10^{-3} M), and with ascorbate (3.0×10^{-3} M) mixed with a solution of H_2O_2 (4.0×10^{-3} M) and catalase (0.16 g/L). The concentrations mentioned apply to the situation before transfer of the solutions to the flat ESR cell, as a minor degree of dilution would have occurred in the flow system. The experiment was performed in the same way each time to achieve the same degree of dilution.

Cyclic Voltammetry Experiment. Experiments were carried out at room temperature with a BAS CV-50W electrochemical analyzer (Bioanalytical Systems, Lafayette, IN). Cyclic voltammograms were acquired with a scan rate of 100 mV/s, a 3.0 mm platinum working electrode, and an Ag/AgCl/KCl (saturated) reference electrode. Ascorbic acid or D-isoascorbic acid was dissolved in phosphate buffer, pH 7.3, and diluted with water (final solution: 0.040 M phosphate, 3.0×10^{-3} M ascorbate or D-isoascorbate, ionic strength 0.16 adjusted with NaCl).

RESULTS

Acid Dissociation. The first pK_a values for ascorbic acid and D-isoascorbic acid were calculated from data points between 25% and 75% neutralization from full titration curves using six individual measurements. A total of eight titrations were made for each acid. $\Delta H^{\ominus}$ and $\Delta S^{\ominus}$ for the acid dissociation and pK_a at 25.0 °C, presented in Table 1, were calculated from van't Hoff plots, which were linear for both acids (accept at the 5% level) and significantly different (1% level) in the temperature range of interest. D-Isoascorbic acid is the more acidic of the epimers. However, the difference is small, and on the basis of the present data, no conclusion can be drawn as to whether the different acidities of ascorbic acid and D-isoascorbic acid are an enthalpy effect, an entropy effect, or a combination thereof.

The pK_a value was used to correct the ascorbate concentration used in further calculations for partial protonization at low pH at the actual temperature.

Kinetics of Reduction. The observed kinetics could be described by (pseudo) first-order reactions for all conditions (Figure 2). k_{obs}, the observed first-order rate constant, did, however, not depend linearly on ascorbate concentration at conditions of constant pH and temperature, and it was concluded that reduction of MbFe(IV)═O by ascorbate was not a simple second-order reaction, as opposed to what has been found for reduction of MbFe(IV)═O by β-lactoglobulin (Østdal et al., 1996). The autoreduction is for all conditions used

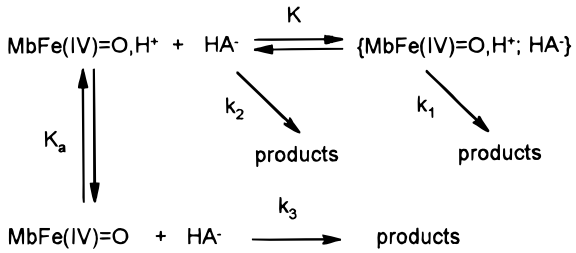

Figure 4. Kinetic scheme for reduction of ferrylmyoglobin by ascorbate in neutral and slightly acidic aqueous solution.

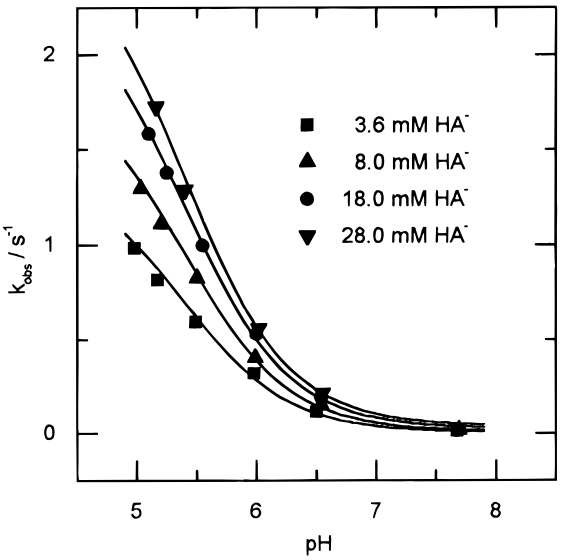

Figure 5. Dependence of k_{obs}, obtained as shown in Figure 2, on pH for four different ascorbate concentrations at 16.0 °C in aqueous solution of ionic strength 0.16 (NaCl). Full lines are calculated according to the kinetic scheme of Figure 4, including correction for ascorbate protonization, cf. eq 2.

significantly slower than the ascorbate reduction, and for the lowest pH (4.8) and the lowest excess of ascorbate (3.6 mM) no more than 0.2% (calculated for 25 °C) ferrylmyoglobin is autoreduced (Mikkelsen and Skibsted, 1995). Excess of H_2O_2 used for transformation of MbFe(III) to MbFe(IV)═O was found not to influence the rate of reduction of MbFe(IV)═O by ascorbate (tested by analysis of variance on observed rate constants for pH 7.37 and 8 mM ascorbate at 25 °C, for excess by factors of 2.9, 5.8, and 9.1).

For constant pH and temperature, and under pseudo-first order conditions, the increase in k_{obs} for MbFe(IV)═O reduction with increasing ascorbate concentration showed some indication of saturation kinetics with k_{obs} approaching a limiting value. However, as may be seen from the results from the experiments presented in Figure 3, a limiting value for k_{obs} is not reached, as k_{obs} above a certain concentration of ascorbate shows a further linear increase rather than a limiting value. Such kinetic behavior is indicative of parallel reactions, and the kinetic scheme of Figure 4 was found to accommodate the observed dependence of k_{obs} on ascorbate concentration and further on pH. The pH dependence of the reduction of MbFe(IV)═O by ascorbate was found to be very similar to that previously observed for reduction by NADH (Mikkelsen and Skibsted, 1995). k_{obs} increased with decreasing pH, as may be seen in Figure 5 for different concentrations of ascorbate. The increase in k_{obs} is less than a factor of 10 when pH is lowered by 1 unit, excluding specific acid catalysis, and the pH dependence of k_{obs} can rather be explained by a model,

in which MbFe(IV)=O exists in a protonated and a deprotonated form, both of which react with ascorbate. This model was previously proposed for the reduction of MbFe(IV)=O by NADH and included the reaction of the protonated as well as the deprotonated form of MbFe(IV)=O by both one- and two-electron reductions (Mikkelsen and Skibsted, 1995). The kinetic scheme of Figure 4 thus includes three parallel reactions, the relative importance of which for conditions of constant temperature depends on ascorbate concentration and pH: (i) a bimolecular and relatively slow reaction between ascorbate and MbFe(IV)=O; (ii) a bimolecular reaction between ascorbate and a protonated form of MbFe(IV)=O; and (iii) an intramolecular reaction of a complex formed between the protonated form of MbFe(IV)=O and ascorbate. The rate of reduction thus depends on protonization and complex formation of ferrylmyoglobin for conditions of excess of ascorbate according to the following rate expression:

$$\frac{-\mathrm{d[MbFe(IV)=O]}}{\mathrm{d}t} = k_1[\mathrm{MbFe(IV)=O,H^+;HA^-}] + k_2[\mathrm{MbFe(IV)=O,H^+}][\mathrm{HA^-}] + k_3[\mathrm{MbFe(IV)=O}] \times [\mathrm{HA^-}] = \left(k_1\frac{K[\mathrm{HA^-}]}{1+K[\mathrm{HA^-}]} + k_2[\mathrm{HA^-}]\right) \times [\mathrm{MbFe(IV)=O,H^+}] + k_3[\mathrm{HA^-}][\mathrm{MbFe(IV)=O}] = \left(\left(k_1\frac{K[\mathrm{HA^-}]}{1+K[\mathrm{HA^-}]} + k_2[\mathrm{HA^-}]\right)\frac{[\mathrm{H^+}]}{[\mathrm{H^+}]+K_a} + k_3[\mathrm{HA^-}]\frac{K_a}{[\mathrm{H^+}]+K_a}\right)[\mathrm{MbFe(IV)=O}]_{total} \quad (1)$$

K is the association constant for binding of ascorbate (HA^-) to the protonated form of ferrylmyoglobin [MbFe(IV)=O,H^+], and K_a is the acid dissociation constant of MbFe(IV)=O,H^+. The concentration of HA^- is in each case corrected for protonization by use of the pK_a determined for ascorbic acid. The following expression for k_{obs} is accordingly obtained:

$$k_{obs} = \left(k_1\frac{K[\mathrm{HA^-}]}{1+K[\mathrm{HA^-}]} + k_2[\mathrm{HA^-}]\right)\frac{[\mathrm{H^+}]}{[\mathrm{H^+}]+K_a} + k_3[\mathrm{HA^-}]\frac{K_a}{[\mathrm{H^+}]+K_a} \quad (2)$$

By nonlinear regression analysis, this model was found to account for the observed pseudo-first-order rate constant at the temperatures 5, 16, and 25 °C. Experiments were also carried out at 35 °C, but the rates obtained at that higher temperature were not reproducible, since the obtained pseudo-first-order rate constants very often decreased with an increase in the time the MbFe(IV)=O was held at 35 °C, as an indication of protein denaturation. Kinetic data at elevated temperature were accordingly not included in the final numerical analysis. The goodness-of-fit may be judged from Figures 3 and 5. In Figure 3 rate constants at constant pH and various ascorbate concentration are seen to be reproduced by the kinetic parameters obtained in the final numeric analysis, and the combination of monomolecular saturation kinetics (k_1 path) and parallel bimolecular reactions (k_2 and k_3 paths) is in agreement with the observed pseudo-first-order rate constants. From Figure 5 it is further seen that the variation in k_{obs} due to change in pH (and ascorbate concentration) is also accommodated by the model of Figure 4.

Nonlinear regression analysis of k_{obs} as function of pH and ascorbate concentration, at each of the three temperatures separately, gave estimates of the kinetic and thermodynamic parameters that, for all but k_2, obeyed the Arrhenius equation (in the case of k_1, k_2, and k_3) or the van't Hoff equation (in the case of K and K_a). The parameters in the Arrhenius equation (for each of the rate constants) and in the van't Hoff equation (for K and K_a) were recalculated by weighted least-squares estimation, where the weights were derived partly from the nonlinear regression analysis at each separate temperature and partly from an ordinary least-squares estimation in the Arrhenius or van't Hoff equation. In the case of K, K_a, k_1, and k_3, the weighting was strongly dominated by the standard errors of estimates calculated in the nonlinear regression analysis at separate temperatures, whereas the standard errors of estimates derived from the ordinary least-squares estimation in the Arrhenius equation were dominating in the case of k_2. Consequently, a t value of approximately 2 is appropriate when one is calculating a 95% confidence interval for k_1, k_3, K, and K_a, since these parameters were determined with more than 100 degrees of freedom, whereas a t value of 12.71 must be used in calculation of the 95% confidence interval for k_2, which was determined with only 1 degree of freedom. The final Arrhenius and van't Hoff plots may be seen in Figure 6.

The poor accuracy of k_2 is easily understood, as the fraction of ferrylmyoglobin being reduced by the k_2 path is small for all conditions, since the k_1 path is dominating at low pH for excess of ascorbate and the k_3 path is dominating at higher pH. The reaction enthalpies, $\Delta H^{\ominus}$, and the reaction entropies, $\Delta S^{\ominus}$, for the association of ascorbate to the protonated ferrylmyoglobin and for the acid dissociation of the protonated ferrylmyoglobin, derived from the van't Hoff plot of parts a and b, respectively, of Figure 6 may be found in Table 2. The activation enthalpies, $\Delta H^{\#}$, and the activation entropies, $\Delta S^{\#}$, for the three parallel reactions were calculated from the Arrhenius plots of Figures 6c−e using transition state theory and may be found in Table 3.

Product Identification. Analysis of absorption spectra of the reaction mixtures after completion of reactions showed that the major reaction product was MbFe(III). However, using the method of Krzywicki (1982), it was shown that approximately 18% MbFe(II)/MbFe(II)O_2 was formed together with MbFe(III) and that the MbFe(II)/MbFe(II)O_2 fraction increased slightly with decreasing pH. Calculation using the method of Miller et al. (1993) including *residual* MbFe(IV)=O gave very similar results with hardly significant levels of MbFe(IV)=O (4 to −1%). From the well-defined isosbestic points in the visible absorption spectra recorded during reduction of MbFe(IV)=O with ascorbate using fast diode array detection (as seen for one reaction mixture in Figure 7), it was concluded that the reaction consisted of a single step and that MbFe(II)/MbFe(II)=O_2 and MbFe(III) are formed in parallel reactions rather than by consecutive reduction of initially formed MbFe(III) to MbFe(II)/MbFe(II)O_2. This latter conclusion is further supported by the rate constants reported by Tsukahara and Yamamoto (1983) and Tsukahara (1986) for reduction of MbFe(III) to MbFe(II) by ascorbate, for which it is estimated that reduction of MbFe(III) under the present conditions is at least a factor of

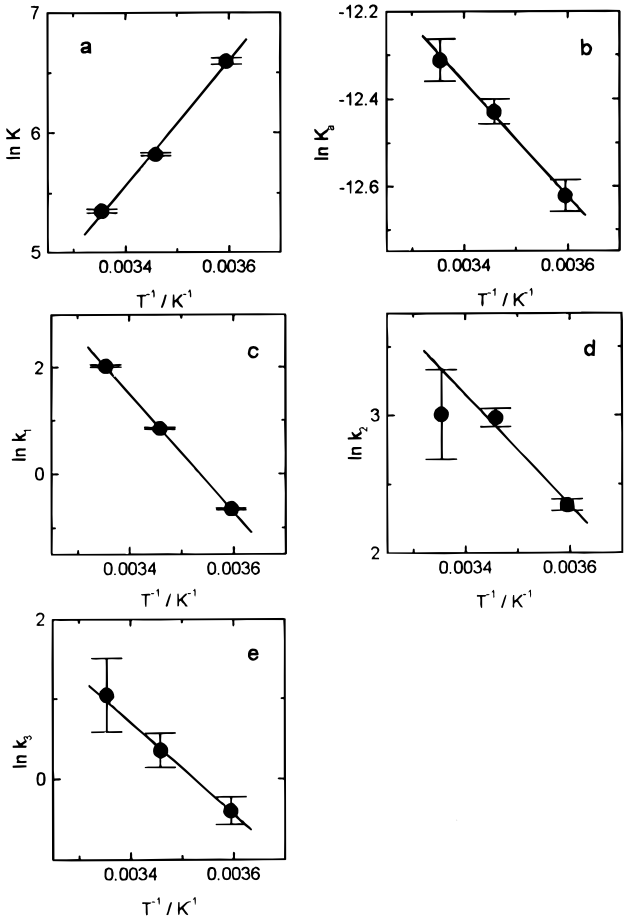

Figure 6. Temperature dependence of kinetic and thermodynamic parameters for reduction of ferrylmyoglobin by ascorbate as given by van't Hoff plots for (a) the association constant for binding of ascorbate to the protonated form of ferrylmyoglobin and for (b) the acid dissociation constant of the protonated form of ferrylmyoglobin, and Arrhenius plots for (c) the first-order rate constant for intramolecular electron transfer from ascorbate to the iron(IV) center in the ascorbate protonated ferrylmyoglobin complex, for (d) the second-order rate constant for the bimolecular reduction of protonated ferrylmyoglobin by ascorbate, and for (e) the second-order rate constant for the bimolecular reduction of ferrylmyoglobin by ascorbate.

Table 2. Association Constant of Ascorbate to Protonated Ferrylmyoglobin (K) and Acid Dissociation Constant[a] of Protonated Ferrylmyoglobin in Aqueous Solution at 25.0 °C with Ionic Strength 0.16 (NaCl) Together with Reaction Enthalpy and Reaction Entropy

	equilibrium constant[b] at 25.0 °C	$\Delta H^{\ominus}$ [b] (kJ·mol^{-1})	$\Delta S^{\ominus}$[b] (J·mol^{-1}·K^{-1})
K (M^{-1})	205 ± 9	−45 ± 3	−100 ± 9
pK_a	5.34 ± 0.02	11 ± 2	−66 ± 7

[a] Presented as pK_a = −log K_a; K_a in M. [b] Estimated from van't Hoff plot of Figure 6a,b according to $-\ln K = \Delta H^{\ominus}/RT - \Delta S^{\ominus}/R$ and given with standard error of estimate.

100 slower than reduction of MbFe(IV)═O to MbFe(III). Similar simultaneous one- and two-electron reductions of MbFe(IV)═O were previously demonstrated for NADH (Mikkelsen and Skibsted, 1995).

In agreement with the dominating one-electron reduction of MbFe(IV)═O, ascorbyl radical was detected by ESR spectroscopy using a flow method. The spectra seen in Figure 8, measured after 60 s of reaction under conditions with a half-life for MbFe(IV)═O of approximately 22 s, matched the spectrum reported by Laroff et al. (1972), with splitting constants of 1.76 G and approximately 0.19 G. MbFe(IV)═O was ESR silent for the magnetic field of interest, while ascorbate alone or together with a H_2O_2/catalase mixture resulted in very weak signals from ascorbyl radicals due to autoxidation, as previously described by Giulivi and Cadenas (1993). A signal of comparable low intensity was observed for a mixture of MbFe(III) and ascorbate, confirming the conclusion that MbFe(III) is not significantly being reduced by ascorbate under the present reaction conditions.

Table 3. Rate Constants and Activation Parameters at 25.0 °C for the Three Parallel Reaction Paths of Figure 4 for Reduction of Ferrylmyoglobin by Ascorbate in Aqueous Solution with Ionic Strength 0.16 (NaCl)

	rate constant[a] at 25.0 °C	$\Delta H^{\#a}$ (kJ·mol^{-1})	$\Delta S^{\#a}$ (J·mol^{-1}·K^{-1})
k_1 (s^{-1})	7.5 ± 0.1	89.6 ± 0.9	72 ± 3
k_2 (M^{-1}·s^{-1})	28 ± 5	31 ± 8	113 ± 28
k_3 (M^{-1}·s^{-1})	2.7 ± 0.8	45 ± 13	−85 ± 46

[a] Estimated from Arrhenius plot of Figure 6c−e according to $k = A\exp(-E_a/RT)$ using transition state theory and given with standard error of estimate.

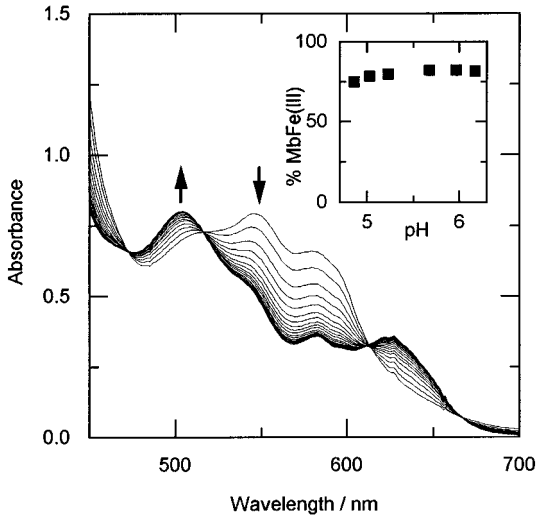

Figure 7. Absorption spectra in the visible region recorded in a 1 cm cuvette at 16.7 °C with 15 s interval following 2 s of mixing time, for an aqueous 9.4×10^{-5} M MbFe(IV)═O, 4.0×10^{-3} M ascorbate solution with pH 7.30 (0.020 M phosphate buffer) and ionic strength 0.16 (NaCl). Fourteen minutes elapsed from initiation of reaction to recording of the final spectrum. (Insert) Final spectra recorded at 25.0 °C, 9.2×10^{-5} M MbFe(IV)═O, 3.6 mM ascorbate, ionic strength 0.16 (NaCl), and 0.020 M phosphate or phthalate buffer analyzed according to the method of Krzywicki (1982) to obtain the distribution between MbFe(III) and MbFe(II)/MbFe(II)O_2, which is shown for conditions of different pH values.

Comparison of Ascorbate and D-Isoascorbate. No significant differences in the kinetics of reduction of ferrylmyoglobin by ascorbate or by D-isoascorbate could be detected, as seen from the observed rate constants presented in Figure 9 for both epimers together with the full line calculated from the kinetic parameters of Tables 2 and 3 determined for ascorbate. The very similar reducing properties of the two epimers were confirmed by cyclic voltammetry as may be seen from Figure 10. This documentation of comparable potentials for ascorbate and D-isoascorbate is in agreement with the findings of Iheanacho et al. (1995).

DISCUSSION

Ferrylmyoglobin has been shown to be reduced by ascorbate in a facile reaction (Figure 2) mainly by one-

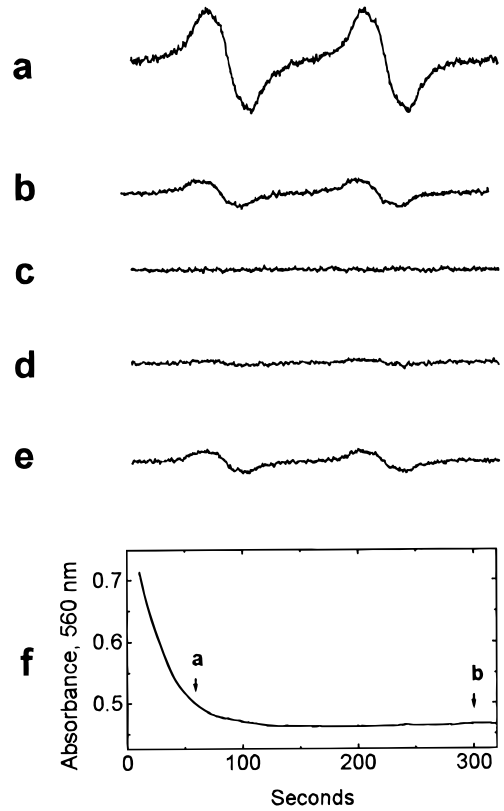

Figure 8. Electron spin resonance spectra of (a) a 1×10^{-4} M MbFe(IV)═O solution together with 3×10^{-3} M ascorbate, recorded 1 min after mixing, and (b) the same mixture recorded after 5 min of mixing; (c) a 1×10^{-4} M MbFe(IV)═O solution; (d) a 4×10^{-3} M H_2O_2 solution with catalase (0.16 g/L) and subsequent addition of ascorbate (3×10^{-3} M); (e) a 5×10^{-5} M MbFe(III) solution together with 3×10^{-3} M ascorbate. (f) shows the absorbance at 560 nm recorded spectrophotometrically for the reaction mixture used in (a) and (b). For all experiments common conditions of aqueous solution with pH 7.3 and ionic strength 0.16 (NaCl) were used. Spectrum e cannot quantitatively be compared to spectra a−d, as the pigment concentration is different. However, the height of the signals in (e) is approximately half of the heigth in (b), in agreement with the results of Guilivi and Cadenas (1993).

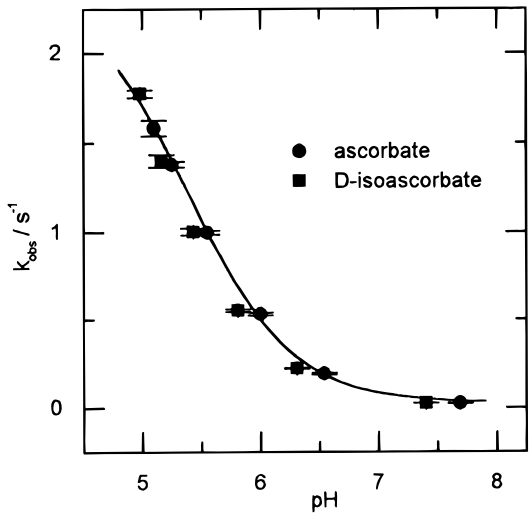

Figure 9. Comparison of pseudo-first-order rate constants for reduction of ferrylmyoglobin by ascorbate and D-isoascorbate [16.0 °C, 18 mM ascorbate/D-isoascorbate, ionic strength 0.16 (NaCl)]. The full line is calculated from the ascorbate parameters of Tables 2 and 3 according to the model of Figure 4.

electron transfer to yield the iron(III) pigment metmyoglobin and to a lesser degree by two-electron transfer

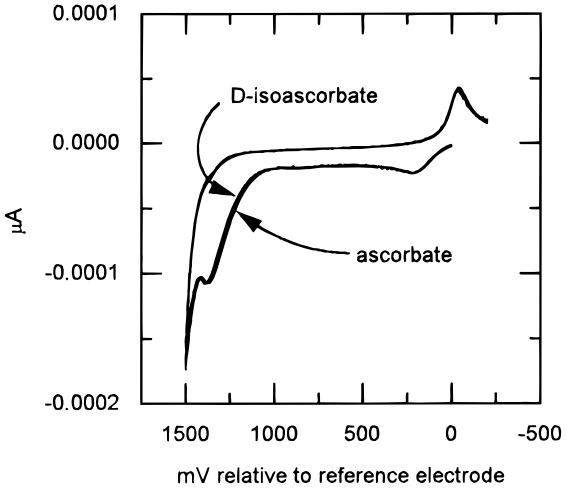

Figure 10. Superimposed cyclic voltammograms of ascorbate and D-isoascorbate in aqueous solution with pH 7.3 and ionic strength 0.16 (NaCl) at room temperature.

to yield the iron(II) pigments myoglobin/oxymyoglobin (Figure 7). Since ferrylmyoglobin is believed to be a prooxidant involved in initiation of lipid peroxidation (Kanner and Harel, 1985a,b) and in protein cross-linking (Hanan and Shaklai, 1995), the efficiency of ascorbate as an antioxidant in meat products may depend at least partly on deactivation of ferrylmyoglobin. In this context it should also be noted that we found no significant differences in the reducing potential (Figure 10) or in the rate of reduction (Figure 9) between ascorbate and D-isoascorbate under conditions of importance for meat products, a finding that may be of importance for a possible substitution of ascorbate with D-isoascorbate. The acid/base properties of the two epimers are also very similar (Table 1), and from the pH profile for deactivation of ferrylmyoglobin (Figures 5 and 9) it is further seen that the deactivation is most efficient under low-pH conditions. Notably, these conditions are also the conditions under which H_2O_2 is produced most rapidly in meats, as may be concluded from Figure 1 (Skibsted et al., 1994). The function of ascorbate/D-isoascorbate as antioxidants seems accordingly to match the conditions developing in meat products during storage.

Ascorbate is oxidized by ferrylmyoglobin mainly in one-electron processes, as has been demonstrated by visible spectroscopy (Figure 7) and by ESR spectroscopy (Figure 8). The ascorbyl radical ($A^{\bullet -}$) formed by one-electron oxidation of ascorbate (HA^-) according to the stoichiometry

$$\text{MbFe(IV)═O} + HA^- + H^+ \rightarrow \text{MbFe(III)} + A^{\bullet -} + H_2O \quad (3)$$

and detected during reaction (Figure 8) is subject to disproportionation to yield dehydroascorbate (A):

$$2A^{\bullet -} + H^+ \rightarrow HA^- + A \quad (4)$$

The deactivation of ferrylmyoglobin shows for the pH conditions developing in meat products a dependence on ascorbate concentration (Figure 3) with a characteristic "leveling off" in rate at concentrations representative for product formulations [$c_{\text{myoglobin}} = 0.05$ mM and $c_{\text{asc}} = 2$ mM (Andersen et al., 1990)]. The mechanism presented in Figure 4 with ascorbate binding to ferrylmyoglobin accommodates these findings, which

should also be of practical importance, as any increase in ascorbate addition above this level has less impact on deactivation of hypervalent iron. In contrast, such addition may enforce prooxidative effects of ascorbate by the Fenton reaction.

The rate of reduction of ferrylmyoglobin by ascorbate has been measured previously, and Giulivi and Cadenas (1993) report a second-order rate constant of $1.6 \pm 0.4 \times 10^6$ $M^{-1}\cdot s^{-1}$ for unspecified conditions. For more well-defined conditions Laranjinha et al. (1995) report an initial rate of $(1.32 \pm 0.1) \times 10^{-5}$ $M\cdot min^{-1}$ valid at the somewhat elevated temperature 37 °C, pH 7.4, for concentrations for both ascorbate and MbFe(IV)=O of 1.0×10^{-5} M. Calculations based on the model of Figure 4 together with the data of Tables 2 and 3 yield a rate of 1.8×10^{-7} $M\cdot min^{-1}$. A more qualitative comparison between different reductants yields a series of decreasing efficiencies with which ferrylmyoglobin is reduced in aqueous solution: cysteine (Romero et al., 1992) > ergothioneine (Romero et al., 1992) > lipoate, glutathione > dihydrolipoate (Romero et al., 1992), caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid (Laranjinha et al., 1995) > Trolox C (Giulivi et al., 1992; Laranjinha et al., 1995), ascorbate, D-isoascorbate, NADH (Mikkelsen and Skibsted, 1995) > menadione–glutathione conjugate (Buffinton et al., 1988), *p*-benzoquinone, 1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, 2,3-epoxy-1,4-naphthoquinone (Buffinton and Cadenas, 1988) > α-tocopherol (Giulivi et al., 1992) > native *β*-lactoglobulin (Østdal et al., 1996). Although the ranking shows a certain pattern with the hydrophilic compounds normally considered to be efficient antioxidants also being most efficient as deactivators of ferrylmyoglobin, it is difficult to make any definitive conclusions, because the experimental conditions have been very different. It should also be noted that only the present investigation of ascorbate (and D-isoascorbate) together with the investigation of NADH (Mikkelsen and Skibsted, 1995) and the investigation of *β*-lactoglobulin (Østdal et al., 1996) has utilized systematic variation of concentration of reductor and pH to obtain mechanistic information. Except for ascorbate (Table 3), activation parameters are only available for *β*-lactoglobulin (Østdal et al., 1996) and for the latter reductant only for the reaction path dominating at neutral pH.

The dependence of rate of reduction of ferrylmyoglobin by ascorbate on pH (Figure 5) and on ascorbate concentration (Figures 3 and 5) was accommodated by the mechanism presented in Figure 4. For temperature conditions of relevance to meat storage the influence of temperature on the rate of reaction could be accounted for (Figure 6) by the reaction enthalpies and reaction entropies of Table 2 and by the activation parameters of Table 3. At higher temperature the kinetics probably were affected by protein denaturation, resulting in less reproducible rates. The protonization of ferrylmyoglobin at low pH resulting in rate increase corresponds to $pK_a = 5.34 \pm 0.02$, a value very similar to the value $pK_a = 4.94 \pm 0.12$ deduced from the rate of reduction of MbFe(IV)=O by NADH at different pH values (Mikkelsen and Skibsted, 1995), and to the value $pK_a \sim 5$ deduced from similar experiments with *β*-lactoglobulin (Østdal et al., 1996), thus confirming that MbFe(IV)=O rather than the individual reductants (ascorbate, NADH or *β*-lactoglobulin) is protonated to facilitate electron transfer. However, the identification of the site of protonization on ferrylmyoglobin will have to await further studies, although a direct protonization of the iron–oxo center

$$\mathrm{MbFe(IV){=}O} + \mathrm{H_3O^+} \rightleftharpoons (^{\bullet+}\mathrm{Mb})\mathrm{Fe(III){-}OH} + \mathrm{H_2O} \quad (5)$$

resulting in formation of a myoglobin radical ($^{\bullet+}$Mb) as a reactive oxidant is in agreement with our experimental findings. A similar mechanism involving a cation radical has recently been suggested for microperoxidase 8 to accommodate the pH dependence of the observed rate of reduction of the compound II form (Low et al., 1996). The presence of a cation-radical form of ferrylmyoglobin could explain the radical signal observed as long as 90 min after addition of H_2O_2 to MbFe(III) (Cooper et al., 1994). The reaction entropy for acid dissociation (reaction from right to left in eq 5: $\Delta S^{\ominus} = -66$ $J\cdot mol^{-1}\cdot K^{-1}$; Table 2) is thus comparable to the likewise negative value ($\Delta S^{\ominus} = -54$ $J\cdot mol^{-1}\cdot K^{-1}$) found for acid dissociation of metmyoglobin (Andersen et al., 1988):

$$\mathrm{MbFe(III)OH_2} + \mathrm{H_2O} \rightleftharpoons \mathrm{MbFe(III)OH} + \mathrm{H_3O^+} \quad (6)$$

Association of ascorbate to the protonated form of ferrylmyoglobin is a highly exothermic process ($\Delta H^{\ominus} = -45$ $kJ\cdot mol^{-1}$; Table 2) and the binding is significantly counteracted by a large negative reaction entropy ($\Delta S^{\ominus} = -100$ $J\cdot mol^{-1}\cdot K^{-1}$), indicating a substantial degree of organization of the protein upon ascorbate binding. However, we found no difference in the Soret absorption band intensity or position ($\lambda_{max} = 414$ nm) when comparing MbFe(IV)=O with and without the presence of ascorbate by stopped-flow spectoscopy (pH 5.5, 9.6 °C, 8.0 mM ascorbate, measured after 3 ms) in agreement with the finding for binding of nitrite (a stronger field ligand) to MbFe(III) (Arendt et al., 1996). When compared to the reaction of eq 5, a negative reaction entropy is expected for a direct binding of the negatively charged ascorbate to the iron(III) center in the protonated form of ferrylmyoglobin resulting in reorganization

$$(^{\bullet+}\mathrm{Mb})\mathrm{Fe(III){-}OH} + \mathrm{HA^-} \rightleftharpoons \{\mathrm{MbFe(IV){=}O,H_2A}\} \quad (7)$$

The electron transfer within the complex

$$\{\mathrm{MbFe(IV){=}O,H_2A}\} \xrightarrow{k_1} \{\mathrm{MbFe(III){-}OH,HA^{\bullet}}\} \quad (8)$$

which then becomes rate-determining has a high enthalpy barrier ($\Delta H^{\#} = 89.6$ $kJ\cdot mol^{-1}$; Table 3), but the process is favored by a positive entropy of activation ($\Delta S^{\#} = 72$ $J\cdot mol^{-1}\cdot K^{-1}$). The positive entropy of activation is indicative of a transition state with loss of structure, and this transition state is probably similar to ($^{\bullet+}$Mb)Fe(III)–OH according to the positive reaction entropy (reaction of eq 7 from right to left) for the electron-transfer process to yield this radical from {MbFe(IV)=O,H_2A}. Apparently, two possible electron-transfer processes are available for the latter complex with ascorbate/ascorbic acid in the heme cleft: (i) abstraction of an electron from the protein to yield a myoglobin cation radical or (ii) abstraction of an electron from the coordinated ascorbic acid to yield the ascorbyl radical. The latter process is further driven by dissociation of the ascorbyl radical (Figure 8) and subsequent disproportionation, in effect making the complex rela-

tively short-lived (half-life of 0.1 s at 25 °C as calculated from the parameters of Table 3). The binding of ascorbate to the hypervalent form of myoglobin is considered to be an important observation, and we intend to study the properties of this complex further at low-temperature conditions by ESR spectroscopy to obtain information of the electron distribution within this protein complex.

The bimolecular reaction paths (corresponding to the k_2 and k_3 rate constants of Figure 4 and Table 3) both have smaller enthalpy barriers and the reaction may take place as the result of an encounter between ascorbate and ferrylmyoglobin by an outer-sphere electron transfer. For the k_2 path a reaction between the protein-radical form $^{\bullet+}$MbFe(III)—OH and ascorbate is suggested in agreement with the small enthalpy of activation observed ($\Delta H^{\#} = 31$ kJ·mol^{-1}, Table 3). For the reaction dominating in neutral solution (k_3 path) it is noteworthy that the parameters of activation ($\Delta H^{\#} = 45$ kJ·mol^{-1} and $\Delta S^{\#} = -85$ J·mol^{-1}·K^{-1}) are similar to the activation parameters for reduction of ferrylmyoglobin by β-lactoglobulin [$\Delta H^{\#} = 45$ kJ·mol^{-1} and $\Delta S^{\#} = -93$ J·mol^{-1}·K^{-1} (Østdal et al., 1996)]. This observation strongly suggests that for these conditions, an intramolecular process in ferrylmyoglobin is becoming rate-determining. We suggest that noncatalyzed electron transfer (in contrast to the acid-catalyzed process of eq 5) is controlling the rate of reduction of ferrylmyoglobin.

$$\text{MbFe(IV)=O} \rightarrow {}^{\bullet+}\text{MbFe(III)—O}^{-} \qquad (9)$$

The negative entropy of activation ($\Delta S^{\#} = -85$ J·mol^{-1}·K^{-1}; Table 3) is expected on the basis of a comparison with the reaction entropy for the reactions of eqs 5 and 6. This latter conclusion appears to be of utmost importance for an understanding of the role of hypervalent iron pigments as initiator of oxidative damage in tissue under physiological conditions, and we are currently investigating the temperature dependence of the reduction of ferrylmyoglobin by a variety of substrates of biological relevance to identify a possible common rate-determining step. In contrast, the lower pH of meat favors another reaction path for deactivation of hypervalent iron compared to physiological conditions. This other mechanism entails saturation kinetics, and no further protection will result from addition of ascorbate/D-isoascorbate above a certain level, above which prooxidative effects rather may become dominant. The binding of ascorbate is increasing with lower temperature (exothermic process), making the saturation effect even more important for frozen storage.

ABBREVIATIONS USED

MbFe(III), metmyoglobin; MbFe(II), myoglobin; MbFe(II)O_2, oxymyoglobin; MbFe(IV)=O, ferrylmyoglobin; $^{\bullet}$MbFe(IV)=O, perferrylmyoglobin; HA^{-}, ascorbate; A, dehydroascorbate; $A^{\bullet-}$, ascorbyl radical; $^{\bullet+}$Mb, myoglobin radical; $\Delta H^{\ominus}$, molar reaction enthalpy; $\Delta S^{\ominus}$, molar reaction entropy; $\Delta H^{\#}$, molar activation enthalpy; $\Delta S^{\#}$, molar activation entropy.

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