Protein Binding in Deactivation of Ferrylmyoglobin by Chlorogenate and Ascorbate

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Kinetics of reduction of iron(IV) in ferrylmyoglobin by chlorogenate in neutral or moderately acidic aqueous solutions (0.16 M NaCl) to yield metmyoglobin was studied using stopped flow absorption spectroscopy. The reaction occurs by direct bimolecular electron transfer with $(2.7 \pm 0.3) \times 10^3$ $M^{-1}\cdot s^{-1}$ at 25.0 °C ($\Delta H^{\#} = 59 \pm 6$ kJ·mol^{-1}, $\Delta S^{\#} = 15 \pm 22$ J·mol^{-1}·K^{-1}) for protonated ferrylmyoglobin ($pK_a = 4.95$) and with 216 ± 50 $M^{-1}\cdot s^{-1}$ ($\Delta H^{\#} = 73 \pm 8$ kJ·mol^{-1}, $\Delta S^{\#} = 41 \pm 30$ J·mol^{-1}·K^{-1}) for nonprotonated ferrylmyoglobin in parallel with reduction of a chlorogenate/ferrylmyoglobin complex by a second chlorogenate molecule with $(8.6 \pm 1.1) \times 10^2$ $M^{-1}\cdot s^{-1}$ ($\Delta H^{\#} = 74 \pm 8$ kJ·mol^{-1}, $\Delta S^{\#} = 59 \pm 28$ J·mol^{-1}·K^{-1}) for protonated ferrylmyoglobin and with 61 ± 9 $M^{-1}\cdot s^{-1}$ ($\Delta H^{\#} = 82 \pm 12$ kJ·mol^{-1}, $\Delta S^{\#} = 63 \pm 41$ J·mol^{-1}·K^{-1}) for nonprotonated ferrylmyoglobin. Previously published data on ascorbate reduction of ferrylmyoglobin are reevaluated according to a similar mechanism. For both protonated and nonprotonated ferrylmyoglobin the binding constant of chlorogenate is ~300 M^{-1}, and the modulation of ferrylmyoglobin as an oxidant by chlorogenate (or ascorbate) leads to a novel antioxidant interaction for reduction of ferrylmyoglobin by ascorbate in mixtures with chlorogenate.

Keywords: *Myoglobin; chlorogenic acid; ascorbic acid; protein binding; antioxidant interaction*

INTRODUCTION

Hypervalent iron compounds derived from myoglobin may catalyze oxidation in living muscles and in meat products (Harel and Kanner, 1985; Newman et al., 1991), and in model systems they are found to oxidize lipids, proteins, and reducing cofactors (Kelman et al., 1994; Rao et al., 1994; Østdal et al., 1996; Mikkelsen and Skibsted, 1995). Oxidized by hydrogen peroxide, metmyoglobin yields perferrylmyoglobin, $^{+\bullet}$MbFe(IV)═O, a protein radical with iron in the 4+ state, which rapidly decays, forming the longer lived ferrylmyoglobin, MbFe(IV)═O (George and Irvine, 1956; Davies, 1991). Ferrylmyoglobin is found to reduce slowly in the absence of external reducing agents to yield oxidatively modified metmyoglobin (King and Winfield, 1963; Mikkelsen and Skibsted, 1995; Kröger-Ohlsen et al., 1999). External reducing agents, however, are capable of reducing ferrylmyoglobin in reactions occurring more rapidly than this so-called autoreduction (Laranjinha et al., 1995; Jørgensen et al., 1997; Kröger-Ohlsen and Skibsted, 1997); hence, they possess an antioxidative effect by deactivating the prooxidant as long as the reactivity of the antioxidant radicals produced is low.

Recent results indicate that MbFe(IV)═O, due to a largely unknown reaction mechanism, rather than $^{+\bullet}$MbFe(IV)═O, initiates lipid oxidation at pH values relevant for meat and biological systems (Rao et al., 1994; Baron et al., 1997). This observation necessitates detailed descriptions of the deactivation of ferrylmyoglobin by various reductants in order to understand the role of myoglobin in oxidative deterioration of foods and biological systems. Among the reducing agents shown to reduce ferrylmyoglobin to metmyoglobin or oxymyoglobin are cinnamic acid derivatives, including chlorogenic acid (Laranjinha et al., 1995). Chlorogenic acid, which is also shown to be an effective reductant of peroxynitrite, superoxide radicals, hydroxyl radicals, and lipid peroxyl radicals (Kono et al., 1997), possesses two ortho phenolic groups as part of a larger conjugated system and is water soluble due to the hydrophilic quinic acid group. An antioxidative effect of chlorogenic acid is thus anticipated from the molecular structure, and the reduction potential of +0.37 V for the oxidized form of chlorogenate at neutral pH (Jørgensen and Skibsted, 1998) indicates that reduction of protein and lipid radicals by chlorogenate is thermodynamically favorable.

The present kinetic study was undertaken to obtain mechanistic information of the reaction between chlorogenate and ferrylmyoglobin and to compare the mechanism for chlorogenate, as an example of a plant phenol, to that of other potential reductants such as ascorbate in the reduction of ferrylmyoglobin. Whereas ascorbate as a vitamin is a nutritive antioxidant, chlorogenate is a non-nutritive antioxidant, and the interaction between the two antioxidants in the reduction of ferrylmyoglobin was further studied to contribute to a better understanding of the mechanisms of antioxidant interaction.

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MATERIALS AND METHODS

Chemicals. Metmyoglobin [MbFe(III), horse heart, type III] and chlorogenic acid [1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate)] were obtained from Sigma Chemical Co. (St. Louis, MO). L-(+)-Ascorbic acid sodium salt was from Fluka Chemie AG (Buchs, Switzerland). Analytical grade hydrogen peroxide was from Riedle-de-Haën (Selze, Germany). HCl (0.0200 M) was purchased from Bie & Berntsen Laboratory (Bie & Berntsen A/S, Rødovre, Denmark). All other chemicals (analytical grade) were from Merck (Darmstadt, Germany). Water was purified through a Millipore Q-Plus purification train (Millipore Corp., Bedford, MA).

Synthesis of Ferrylmyoglobin. MbFe(III) dissolved in 5.0 mM phosphate buffer (ionic strength 0.16 adjusted with NaCl) was purified on a Shephadex G25 column (40 × 2.5 cm, Pharmacia Biotech AB, Uppsala, Sweden). The eluted MbFe(III) was diluted with the phosphate buffer to yield a ~0.1 mM solution. Ferrylmyoglobin was formed by reaction of MbFe(III) with a 3 M excess of H_2O_2 for 3 min. Excess H_2O_2 was removed by separation on a Shephadex G25 PD-10 column (Pharmacia Biotech AB). The identity of the eluted MbFe(IV)═O (~0.1 mM) was confirmed by absorption spectroscopy (Whitburn et al., 1982), and the solution was used immediately.

pH Measurements. 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 Hamilton 640.238-100 combination glass electrode (Hamilton Bonaduz AG, Bonaduz, Switzerland) connected to a Metrohm 713 pH-meter (Metrohm, Herisau, Switzerland).

Kinetic Experiments. Stock solutions of sodium chlorogenate (166.7 mM) were prepared by neutralizing the free acid in water with NaOH and diluting with water. A stock solution was used within 2 days, after which time no degradation of the chlorogenate molecule could be detected spectrophotometrically. Stock solutions of sodium ascorbate were prepared by dissolving sodium ascorbate in water immediately before use. From the stock solutions buffered sodium chlorogenate or sodium ascorbate solutions were made shortly before use by appropriate dilution with phthalate or phosphate buffer solutions and adjustment to a final ionic strength of 0.16 with NaCl. MbFe(IV)═O solution and chlorogenate or ascorbate solution or solution with both reductants were placed in each syringe of a DX-17MW stopped flow spectrofluorometer (Applied Photophysics, London, U.K.), and the reactions were followed by absorbance measurements at 580 nm as seen in Figure 1. Pseudo-first-order rate constants for the reactions were calculated by nonlinear regression analysis (the Marquardt−Levenberg algorithm). In the reaction mixtures the chlorogenate or ascorbate concentration was in excess relative to MbFe(IV)═O by at least a factor of 25, the buffer concentration was 20 mM, and the ionic strength was 0.16 ± 0.01 adjusted with NaCl. In the study of the reduction of MbFe(IV)═O by chlorogenate a total of 119 different combinations of pH, chlorogenate concentration, and temperature resulted in 1252 individual stopped flow experiments. To investigate the reduction of MbFe(IV)═O by ascorbate and chlorogenate when present together, 9 combinations of ascorbate and chlorogenate concentrations resulted in 90 stopped flow experiments (pH 6.08, 25.0 °C). For each combination pH was measured in thermostated 1:1 mixtures of the chlorogenate/ascorbate and MbFe(IV)═O solutions. Additional stopped flow experiments were performed at 560 nm, but no significant differences from the observed rate constants at 580 nm were observed.

Identification of the Reaction Product. The myoglobin reaction product was identified by recording absorption spectra in the region 450−700 nm during the reaction between MbFe(IV)═O and chlorogenate (Figure 2) using an HP8453 UV−vis diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) or the DX-17MW stopped flow spectrofluorometer (Applied Photophysics) equipped with a photodiode array detector. Then, in accordance with the method used by Jørgensen and Skibsted (1998), the distribution between

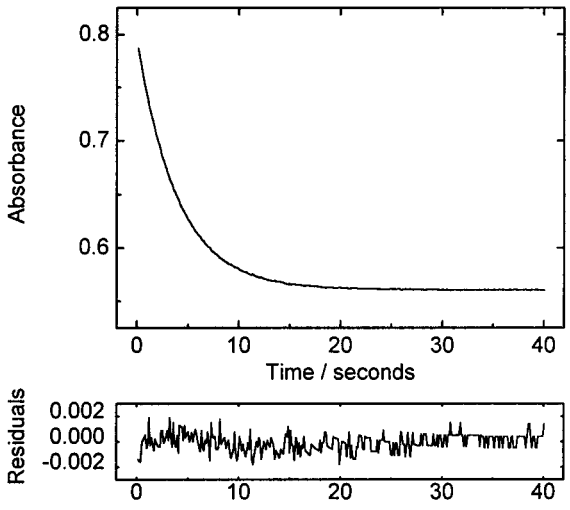

Figure 1. Relative absorbance at 580 nm during reaction between 0.0500 mM MbFe(IV)═O and 15.0 mM chlorogenate (total concentrations) at pH 6.61 (20 mM phosphate buffer) and 5.0 °C at ionic strength 0.16 (NaCl) using the stopped flow technique. Lower panel shows residuals from a nonlinear regression analysis: $A(t) = a + b \exp(-k_{obs}t)$, from which a pseudo-first-order rate constant $k_{obs} = 0.250 \pm 0.001$ s^{-1} was obtained.

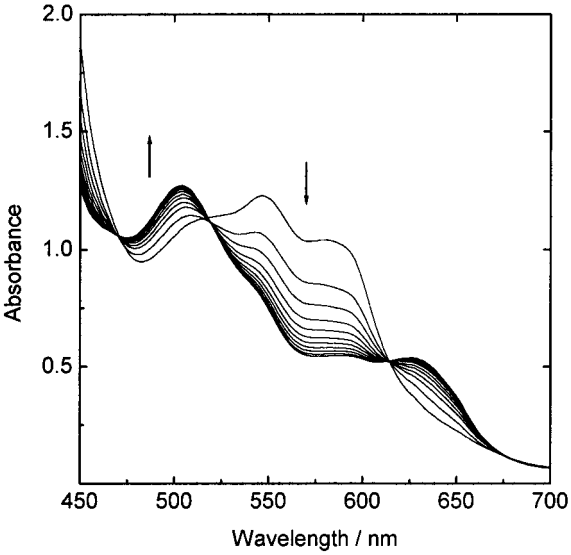

Figure 2. Absorption spectra in the visible region of an aqueous 120 μM MbFe(IV)═O/0.05 mM chlorogenate solution with pH 7.19 (20 mM phosphate buffer) and ionic strength 0.16 (NaCl). Spectra are recorded at 30 s intervals following 2 s of mixing time in a 1 cm cuvette at 25.1 °C. Five minutes elapsed from initiation of the reaction to recording of the final spectrum.

MbFe(III) and MbFe(II)O_2 in the product solution was calculated from the spectral data. Reaction stoichiometry was determined by optical titrations as described by Jørgensen and Skibsted (1998) using the HP8453 UV−vis diode array spectrophotometer.

Water/Octanol Partition Experiments. Partition experiments were designed as recommended by Hansch and Leo (1995). Solutions of chlorogenic acid in aqueous 0.0100 M HCl saturated with octanol were placed together with octanol saturated with aqueous 0.0100 M HCl in the ratio 4:1 in closed bottles. The bottles were placed in a water bath (at 25 °C) with constant magnetic stirring, and the chlorogenic acid was allowed to equilibrate between the two phases for 1 h. After equilibration, the remaining chlorogenic acid in the water phase was determined spectrophotometrically at 261 nm (Cary 13, UV−vis spectrophotometer, Varian, Harbor City, CA) in a

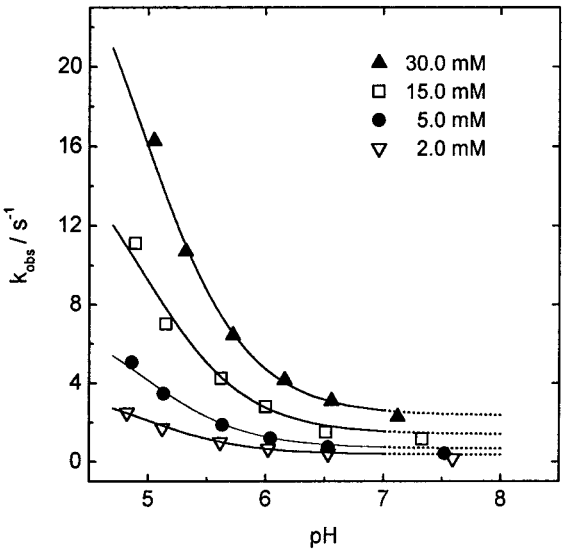

Figure 3. Dependence of k_{obs} obtained as shown in Figure 1 on pH for four different chlorogenate concentrations at 25.0 °C in aqueous solution of ionic strength 0.16 (NaCl). Full lines are calculated according to the estimated parameters (Tables 1 and 2) for the final kinetic model in eq 4. Dotted lines indicate extrapolation of parameters for the reaction model found to be valid for pH values <7.0.

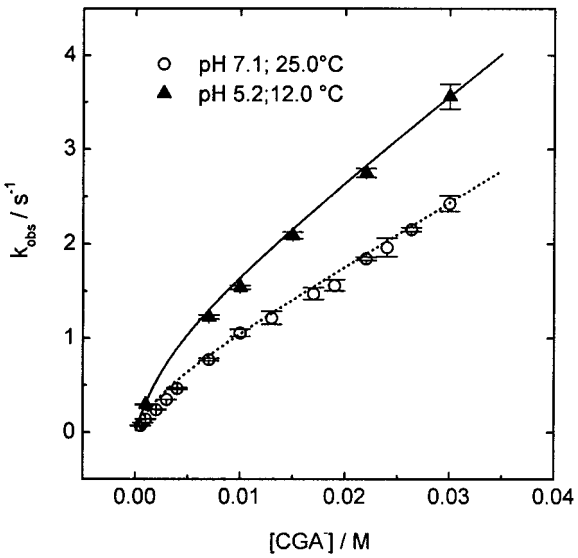

Figure 4. Pseudo-first-order rate constants obtained as shown in Figure 1 as a function of chlorogenate concentration at pH 5.2 and 12.0 °C and at pH 7.1 and 25.0 °C, for both conditions at ionic strength 0.16 (NaCl). Experimental points are compared with the lines as calculated from the final kinetic parameters given in Tables 1 and 2. The dotted line indicates extrapolation of parameters for the reaction model found to be valid for pH values <7.0.

0.1 cm quartz cuvette using a standard curve (0.03−3.0 mM chlorogenic acid in aqueous 0.0100 M HCl saturated with octanol).

Simulation of Reduction Kinetics. Simulation of reduction kinetics was carried out using the Pro/K software package (Applied Photophysics).

RESULTS

The observed kinetics for the reduction of MbFe(IV)═O by chlorogenate, ascorbate, or mixtures of chlorogenate and ascorbate in excess could be described by (pseudo)-first-order reactions for all conditions (as shown in Figure 1 for one example). The reaction product of MbFe(IV)═O formed by reduction by chlorogenate, for which a final absorption spectrum is shown in Figure 2, was identified as MbFe(III), and <5% MbFe(II)O_2 was formed from MbFe(IV)═O in a parallel or subsequent reaction, thus indicating minor influence on the calculated rate constants for the main reduction. k_{obs} increased with decreasing pH, as may be seen in Figure 3 for different concentrations of chlorogenate. This pH dependence of the reduction of MbFe(IV)═O by chlorogenate is very similar to that previously observed for the reduction by NADH (Mikkelsen and Skibsted, 1995) and by ascorbate (Kröger-Ohlsen and Skibsted, 1997). Thus, the pH dependence of the reduction is described by a reaction model including an acid−base equilibrium of ferrylmyoglobin with an acid dissociation constant, K_a, rather than by a model in which the reaction is specifically acid catalyzed (Figure 3). As may be further seen in Figure 4, k_{obs} did not depend linearly on the chlorogenate concentration at conditions of constant pH and temperature, and the mechanism of the reaction between MbFe(IV)═O and chlorogenate is therefore not explained by a simple second-order reaction. Autoreduction of MbFe(IV)═O, being specifically acid catalyzed, is for all experimental conditions used significantly slower than the reduction by chlorogenate. At pH 4.9, which is the lowest pH value included in this investigation, the half-life, $t_{1/2}$, of the autoreduction of MbFe(IV)═O is 2 min (Mikkelsen and Skibsted, 1995), and autoreduction constitutes no more than 2.6% of the total reduction for the slowest reaction with chlorogenate at this pH (with a chlorogenate concentration of 2.0 mM and a temperature of 5.0 °C). At higher pH values the difference between $t_{1/2}$ for the autoreduction and $t_{1/2}$ for the reduction by chlorogenate is even larger.

The deviation from linearity in Figure 4 may be the result of a dimerization of the chlorogenate molecules in aqueous solution as shown in

$$2CGA^- \rightleftarrows (CGA{-}CGA)^{2-} \qquad (1)$$

CGA^- is chlorogenate, and $(CGA{-}CGA)^{2-}$ is a chlorogenate dimer. If the dimerized form reduces MbFe(IV)═O at a rate other than that at which chlorogenate does, the value of k_{obs} will be affected as dimerization occurs. Such a dimerization in aqueous solution is among other molecules with both a hydrophilic and a hydrophobic part observed for the pigment carminic acid, where the phenomenon was recognized from derivations from Lambert Beer's law for the absorption of visible light (Stapelfeldt et al., 1993). The degree of dimerization decreases with increasing dilution, and measurement of the UV−visible absorbance of aqueous solutions of chlorogenate in a relevant concentration region proved not to be possible because chlorogenate absorbs strongly in the UV area; an upper limit of 4 mM was found for reliable absorption measurements, even for a light path of 0.1 cm. Instead, water/octanol partition experiments were carried out, in which a dimerization or polymerization of chlorogenate in the water phase will result in a dependence of the calculated partition coefficient, P_{CGA}, for the distribution of chlorogenate between the octanol and the water phase on the concentration of chlorogenate (apparent deviation from Nernst's distribution law). For conditions of no dimerization of chlorogenate the following equation describes the

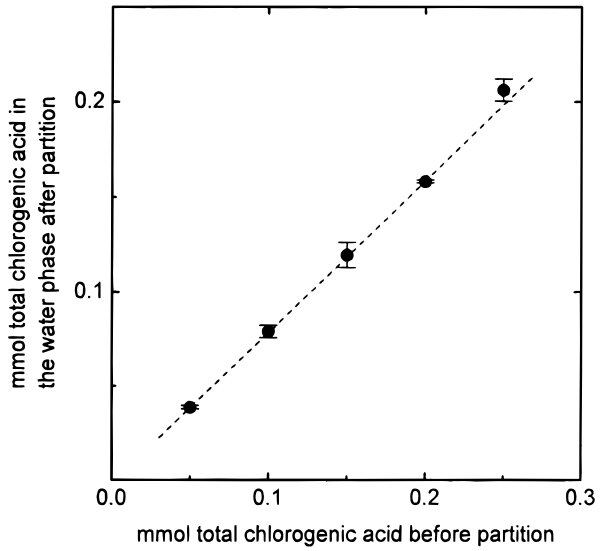

Figure 5. Partition of chlorogenic acid between 0.0100 M HCl and octanol at 25 °C. The linear regression confirms that eq 2 is obeyed, and a water/octanol partition coefficient, $\log(P_{CGA})$, of -0.09 ± 0.10 for chlorogenic acid at pH 2.0 and 25 °C is calculated.

distribution between the two phases:

$$n_{CGA_w^-} = \left(1 + \frac{P_{CGA}V_o}{V_w}\right)^{-1} n_{CGA^-}^{total} \quad (2)$$

In eq 2 n_{CGA_w} is the number of moles of chlorogenate in the water phase, n_{CGA}^{total} is the total number of moles of chlorogenate in the two phases, and V_o and V_w are the volumes of the octanol phase and the water phase, respectively. However, if dimerization occurs, eq 2 will no longer be valid, resulting in a deviation from linearity when n_{CGA_w} is plotted against n_{CGA}^{total} for conditions of constant volumes V_o and V_w. Chlorogenate is an anion and is expected to have little solubility in the octanol phase, and the experiments were therefore carried out at pH 2.0, where the molecule exists only as chlorogenic acid (CGA^- in eq 2 replaced by HCGA). The results from a series of water/octanol partition experiments are shown in Figure 5, and no deviation from linearity is observed. Hence, it was concluded that chlorogenic acid does not dimerize in aqueous solution and further that chlorogenate, which due to the presence of a negative charge and charge repulsion is assumed to have a lower tendency to dimerize than chlorogenic acid, does not dimerize in aqueous solutions in the concentration range used in the kinetic experiments.

A deviation from linearity when k_{obs} is plotted against the concentration of the reducing agent (as in Figure 4) was previously observed for the reduction of MbFe(IV)=O by ascorbate (Kröger-Ohlsen and Skibsted, 1997). This deviation was explained by a binding of ascorbate to protonated ferrylmyoglobin in an MbFe(IV)=O,H^+–ascorbate complex, resulting in the observed saturation kinetics with k_{obs} almost approaching a limiting value. The same extent of saturation kinetics is not seen for chlorogenate reduction (Figure 4), and therefore a model in which chlorogenate binds to ferrylmyoglobin prior to reduction does not solely account for the observed kinetics. Instead, a reaction scheme with reaction of a ferrylmyoglobin–chlorogenate complex with another chlorogenate molecule in parallel with direct reduction included could account for the further linear increase in k_{obs} with higher chlorogenate concentrations (Figure 4). On the basis of these considerations a general kinetic scheme (Figure 6) of the reduction of ferrylmyoglobin by electron-donating agents is proposed. From this generalized scheme different rate expressions taking one or more of the six reaction paths in Figure 6 into account were derived for conditions of constant temperature and excess chlorogenate (or other reductants) relative to ferrylmyoglobin. To find a suitable kinetic model to account for the observed kinetics the parameters of the resulting rate expressions were fitted to the experimental data by nonlinear regression analysis. Preliminary analysis was indicative of the presence of variance heterogeneity among the observations, and therefore a logarithm transformation was applied on both sides of the statistical model. Moreover, all of the observations with the same pH values and chlorogenate concentration were considered correlated. This assumption had, however, little effect on the final parameter estimates but resulted in more precise estimates of standard errors and thereby in a more satisfactory estimation from a statistical point of view. The results did not seem to depend on the chosen correlation structure, and therefore a more general correlation structure (autocorrelation and compound symmetry) was chosen (Diggle et al., 1994). The model including the reactions paths k_1, k_3, k_4, and k_6 in Figure 6 was found to accommodate the experimental observations for k_{obs} at various pH values and chlorogenate concentrations when pH <7.0. The final model thus includes four reactions, the relative importance of which for conditions of constant temperature depends on chlorogenate concentration and pH: (i) a bimolecular reaction between chlorogenate and a protonated form of MbFe(IV)=O; (ii) a bimolecular reaction between chlorogenate and a complex formed between a protonated form of MbFe(IV)=O and chlorogenate; (iii) a bimolecular reaction between chlorogenate and MbFe(IV)=O; and (iv) a bimolecular reaction between chlorogenate and a complex formed between MbFe(IV)=O and chlorogenate. The rate of reduction therefore depends on protonization and a complex formation of ferrylmyoglobin for conditions of excess chlorogenate according to the following rate expression:

$$\begin{aligned}\frac{-dC_{MbFe(IV)=O}^{total}}{dt} &= k_1[MbFe(IV)=O,H^+]\cdot[CGA^-] + \\ &\quad k_3[MbFe(IV)=O,H^+;CGA^-]\cdot[CGA^-] + \\ &\quad k_4[MbFe(IV)=O]\cdot[CGA^-] + \\ &\quad k_6[MbFe(IV)=O;CGA^-]\cdot[CGA^-] \\ &= \left(\left(k_1\frac{[CGA^-]}{1+K_p[CGA^-]} + k_3\frac{K_p[CGA^-]^2}{1+K_p[CGA^-]}\right)\cdot\right. \\ &\quad \frac{[H^+]}{[H^+]+K_a} + \left(k_4\frac{[CGA^-]}{1+K[CGA^-]} + \right. \\ &\quad \left.\left. k_6\frac{K[CGA^-]^2}{1+K[CGA^-]}\right)\cdot\frac{K_a}{[H^+]+K_a}\right)C_{MbFe(IV)=O}^{total} \quad (3)\end{aligned}$$

K_p is the association constant for binding of chlorogenate (CGA^-) to the protonated form of ferrylmyoglobin [MbFe(IV)=O,H^+], K is the association constant for binding of CGA^- to the nonprotonated form of ferrylmyoglobin [MbFe(IV)=O], and K_a is the acid dissociation

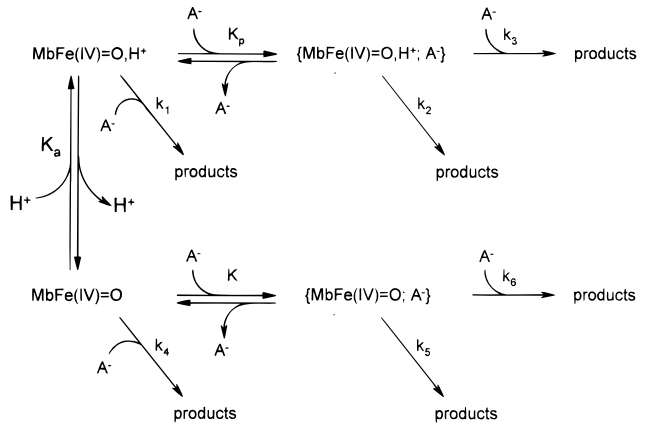

Figure 6. General kinetic scheme for reduction of ferrylmyoglobin by electron-donating species in neutral and slightly acidic solutions.

constant of MbFe(IV)═O,H^+. k_1, k_3, k_4, and k_6 are the rate constants for the four pathways in Figure 6 contributing significantly to the actual model. The following expression for k_{obs} is accordingly obtained:

$$k_{obs} = \left(k_1\frac{[CGA^-]}{1 + K_p[CGA^-]} + k_3\frac{K_p[CGA^-]^2}{1 + K_p[CGA^-]}\right)\frac{[H^+]}{[H^+] + K_a} + \left(k_4\frac{[CGA^-]}{1 + K[CGA^-]} + k_6\frac{K[CGA^-]^2}{1 + K[CGA^-]}\right)\frac{K_a}{[H^+] + K_a} \quad (4)$$

Nonlinear regression analysis of the model corresponding to eq 4 at each of the four different temperatures included in the investigation gave an estimation of the rate and the equilibrium constants, for which the temperature dependence should be accounted for by the Arrhenius and van't Hoff equations, respectively.

The values of the estimates of the rate and equilibrium constants are plotted in the Arrhenius and van't Hoff plots shown in Figure 7 for each parameter in the model corresponding to eq 4. The full lines in the plots show the ordinary linear regression analysis between the rate or equilibrium constants estimated from each of the four temperatures. The final activation parameters in the Arrhenius equation and the final reaction parameters in the van't Hoff equation were estimated by adapting the linear dependence of the logarithm of the parameters on the reciprocal temperature directly in the eq 4 and repeating the estimation of all parameters and their temperature dependence including observations at all four temperatures in the same calculation. The results are shown as the dotted lines in the plots in Figure 7. By incorporating the temperature dependence directly into eq 4, introduction of variability due to an estimation of the rate and equilibrium constants at each temperature, separately, was thus avoided. Furthermore, it was possible to test the linearity of the estimates at different temperatures (temperature dependence according to the Arrhenius and van't Hoff equations) by a likelihood ratio test. The hypothesis of such linearity for all of the parameters was not rejected, although Figure 7 shows small deviations in some cases. Consequently, on the basis of the statistical analysis, the model given by eq 4 was found to account for the observed pseudo-first-order rate constants at all of the temperatures 5, 12, 19, and 25 °C, when pH <7.0. The goodness of fit may be judged from Figures 3 and 4. From Figure 3 it is seen that values of k_{obs} at constant chlorogenate concentrations and various pH values are reproduced by the kinetic parameters obtained in the numerical analysis, and the combination of the four reaction paths k_1, k_3, k_4, and k_6 in Figure 6 are in agreement with the observed pseudo-first-order rate constants at pH <7.0. Furthermore, in Figure 4 the variation in k_{obs} due to changes in chlorogenate concentration is accommodated by the model corresponding to eq 4 when the two concentration profiles with constant pH values of 5.2 and 7.1, respectively, are considered. The estimated values for the equilibrium constants at 25 °C, the reaction enthalpies, $\Delta H°$, and the reaction entropies, $\Delta S°$ are given in Table 1; the rate constants at 25 °C together with activation enthalpies, $\Delta H^{\#}$, and the activation entropies, $\Delta S^{\#}$, for the four reaction paths may be found in Table 2. Unfortunately, it was not possible to propose a kinetic scheme resulting in a model describing the variations in experimental data in the full pH interval investigated. This is probably due to the chlorogenate dianion, which, because the pK_{c2} value of chlorogenic acid is 8.12 (Timberlake, 1959), becomes important when pH approaches this value. The chlorogenate dianion may react differently and probably more quickly as reductant in some of the four reaction paths than chlorogenate does, thus resulting in k_{obs} values different from those which would have been observed if chlorogenate alone was dominating the reaction solutions at higher pH values. To elucidate this problem, more rate data above pH 7.0 are clearly needed, optimally ranging well above pK_{c2} for chlorogenic acid, from which it would be possible to account for reactions of both chlorogenate and the chlorogenate dianion with MbFe(IV)═O. Such an approach, which was considered out of the scope of the present study with its focus on meat systems, would result in a much more complicated expression for k_{obs} than the one shown in expression 4. Stoichiometry experiments (not shown) indicated a dominating stoichiometry of 2:1 [MbFe(IV)═O relative to chlorogenate]; that is, chlorogenate molecules are through one-electron transfers to MbFe(IV)═O groups oxidized to semiquinone radicals, which are expected to disproportionate or to be further oxidized to quinones by additional one-electron transfers to another MbFe(IV)═O molecule. Recently, formation of semiquinone radicals has also been proposed by Giulivi and Cadenas (1998) in the reduction of ferrylmyoglobin by adrenaline. However, the stoichiometry observed in the present study has to be explored further including ESR spectroscopy, as the preliminary experiments indicate a different stoichiometry when pH approaches a physiological value, probably because the chlorogenate dianion at these pH values affects the reaction stoichiometry through stabilization of the semiquinone radical in alkaline solution.

Kinetic data from the previous study of reduction of ferrylmyoglobin by ascorbate (Kröger-Ohlsen and Skibsted, 1997) were reevaluated according to the general reaction scheme in Figure 6, because the model originally proposed (erroneously) did not account for lowering in the concentration of free ferrylmyoglobin due to complex binding of ferrylmyoglobin with ascorbate. It was found that the more generalized reaction mecha-

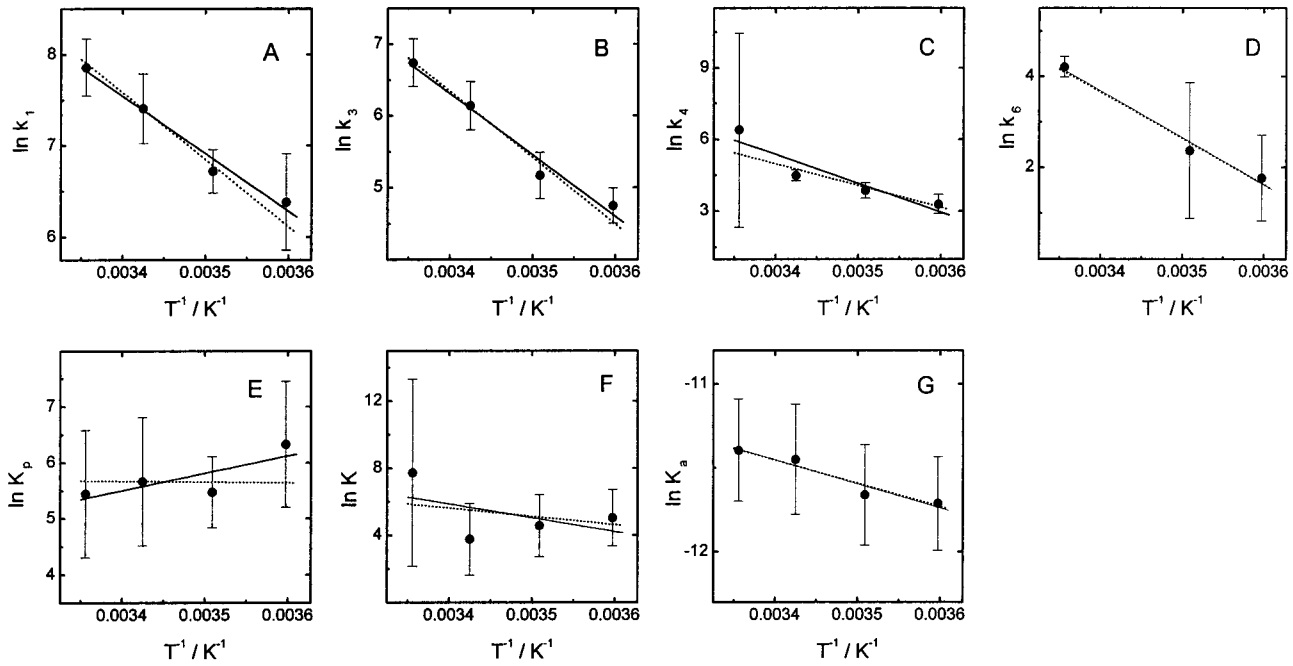

Figure 7. Temperature dependence of kinetic and thermodynamic parameters for reduction of ferrylmyoglobin by chlorogenate. Arrhenius plots are given for (A) second-order rate constant for the bimolecular reduction of protonated ferrylmyoglobin by chlorogenate, for (B) second-order rate constant for the reaction between the complex of chlorogenate with protonated ferrylmyoglobin and another molecule of chlorogenate, for (C) second-order rate constant for the bimolecular reduction of ferrylmyoglobin by chlorogenate, and for (D) second-order rate constant for the reaction between the chlorogenate ferrylmyoglobin complex and another molecule of chlorogenate. van't Hoff plots are given for (E) association constant for binding of chlorogenate to the protonated form of ferrylmyoglobin, for (F) association constant for binding of chlorogenate to the nonprotonated form of ferrylmyoglobin, and for (G) acid dissociation constant of the protonated form of ferrylmyoglobin. Full lines are obtained by linear regression from the data at each temperature (shown with 95% confidence intervals). Dotted lines are calculated from the reaction and activation parameter values (Tables 1 and 2) obtained when the temperature dependence is directly included in the overall numeric analysis. The value for k_6 at 19.0 °C is not significantly estimated and is therefore not included.

nism proposed for chlorogenate also gave a better description of the ascorbate data than the model previously used, with the modification that complex formation occurs at acidic pH only, as was also the case in the original mechanism (Kröger-Ohlsen and Skibsted, 1997). The expression for k_{obs} for the reduction of ferrylmyoglobin by excess ascorbate is therefore by analogy with eq 4

$$k_{obs} = \left(k_1 \frac{[\text{ascorbate}]}{1 + K_p[\text{ascorbate}]} + k_3 \frac{K_p[\text{ascorbate}]^2}{1 + K_p[\text{ascorbate}]} \right) \frac{[H^+]}{[H^+] + K_a} + k_4[\text{ascorbate}] \frac{K_a}{[H^+] + K_a} \quad (5)$$

Final parameter estimates are given in Tables 1 and 2, which should replace the parameters previously published by Kröger-Ohlsen and Skibsted (1997). The modification based on the more generalized model has minor effects on the estimated equilibrium constants K_a and K_p and their thermodynamic parameters, except for the reaction enthalpy and entropy of K_p, which in the modified model is characterized by a less negative reaction enthalpy and a negative reaction entropy. The rate constant dominating at alkaline pH (k_4) seems unchanged by the modification of the model; however, the activation parameters for this parameter could not be estimated satisfactorily. For lower pH reaction paths other than those originally published were found to dominate the reduction, and intramolecular reduction of the ascorbate complex of protonated MbFe(IV)═O is concluded to be insignificant and replaced by a reaction in which a second ascorbate is required for reduction of the initially formed complex.

Table 1. Equilibrium Constants at 25.0 °C and Reaction Enthalpy and Reaction Entropy for the Equilibria of Importance for the Reduction of Ferrylmyoglobin by Chlorogenate and Ascorbate in Aqueous Solution with Ionic Strength 0.16 (NaCl)[a]

equilibrium	reducing agent	equilibrium constant at 25.0 °C[b]	$\Delta H°$ [b] (kJ·mol^{-1})	$\Delta S°$ [b] (J·mol^{-1}·K^{-1})
K_p (M^{-1})	chlorogenate	291 ± 129	−1 ± 21	51 ± 72
	ascorbate	217 ± 12	−27 ± 4	−45 ± 28
K (M^{-1})	chlorogenate	343 ± 247	39 ± 26	186 ± 92
	ascorbate	ns		
pK_a	chlorogenate	4.95 ± 0.05[c]	9 ± 7	−56 ± 26
	ascorbate	5.29 ± 0.01[c]	12 ± 3	−61 ± 19

[a] For identification of equilibria, see Figure 6. ns indicates nonsignificant binding. Parameters for ascorbate are recalculated on the basis of kinetic data previously published (Kröger-Ohlsen and Skibsted, 1997). [b] Estimated using the van't Hoff equation and given with standard error of estimate. [c] Acid dissociation constants presented as p$K_a = -\log K_a$.

With the rather detailed knowledge of reduction of MbFe(IV)═O by chlorogenate and ascorbate, it was further studied how they interact when both are present as reductants for MbFe(IV)═O. Ferrylmyoglobin was mixed either with ascorbate alone or with ascorbate together with chlorogenate in a constant concentration. As may be seen in Figure 8, a negative effect on reduction rate was found for the combined action of

Table 2. Rate Constants and Activation Parameters at 25.0 °C for the Reaction Paths of Importance for the Reduction of Ferrylmyoglobin by Chlorogenate or Ascorbate in Aqueous Solution with Ionic Strength 0.16 (NaCl)[a]

reaction path	reducing agent	rate constant[b] at 25.0 °C	$\Delta H^{\#b}$ (kJ·mol^{-1})	$\Delta S^{\#b}$ (J·mol^{-1}·K^{-1})
k_1 ($M^{-1}\cdot s^{-1}$)	chlorogenate	2714 ± 339	59 ± 6	15 ± 22
	ascorbate	1732 ± 18	62 ± 5	24 ± 18
k_2 (s^{-1})	chlorogenate	ns		
	ascorbate	ns		
k_3 ($M^{-1}\cdot s^{-1}$)	chlorogenate	858 ± 106	74 ± 8	59 ± 28
	ascorbate	26 ± 7	40 ± 14	−84 ± 48
k_4 ($M^{-1}\cdot s^{-1}$)	chlorogenate	216 ± 50	73 ± 8	41 ± 30
	ascorbate	2.9 ± 0.7	51 ± 35	−63 ± 120
k_5 (s^{-1})	chlorogenate	ns		
	ascorbate	ns		
k_6 ($M^{-1}\cdot s^{-1}$)	chlorogenate	61 ± 9	82 ± 12	63 ± 41
	ascorbate	ns		

[a] For identification of reaction paths, see Figure 6, n.s. indicates nonsignificant reduction path. Parameters for ascorbate are recalculated on the basis of kinetic data previously published (Kröger-Ohlsen and Skibsted, 1997). [b] Estimated using the transition state theory and given with standard error of estimate.

ascorbate and chlorogenate on MbFe(IV)═O, resulting in smaller values for the observed rate constants than expected if the individual contributions of the two reductants were additive. The values of k_{obs} measured when ascorbate was alone as reductant were in agreement with the values predicted from the above expression of eq 5 with the parameters of Tables 1 and 2. Preliminary simulations of reduction kinetics of ferrylmyoglobin by ascorbate and chlorogenate (either alone or together), using the reaction mechanisms proposed in the present study, reproduced the lack of additivity observed experimentally, thus inviting further speculations.

DISCUSSION

The heme group in myoglobin is identical to the heme group in peroxidases, and although the physiological function of myoglobin as an oxygen binding protein is different from that of peroxidases, myoglobin is able to undergo reaction cycles similar to those undergone by peroxidases. The myoglobin molecule is, however, altered during the reaction following activation by peroxides, and myoglobin is accordingly often classified as a pseudoperoxidase (King and Winfield, 1963). Peroxidation of lipids seems to be initiated by MbFe(IV)═O, which is an analogue of peroxidase compound **II**, rather than by $^{+\bullet}$MbFe(IV)═O, the analogue to compound **I** (Baron et al., 1997). Deactivation of MbFe(IV)═O by different groups of antioxidants accordingly deserves further attention, especially because the mechanism of reduction of the hypervalent iron is largely unknown (Everse, 1998).

Reduction of MbFe(IV)═O by ascorbate as an important nutritive antioxidant has previously been found to occur through several parallel reactions and to be accelerated by decreasing pH (Kröger-Ohlsen and Skibsted, 1997). Chlorogenate, an example of a non-nutritive antioxidant, was for all conditions investigated found to be a more efficient reductant than ascorbate. This is an important observation, because ascorbate thermodynamically is a better reductant than chlorogenate. By

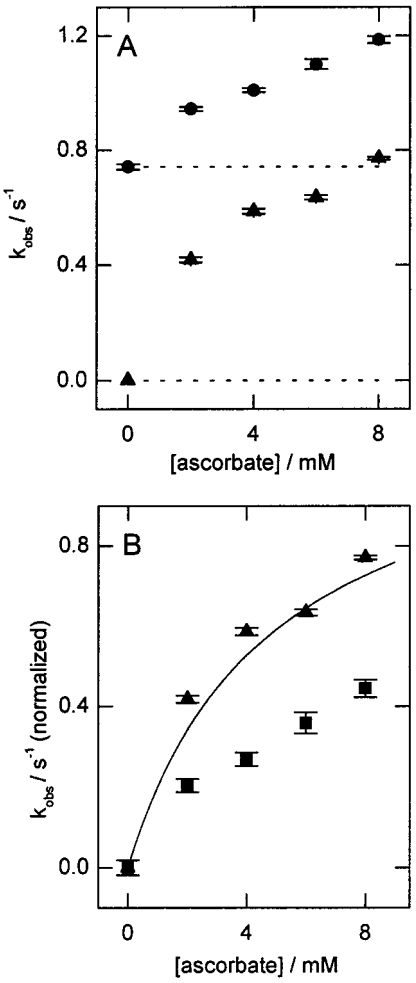

Figure 8. Reduction of MbFe(IV)═O by ascorbate in the presence of chlorogenate at 25.0 °C, pH 6.08, I = 0.16: (A) observed pseudo-first-order rate constants for reduction by ascorbate (triangles) and by ascorbate in the presence of 2.5 mM chlorogenate (circles) (dotted lines, virtual baselines for the contribution by chlorogenate to k_{obs}); (B) observed pseudo-first-order rate constants for the reduction by ascorbate (triangles, same points as shown in panel A). k_{obs} was determined for the reduction by ascorbate in the presence of chlorogenate and normalized by subtraction of the contribution from chlorogenate (squares, virtual baselines subtracted). The full line was calculated from the ascorbate parameters of Tables 1 and 2 according to eq 5.

cyclic voltammetry the reduction potential for the oxidized form of ascorbate and chlorogenate was thus found to have the values +0.22 V and +0.37 V, respectively, in aqueous solution with pH 7.4 at 25 °C (Jørgensen and Skibsted, 1998).

On the basis of the dependence of the observed pseudo-first-order rate constant for the reduction of MbFe(VI)═O by chlorogenate on pH (in the region of relevance for the living muscle and for meat), chlorogenate concentration (for conditions of excess chlorogenate), and temperature, the generalized reaction scheme of Figure 6 is suggested for the reduction of MbFe(IV)═O by various reductants. By a detailed numerical analysis based on calculation at each temperature followed by an analysis including all temperatures in the final calculations, it was concluded that protonated MbFe(IV)═O was reduced in a bimolecular reaction (k_1) in parallel with a reaction in which one chlorogenate is forming a complex with MbFe(IV)═O prior to reduction by a second molecule of chlorogenate

(k_3). The protonated form of MbFe(IV)═O was found to be in an acid−base equilibrium with the nonprotonated form with a pK_a of 4.95 ± 0.05 (a value very similar to the values of pK_a = 5.30 and pK_a = 4.94 previously found in studies of the kinetic of reduction by ascorbate and NADH, respectively), and the nonprotonated MbFe(IV)═O was further found to be reduced in a direct bimolecular reaction (k_4) and in a reaction between a chlorogenate complex of nonprotonated MbFe(IV)═O and a second molecule of chlorogenate (k_6). For both of the two complexes of ferrylmyoglobin with chlorogenate, reduction by intramolecular transfer from chlorogenate was found to be insignificant (k_2 and k_5 in Figure 6).

When the rate data previously reported for ascorbate reduction of MbFe(IV)═O were analyzed according to the more general model of Figure 6, the same reactions were found to be significant for ascorbate as for chlorogenate except that reduction of a complex between nonprotonated MbFe(IV)═O by a second molecule of ascorbate could not be detected.

The protonization of MbFe(IV)═O enhances autoreduction to MbFe(III), and an ESR signal (with g = 2.003) has been assigned to a radical intermediate resulting from protonization of the ferryl group (Kröger-Ohlsen et al., 1999):

$$H^+ + \text{MbFe(IV)}{=}\text{O} \rightarrow {}^{+\bullet}\text{MbFe(III)}{-}\text{OH} \qquad (6)$$

Intramolecular electron transfer to the iron(IV) center was found to be surprisingly slow in MbFe(IV)═O without initial protonization (Fenwick et al., 1997), and as may be concluded from the results for ascorbate and chlorogenate, protonization also facilitates intermolecular electron transfer from the external reductant. Protonization also appears to have a leveling effect on the rate of electron transfer for chlorogenate and ascorbate, because direct reduction (the k_1 path) of protonated ferrylmyoglobin by chlorogenate occurs with a comparable rate as for ascorbate in contrast to what is the case for the nonprotonated form (k_4 path), for which reduction by chlorogenate is almost 2 orders of magnitude more rapid than for ascorbate. A mechanistic explanation for this leveling effect caused by protonization is not available, but on the basis of the very similar activation parameters for the k_1 path for chlorogenate and ascorbate, it may be speculated that a cation radical formed on the protein surface by the reaction of eq 6 is less discriminating against different anionic reductants as the result of an important electrostatic contribution (charge neutralization). A further consequence of the effect of protonization is that the prooxidative effect of ferrylmyoglobin is more pronounced in meat than in the living muscle tissue.

Besides the direct reduction of MbFe(IV)═O and its conjugate acid, another type of reduction is important in which one molecule of reductant is forming a complex with the hypervalent heme pigment prior to reduction by a second molecule. This mechanism finds a parallel to what has been observed for oxidation of iodide by the compound **II** form of lactoperoxidase (Maguire and Dunford, 1972). At least two aspects of this mechanism deserve attention. First, the reductant bound to the strong oxidant does not seem to reduce the iron(IV) center by an intramolecular electron transfer (k_2 path), because for both chlorogenate and ascorbate reduction of the complex of the protonated form of MbFe(IV)═O is not significant. Second, the ascorbate or the chlorogenate complex of the protonated MbFe(IV)═O is being reduced by the second molecule at a considerably slower rate than that for reduction of the noncomplexed, protonated form of MbFe(IV)═O. The same difference is noted for reduction of the nonprotonated MbFe(IV)═O by chlorogenate. Ascorbate was not found to bind to the nonprotonated form of MbFe(IV)═O in contrast to chlorogenate, most likely due to the higher hydrophilicity of ascorbate compared to chlorogenate.

The absence of intramolecular electron transfer from the bound ascorbate or chlorogenate to the iron(IV) center indicates that the binding site for the reductant is different from the site for electron transfer into the protein. With the present knowledge it is not possible to identify the two sites on the myoglobin. However, the binding of one mole of reductant modulates the kinetics significantly, and an understanding of this effect of binding of a plant phenol as chlorogenate or of ascorbate to the hypervalent heme pigment on the efficiency of MbFe(IV)═O as an oxidant is an important goal for our current research. For chlorogenate it is seen that this effect largely is due to an increased enthalpy of activation, as $\Delta H^{\#}$ is increased by 15 kJ·mol^{-1} for the protonated form and by 9 kJ·mol^{-1} for the nonprotonated form.

The mechanism entailing binding of the antioxidants to the heme pigment results in saturation kinetics, and for ascorbate this binding is exothermic with an increased binding for decreasing temperature. Hence, MbFe(IV)═O is more easily saturated by ascorbate at low temperatures such as found in chilled meat, resulting in a relatively slow deactivation for such conditions in products such as cured meat. However, the binding of an antioxidant without direct deactivation may be identified as an antioxidant mechanism because the reactivity of the prooxidant hereby is lowered. A further consequence of the binding of reducing compounds by MbFe(IV)═O is the less than additive effect observed when ascorbate and chlorogenate were allowed to react simultaneously with MbFe(IV)═O. This is clearly an interesting finding, because this situation is more realistic than model systems containing only one reducing compound at a time; furthermore, the mechanisms of interaction between antioxidants is currently receiving considerable attention. The rationale behind this antioxidant interaction is that the chlorogenate complex of the protonated or nonprotonated MbFe(IV)═O has a highly reduced reactivity against another chlorogenate and (most likely) also ascorbate. A more detailed description of this novel type of antioxidant interaction, which is different from that found for the synergistic interaction between chlorogenate and ascorbate in the deactivation of H_2O_2 by a plant peroxidase (Yamasaki and Grace, 1998), should include determination of rate constants for reduction of the chlorogenate complex by ascorbate and vice versa. This will, however, require new types of experiments based on sequential stopped flow with stepwise mixing of the heme pigment with the interacting antioxidants.

ABBREVIATIONS USED

MbFe(III), metmyoglobin; MbFe(II)O_2, oxymyoglobin; $^{+\bullet}$MbFe(IV)═O, perferrylmyoglobin; MbFe(IV)═O, ferrylmyoglobin; CGA^-, chlorogenate; HCGA, chlorogenic acid; $\Delta H°$, molar reaction enthalpy; $\Delta S°$, molar reaction entropy; $\Delta H^{\#}$, molar activation enthalpy; $\Delta S^{\#}$, molar activation enthalpy.

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