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Kinetics of Reduction of Ferrylmyoglobin by (−)-Epigallocatechin Gallate and Green Tea Extract

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The hypervalent heme pigment ferrylmyoglobin, a potential prooxidant in muscle tissue and meat, is efficiently reduced by epigallocatechin gallate (EGCG) from green tea and by green tea polyphenol extract (GTP) in neutral or moderately acidic aqueous solution (0.16 M NaCl) to yield metmyoglobin in two parallel processes. The second-order rate constant for direct reduction at pH 7.4 and 25 °C was found to have the value 1170 ± 83 $M^{-1}\cdot s^{-1}$ and activation parameters $\Delta H^{\#} = 70.6 \pm 7.2$ $kJ\cdot mol^{-1}$ and $\Delta S^{\#} = 50.7 \pm 24.1$ $J\cdot mol^{-1}\cdot K^{-1}$ for EGCG and the value 2300 ± 77 $M^{-1}\cdot s^{-1}$ and parameters $\Delta H^{\#} = 60.6 \pm 2.6$ $kJ\cdot mol^{-1}$ and $\Delta S^{\#} = 23 \pm 9$ $J\cdot mol^{-1}\cdot K^{-1}$ for GTP (based on EGCG concentration). For decreasing pH, the rate increased moderately due to a parallel reduction of protonated ferrylmyoglobin. At physiological pH, EGCG is more efficient in deactivating ferrylmyoglobin than other plant phenols investigated, and the relatively high enthalpy and positive entropy of activation suggest an outer-sphere electron transfer mechanism. The interaction between EGCG and other tea catechins in GTP could be responsible for the even stronger ability for GTP to deactivate ferrylmyoglobin.

KEYWORDS: Ferrylmyoglobin; (−)-epigallocatechin gallate; green tea extract

INTRODUCTION

The hypervalent heme pigment ferrylmyoglobin [MbFe(IV)═O] and its protein radical form, perferrylmyoglobin, both formed by reaction of metmyoglobin with hydrogen peroxide in vivo under ischemic conditions, are powerful oxidizing agents. They are believed to be able to oxidize a number of compounds present in the living cell under reperfussion or in meat products during storage, causing cellular damage in vivo or in vitro (*1*−*5*). Hypervalent myoglobins have been shown to initiate lipid oxidation at pH values relevant to meat and biological systems (*2*) and to induce oxidation of proteins and membrane structures (*3*−*5*). On the other hand, hypervalent myoglobin species are formed in a hydrogen peroxide consuming step, and the resulting pseudoperoxidase cycle could also therefore prevent hydrogen peroxide from entering the Fenton reaction.

It is generally accepted that formation of the protein radical, perferrylmyoglobin, precedes formation of MbFe(IV)═O (*6*) and that the protein radical is unstable and decays rapidly to yield MbFe(IV)═O (*7*, *8*), which transforms to metmyoglobin, MbFe(III), by a rather slow acid-catalyzed autoreduction in the absence of external reductants (*9*, *10*). However, in the presence of external reductants, ferrylmyoglobin can be deactivated by a large number of compounds, such as ascorbate (*10*, *11*), glutathione (*8*), and flavonoids (*9*). However, the mechanisms for such deactivations of hypervalent myoglobin are only poorly understood, and nonadditive effects of a plant phenol and ascorbate in deactivation have been demonstrated (*11*). To understand the role of myoglobin in oxidative deterioration of foods and its damaging effect in biological systems under oxidative stress, it is necessary to establish the mechanisms responsible for the deactivation of ferrylmyoglobin by various reductants including non-nutritive antioxidants such as plant phenols.

(−)-Epigallocatechin gallate (EGCG), one of the main catechins isolated from green tea, has been found to act as an effective chain-breaking antioxidant in vitro (*12*) and in vivo (*13*). EGCG has thus been found to inhibit carcinogen-induced tumors in the skin, lung, duodenum, liver, and colon in rodents (*14*), by a mechanism believed to be related to the antioxidative properties of EGCG. The reactivity of ECGC with biologically relevant oxidants has drawn a great deal of attention, and products of the reaction of EGCG with peroxidases, peroxyl radicals, and H_2O_2 have been reported in order to elucidate the specific mechanisms of catechin antioxidant reactions (*15*, *16*). However, such specific mechanisms remain unclear.

The present kinetic study was undertaken to obtain information on the kinetics and mechanism of reduction of MbFe(IV)═O by EGCG and by green tea polyphenol extract (GTP). EGCG and GTP were chosen as reductants, because EGCG is not only soluble in water but also one of the most efficient natural antioxidants studied and of interest for human nutrition. GTP, a mixture mainly containing EGCG and other tea catechins, should also be considered for use in meat products as a natural additive with antioxidative properties.

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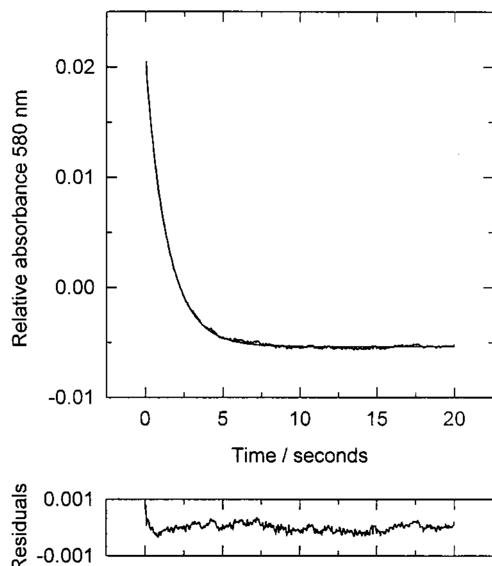

Figure 1. Relative absorbance at 580 nm during reaction between 0.010 mM MbFe(IV)=O and 0.4 mM EGCG at pH 5.00 (25 mM phosphate buffer) and 25.0 °C at ionic strength 0.16 (NaCl) using the stopped-flow technique. (Lower panel) Residuals from a nonlinear regression analysis: $A(t) = a + b \exp(-k_{obs}t)$, from which a pseudo-first-order rate constant $k_{obs} = 3.210 \pm 0.012$ s^{-1} was obtained.

MATERIALS AND METHODS

Chemicals. Metmyoglobin [MbFe(III), horse heart, type III], (−)-EGCG, and hydrogen peroxide were obtained from Sigma Chemical Co. (St. Louis, MO). GTP was donated by the laboratory of Food Chemistry at the Department of Food Science and Technology, Huazhong Agricultural University, People's Republic of China. 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 (pH 7.4)] was purified on a Sephadex G-25 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 based on $\epsilon_{525} = 7700$ $M^{-1}\cdot cm^{-1}$ (*17*). After reaction of MbFe(III) with a 3 times molar excess of H_2O_2 for 3 min, the concentration of the ferrylmyoglobin was determined spectrophotometrically (*18*). The excess of H_2O_2 was removed by separation on a Shephadex G-25 PD-10 column (Pharmacia Biotech AB). The identity of the eluted MbFe(IV)=O was confirmed spectrophotometrically (*19*), and the solution was immediately used.

pH Measurement. 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 23B-100 combination glass electrode (Hamilton Bonaduz AG, Bonaduz, Switzerland) connected to a Metrohm 113 pH-meter (Metrohm, Herisau, Switzerland).

Kinetic Experiment. Solutions of EGCG and GTP were freshly prepared before use by dissolving EGCG or GTP in an NaCl-containing phosphate buffer. MbFe(IV)=O and EGCG or GTP solutions were placed in each syringe of a DX-17MW stopped-flow spectrofluorometer equipped with a diode array detector (Applied Photophysics, London, U.K.), and the reactions were followed by absorbance measurements at 580 nm as seen in **Figures 1** and **3**. Additional stopped-flow experiments were performed at 560 nm, but no significant differences from the rate constants calculated from absorbency changes at 580 nm were observed. Pseudo-first-order rate constants for the reactions were calculated by nonlinear regression analysis (the Marquardt−Levenberg algorithm). In the reaction mixture, the concentration of EGCG or GTP was in excess relative to MbFe(IV)=O by at least a factor of 20 and the buffer concentration was 25 mM; the ionic strength was 0.16 ± 0.01 adjusted with NaCl. For each combination, pH was measured in thermostated 1:1 mixtures of the MbFe(IV)=O and EGCG or GTP solutions. The influences of pH, temperature, and EGCG or GTP concentration on pseudo-first-order rate constants were systematically investigated (**Figure 5−8**).

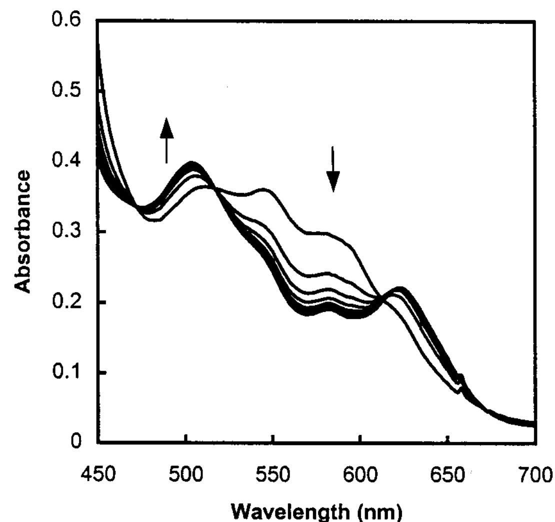

Figure 2. Absorption spectra in the visible region of an aqueous 29 μM MbFe(IV)=O, 0.02 mM EGCG solution with pH 7.4 (25 mM phosphate buffer) and ionic strength 0.16 (NaCl). Spectra are recorded with a 30 s interval following 2 s of mixing time in a 1 cm cuvette at 25 °C. Five minutes elapsed from initiation of the reaction to recording of the final spectrum. The arrows indicate increasing or decreasing absorption. The product spectrum corresponds to MbFe(III) with <6% $MbFe(II)O_2$ present.

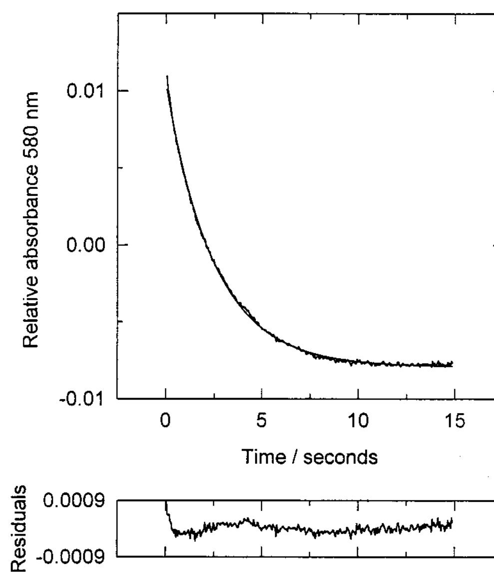

Figure 3. Relative absorbance at 580 nm during reaction between 0.010 mM MbFe(IV)=O and GTP containing 0.072 mM EGCG at pH 7.4 (25 mM phosphate buffer) and 35 °C at ionic strength 0.16 (NaCl) using the stopped-flow technique. (Lower panel) residuals from a nonlinear regression analysis: $A(t) = a + b \exp(-k_{obs}t)$, from which a pseudo-first-order rate constant $k_{obs} = 0.771 \pm 0.006$ s^{-1} was obtained.

Identification of the Reaction Product. Solutions of MbFe-(IV)=O and EGCG or GTP were mixed in the thermostated cell of an HP 8542 UV−vis diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA), and the heme reaction product was identified from absorption spectra in the region 450−470 nm (**Figures 2** and **4**). For each experiment, the relative concentrations of MbFe(II), MbFe(II)=O_2, and MbFe(IV)=O in the reaction mixture were calculated according to the method of Miller et al. (*20*). This method is based on absorbance measurement at three wavelengths (corrected for background absorption at 700 nm) resulting in three linear equations with three unknowns (the three concentrations).

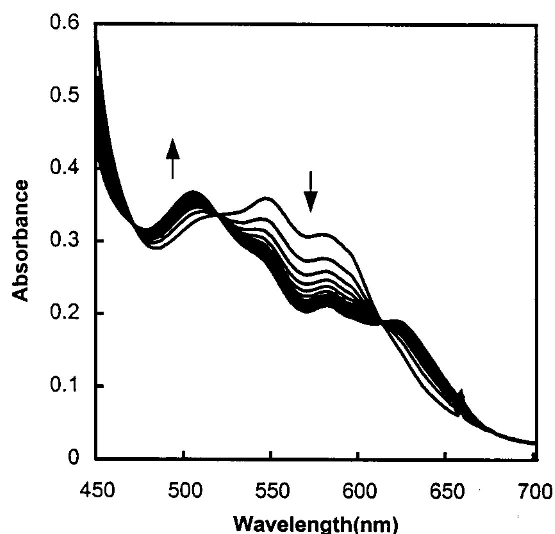

Figure 4. Absorption spectra in the visible region of an aqueous 27 μM MbFe(IV)═O and GTP solution containing 0.0036 mM EGCG with pH 7.4 (25 mM phosphate buffer) and ionic strength 0.16 (NaCl). Spectra are recorded with a 30 s interval following 2 s of mixing time in a 1 cm cuvette at 25 °C. Five minutes elapsed from initiation of the reaction to recording of the final spectrum. The arrows indicate increasing or decreasing absorption. The product spectrum corresponds to MbFe(III) with <6% $MbFe(II)O_2$ present.

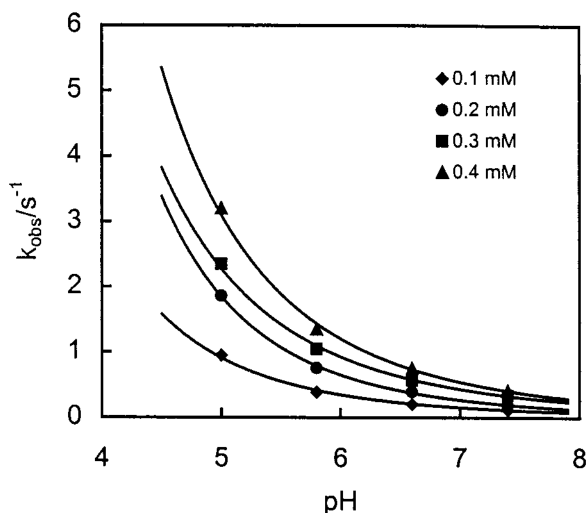

Figure 5. Dependence of k_{obs}, obtained as shown in **Figure 1**, on pH for four different EGCG concentrations at 25.0 °C in an aqueous solution of ionic strength 0.16 (NaCl).

Determination of EGCG Content in GTP. The content of EGCG in GTP was analyzed according to the method of Amarowicz and Shahidi using HPLC (*20*). The pure EGCG was used as standard, and the extract was found to contain 48.5% EGCG. The total content of phenols in GTP was 72.6% expressed as gallic acid equivalent, analyzed as previously described (*22*).

Data Analysis. Pseudo-first-order rate constants k_{obs} for reduction of MbFe(IV)═O by EGCG or GTP were calculated using the PC Pro-K Global Analysis software obtained from Applied Photophysics Ltd. (Leatherhead, U.K.).

RESULTS

The observed kinetics for reduction of MbFe(IV)═O by EGCG or GTP could for excess of EGCG or GTP be described by (pseudo) first-order reactions for all experimental conditions, as shown in **Figures 1** and **3** for one example for each reductant.

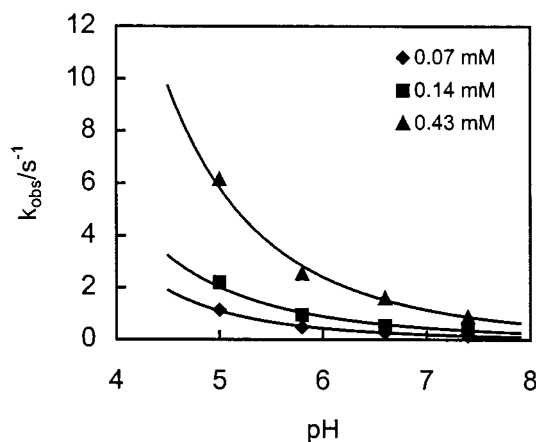

Figure 6. Dependence of k_{obs}, obtained as shown in **Figure 3**, on pH for GTP for three concentrations at 25.0 °C in an aqueous solution of ionic strength 0.16 (NaCl).

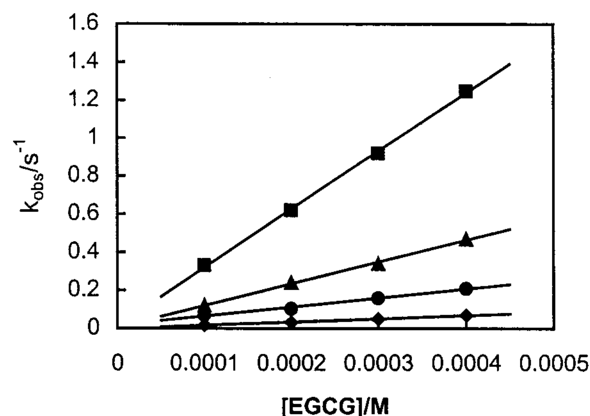

Figure 7. Observed pseudo-first-order rate constants obtained as shown in **Figure 1** for reduction of MbFe(IV)═O by EGCG as a function of EGCG concentration at different temperatures (35, 25, 15, and 5 °C, shown from top to bottom), pH 7.4, and I = 0.16.

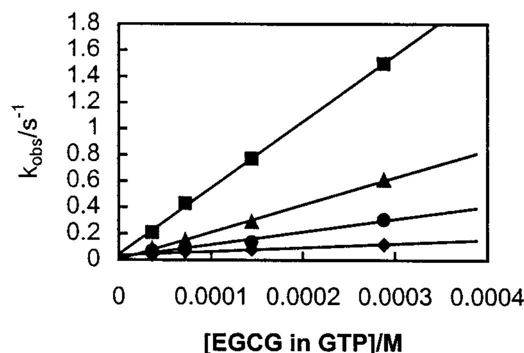

Figure 8. Observed pseudo-first-order rate constants obtained as shown in **Figure 3** for reduction of MbFe(IV)═O by GTP as a function of EGCG concentration at different temperatures (35, 25, 15, and 5 °C, shown from top to bottom), pH 7.4, and I = 0.16.

The reaction products formed from reduction of MbFe(IV)═O by both EGCG and GTP were spectrally identified as MbFe(III), as seen in **Figures 2** and **4**, respectively, and <6% $MbFe(II)O_2$ was formed from MbFe(IV)═O in a parallel or subsequent reaction, thus having minor influence on the calculated rate constants for reduction of MbFe(IV)═O by EGCG or GTP.

The rate of reduction of MbFe(IV)═O by EGCG or GTP was found to depend on pH. The pH effect was studied for four different concentrations of EGCG and for three different

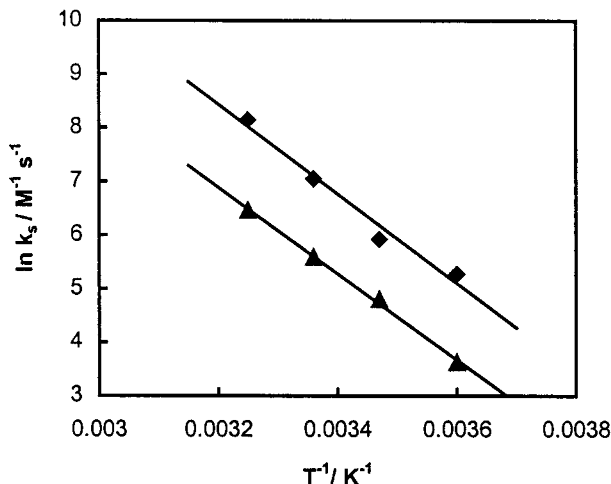

Figure 9. Arrhenius plot for reduction of MbFe(IV)═O by EGCG (◆) and GTP (▲) in an aqueous solution of pH 7.4 and ionic strength 0.16 from which the activation parameters $\Delta H^{\#} = 70.6 \pm 7.2$ kJ·mol^{-1} and $\Delta S^{\#} = 51 \pm 24$ J·mol^{-1}·K^{-1} for EGCG and $\Delta H^{\#} = 60.6 \pm 2.6$ kJ·mol^{-1} and $\Delta S^{\#} = 23 \pm 9$ J·mol^{-1}·k^{-1} for GTP were calculated.

concentrations of GTP, and the variation of rate constant with pH is shown in **Figures 5** and **6**. The pH dependence of reduction of MbFe(IV)═O by EGCG or GTP was found to be very similar to that previously observed for reduction by NADH (*23*) and chlorogenate (*11*). The pH dependence of reduction of MbFe(IV)═O by EGCG or GTP, however, is different from the pH dependence observed for autoreduction of MbFe-(IV)═O, which could be described as a specific acid-catalyzed reaction (*23*). The pH dependence of k_{obs} can rather be explained by a model, which originally was proposed for the reduction of MbFe(IV)═O by NADH, including an acid−base equilibrium of ferrylmyoglobin with an acid dissociation constant, K_a, and with two parallel reaction paths with the protonated form of MbFe(IV)═O reacting more rapidly than the nonprotonated one.

The reaction path dominating at neutral pH was studied in more detail, and as may be seen in **Figures 7** and **8**, the observed (pseudo) first-order rate constant, k_{obs}, was found to depend linearly on the EGCG or GTP concentration at conditions of constant pH and temperature; it could be concluded that reactions of MbFe(IV)═O by EGCG or GTP were both simple second-order reactions in agreement with the reduction of MbFe(IV)═O by β-lactoglobulin, I^-, and NO_2^- (*24*). The observed first-order rate constant k_{obs} can thus be expressed as

$$k_{obs} = k_{auto} + k_{b,1}[\mathrm{EGCG}] \text{ or } [\mathrm{EGCG\ in\ GTP}] \qquad \text{(i)}$$

where $k_{b,1}$ is the second-order rate constant for reduction of the nonprotonated ferrylmyoglobin by EGCG or GTP and k_{auto} is the rate constant for parallel autoreduction of ferrylmyoglobin [$\sim 2 \times 10^4$ s^{-1} at pH 7.4 and 25 °C (*23*)]. It is obvious from **Figures 7** and **8** that autoreduction for all conditions investigated is significantly slower than the EGCG or GTP reduction, and at the same concentration of EGCG, GTP is seen to be a more efficient reductant than EGCG for the same concentrations of EGCG.

The observed pH and concentration dependence for the reduction of MbFe(IV)═O by EGCG and GTP can be accommodated by the reaction scheme proposed in **Figure 10** for conditions of neutral or slightly acidic solution. This model including the one-electron reduction reaction paths $k_{a,1}$ and $k_{b,1}$ was found to accommodate the experimental observations for k_{obs} at various pH values and EGCG concentrations when the

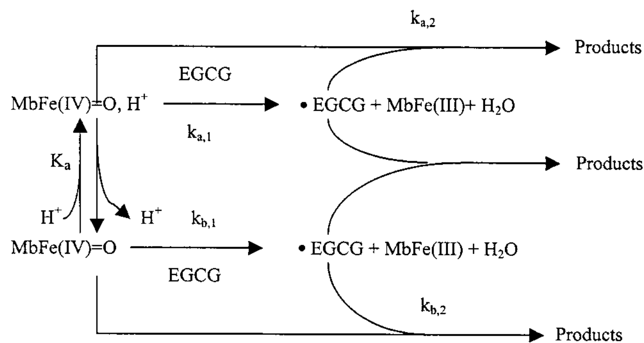

Figure 10. Kinetic scheme adapted for reduction of MbFe(IV)═O by EGCG in neutral and slightly acidic solutions.

pH was <7.4. The phenoxyl radicals of EGCG initially formed may either disproportionate or, as suggested in **Figure 10**, reduce another MbFe(IV)═O in a subsequent and slower reaction of no importance under the present conditions with excess of EGCG. The model for the actual set of conditions, therefore, includes two main reactions, the relative importance of which for conditions of constant temperature depends on the EGCG concentration and pH: (i) a biomolecular reaction between EGCG and a protonated form of MbFe(IV)═O and (ii) a bimolecular reaction between EGCG and a nonprotonated MbFe(IV)═O according to the following rate equation:

$$-\frac{d[\mathrm{MbFe(IV)\!=\!O}]_{\mathrm{TOTAL}}}{dt} = k_{a,1}[\mathrm{MbFe(IV)\!=\!O,H^+}][\mathrm{EGCG}] + k_{b,1}[\mathrm{MbFe(IV)\!=\!O}][\mathrm{EGCG}] = \left(k_{a,1}[\mathrm{EGCG}]\frac{[\mathrm{H^+}]}{K_a + [\mathrm{H^+}]} + k_{b,1}[\mathrm{EGCG}]\frac{[K_a]}{K_a + [\mathrm{H^+}]}\right)c^{\mathrm{total}}_{\mathrm{MbFe(IV)=O}} \qquad \text{(ii)}$$

K_a is the acid dissociation constant of MbFe(IV)═O,H$^+$, which has a value corresponds to p$K_a \sim 5$, and $k_{a,1}$ and $k_{b,1}$ are the rate constants for the two pathways in **Figure 10** contributing significantly to the rate of reduction of MbFe(IV)═O. Under (pseudo) first-order conditions with an excess of EGCG, the following expression for k_{obs} is therefore obtained:

$$k_{obs} = k_{a,1}[\mathrm{EGCG}]\frac{[\mathrm{H^+}]}{K_a + [\mathrm{H^+}]} + k_{b,1}[\mathrm{EGCG}]\frac{[K_a]}{K_a + [\mathrm{H^+}]} \qquad \text{(iii)}$$

The temperature dependence for the reduction rate of the nonprotonated MbFe(IV)═O by EGCG or GTP was studied for the four temperatures of 5, 15, 25, and 35 °C at pH 7.4, and regression analysis of the second-order rate constants for the four temperatures as a function of temperature according to the Arrhenius equation (**Figure 9**) resulted in the activation parameters shown in **Table 1** for the reaction path dominating at neutral pH ($k_{b,1}$ path).

DISCUSSION

The green tea polyphenol EGCG was found to be an efficient reductor of ferrylmyoglobin, the activated heme pigment, which is known as an initiator of lipid and protein oxidation during oxidative stress. EGCG, which is freely soluble in aqueous solution, is thus a potential protector of tissue and membranes against oxidative damage. The overall pattern of deactivation of MbFe(IV)═O by EGCG was similar to that observed for

Table 1. Rate Constants and Activation Parameters for Deactivation of MbFe(IV)═O by Plant Phenols in Aqueous Solution at pH 7.4 and 25.0 °C

reducing compound or extract	$E°$ (V)	rate constant ($M^{-1} \cdot s^{-1}$)	$\Delta H^{\#}$ ($kJ \cdot mol^{-1}$)	$\Delta S^{\#}$ ($J \cdot mol \cdot K^{-1}$)
(–)-EGCG[a]	0.43	1170 ± 83	70.6 ± 7.2	51 ± 24
green tea extract	(0.43)	2300 ± 77	60.6 ± 2.6	23 ± 9
chlorogenate[b]	0.37	216 ± 50	73 ± 8	41 ± 30
rutin[c]	0.40	105 ± 1	65 ± 3	13 ± 9
apigenin[c]	0.71	125 ± 6	69 ± 1	23 ± 4

[a] Present work, the values for green tea extract are based on a concentration of (–)-epigallocatechin gallate. [b] Carlsen et al. (*11*). [c] Jørgensen and Skibsted (*9*).

simple plant phenols and other water soluble antioxidants such as ascorbate with an increasing rate for decreasing pH (*9*). At pH values typically of meat products, EGCG is thus more efficient as a reductor than at physiological pH, and tea extracts rich in EGCG should be further explored for use as an additive to meat products for protection against pseudoperoxidase-mediated hydrogen peroxide oxidation resulting in rancidity and warmed-over flavor. Tea catechins have thus been efficiently found to protect cooked beef and chicken against oxidative deterioration (*25*, *26*).

At least two parallel reactions are responsible for the reduction of MbFe(IV)═O by EGCG (reaction scheme of **Figure 10**). One reaction, dominating at physiological pH, is a second-order reaction between MbFe(IV)═O and EGCG. Another reaction is the moderately faster reaction between a protonated form of MbFe(IV)═O and EGCG, which will dominate for the pH conditions of relevance for meat. The reaction dominating at physiological pH was studied in most detail for EGCG in order to allow a comparison with other flavonoids studied (*9*, *27*, *28*). The linear dependence of the observed (pseudo) first-order rate constant on EGCG concentration for conditions of excess of EGCG is in accordance with a bimolecular mechanism with no initial binding of EGCG to MbFe(IV)═O prior to electron transfer (*11*). At pH <7.4, the noncharged form of EGCG is dominating (EGCG has $pK_{a,1} = 7.8$ and $pK_{a,2} = 8.0$), in contrast to chlorogenate, for which the anionic form was found to bind to MbFe(IV)═O, complicating the observed kinetics (*11*). The observation of MbFe(III) as product shows that the process is a one-electron transfer, and the observation of simple second-order kinetics further indicates that the subsequent reaction of the phenoxyl radical of EGCG does not involve MbFe(IV)═O, but rather is a disproportionation or dimerization, or that the reaction of the phenoxyl radical with MbFe(IV)═O is slow.

The one-electron oxidized form of EGCG has a standard reduction potential of $E° = +0.42$ V, which is well below the potential $E° = +0.85$ V for MbFe(IV)═O at pH 7 and the potential of perferrylmyoglobin (*9*, *27*, *28*). The electron transfer from EGCG to MbFe(IV)═O thus has a driving force comparable to the electron transfer from vitamin E and Trolox to MbFe(IV)═O (*9*). The rate is, however, higher by a factor of 10, mainly due to a favorable entropy of activation. The positive entropy of activation most likely reflects a lower degree of hydration of the protein during the encounter with the polyphenol and subsequent electron transfer.

The reaction between a number of flavonoids and the superoxide anion radical has been studied by Jovanovic et al. (*27*, *28*). It is interesting to compare the reduction of superoxide by flavonoids with the reduction of ferrylmyoglobin by flavonoids because the standard reduction potential for ($O_2^{\bullet-}$, $2H^+$)/H_2O_2 at pH 7.0, $E° = +0.94$ V, is very similar to the standard reduction potential $E° = +0.85$ V (pH 7.4) for MbFe(IV)═0/MbFe(III). For the superoxide anion radical rather low activation enthalpies, ranging from 9.6 to 15.1 $kJ \cdot mol^{-1}$, and negative activation entropies, from −105 to −117 $J \cdot mol^{-1} \cdot K^{-1}$, were found for the reaction with rutin and Trolox (pH 10) and for methylgallate at pH 7, suggesting an inner-sphere electron transfer mechanism for the reduction. On the contrary, the relatively high activation enthalpies found for EGCG and GTP in the present study for the reduction of MbFe(IV)═0 together with positive activation entropies suggest an outer-sphere electron transfer reaction mechanism for the reduction of ferrrylmyoglobin by EGCG. The present finding is in agreement with the result of a previous study of other flavonoids (*9*).

The experimental results showed that GTP, like EGCG, has a strong ability to deactivate ferrylmyoglobin. As may be seen from **Figures 5** and **6**, at the condition of the same concentration, pH, and temperature, k_{obs} of GTP was ~2 times larger than that of EGCG, which indicated that except for EGCG in GTP, the other tea catechins such as epicatechin make significant contributions to the deactivation of ferrylmyoglobin. It is notable that the interactions between the tea catechins is reflected in a lower $\Delta H^{\#}$, which shows that it is not simply a matter of a higher total phenol concentration. The identification of oxidation products formed by reduction of EGCG with MbFe(IV)═O could provide insight into the specific mechanism of the protective reactions. Such studies would also help to explain the observation that EGCG (and GTP) is the most efficient deactivator of hypervalent heme pigment studied so far.

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