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Deactivation of Ferrylmyoglobin by Vanillin as Affected by Vanillin Binding to β-Lactoglobulin

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ABSTRACT: Vanillin was found to be efficient as a deactivator of ferrylmyoglobin with a second-order rate constant of $k_2 = 57 \pm 1$ L mol^{-1} s^{-1} for reduction to metmyoglobin with $\Delta H^{\ddagger} = 58.3 \pm 0.3$ kJ mol^{-1} and $\Delta S^{\ddagger} = -14 \pm 1$ J mol^{-1} K^{-1} in aqueous pH 7.4 solution at 25 °C. Binding to β-lactoglobulin (βLG) was found to affect the reactivity of vanillin at 25 °C only slightly to $k_2 = 48 \pm 2$ L mol^{-1} s^{-1} ($\Delta H^{\ddagger} = 68.4 \pm 0.4$ kJ mol^{-1} and $\Delta S^{\ddagger} = 17 \pm 1$ J mol^{-1} K^{-1}) for deactivation of ferrylmyoglobin. Binding of vanillin to βLG was found to have a binding stoichiometry vanillin/βLG > 10 with $K_A = 6 \times 10^2$ L mol^{-1} and an apparent total $\Delta H°$ of approximately −38 kJ mol^{-1} and $\Delta S° = -55.4 \pm 4$ J mol^{-1} K^{-1} at 25 °C and $\Delta C_{p,\,obs} = -1.02$ kJ mol^{-1} K^{-1} indicative of increasing ordering in the complex, as determined by isothermal titration microcalorimetry. From tryptophan fluorescence quenching for βLG by vanillin, approximately one vanillin was found to bind to each βLG far stronger with $K_A = 5 \times 10^4$ L mol^{-1} and a $\Delta H° = -10.2$ kJ mol^{-1} and $\Delta S° = 55$ J mol^{-1} K^{-1} at 25 °C. The kinetic entropy/enthalpy compensation effect seen for vanillin reactivity by binding to βLG is concluded to relate to the weakly bound vanillin oriented through hydrogen bonds on the βLG surface with the phenolic group pointing toward the solvent, in effect making both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ more positive. The more strongly bound vanillin capable of tryptophan quenching in the βLG calyx seems less or nonreactive.

KEYWORDS: Ferrylmyoglobin, antioxidant, protein, complex, vanillin

INTRODUCTION

Epidemiologic studies show that the cause and prevention of diseases are closely linked to the diet and lifestyle of populations.[1−3] There is increasing evidence that the Mediterranean diet, rich in fruits, vegetables, and olive oil is associated with a low incidence of cardiovascular diseases.[4] Among benefits of such diets rich in phenolic compounds, antioxidant activity and cancer prevention have been highlighted.[5,6] These phenolic compounds present in the diets seem active in the gastrointestinal tract, where their concentration increases to around the millimolar level, in contrast to their concentration in the bloodstream that does not exceed the micromolar level.[7] However, the bioavailability and activity in the gastrointestinal tract can still be affected by other constituents present in food or in the same meal, as reported by Serafini et al.,[8] who demonstrated that absorption of compounds, such as epicatechin, present in chocolate is reduced by the presence of milk components in the product. Similarly, absorption of caffeic and ferulic acids from blueberry fruits has been found to be impaired by milk.[9] Milk-based beverages containing bioactive compounds has a growing market share, but the functionality of such components may be affected by other constituents.[10,11]

β-lactoglobulin (βLG) is a major ruminant whey protein classified as a lipocalin, which is a protein with the ability to bind hydrophobic, low-molecular-weight molecules. Phenolic compounds, added to dairy products, may also bind to proteins, such as βLG, with effects on their antioxidant properties.

During the last few decades, studies have demonstrated a direct relationship between the high consumption of red meat and cancer incidence in the gastrointestinal tract.[12−14] During digestion of red meat, the reactive species ferrylmyoglobin [$MbFe^{IV}{=}O$] and perferrylmyoglobin [$\bullet MbFe^{IV}{=}O$] are formed in the gastrointestinal tract from the reaction of meat pigments with peroxides and hydroperoxides.[15,16] These hypervalent myoglobins are capable of inducing radical processes but may be deactivated by plant phenolics when present together with meat. Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one such phenolic compound, which, moreover, is among the most used flavorings.[17] Vanillin[17] has been found to be a good antioxidant,[18] under certain conditions, while other studies have reported low antioxidant activity.[19]

The objective of the present study was accordingly to investigate the influence of the presence of the whey protein βLG on the deactivation of ferrylmyoglobin as a pro-oxidant by vanillin as an example of a phenolic antioxidant.

MATERIALS AND METHODS

Chemicals. Catalase from bovine liver, βLG (purity >90%), and myoglobin from horse heart (purity >90%) were obtained from Sigma-Aldrich (Steinheim, Germany). Myoglobin was purified using the procedures described in the literature;[20] vanillin (purity 99%) and hydrogen peroxide (30%, v/v), were obtained from Sigma-Aldrich (Steinheim, Germany) and used without further treatment. $KHPO_4$, KH_2PO_4, and NaCl were all of analytical grade and supplied by J. T. Baker (Phillipsburg, NJ). Ethanol and chloroform (HPLC grade)

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were obtained from Tedia (Fairfield, OH) and used without further treatment. The water used was first distilled and then deionized to 18.2 MΩ cm^{-1} at 25 °C in a Milli-Q system from Millipore Co. (Billerica, MA).

Fluorescence Spectroscopy. A Hitachi F-4500 fluorescence spectrometer (Hitachi High-Technologies Corporation, Tokyo, Japan) was used with quartz cells with four polished faces and an optical path of 1.0 cm (Hellma GmbH and Co. KG, Müllheim, Germany), applying slit of excitation and emission of 10 nm, wavelength of excitation of 280 nm, and emission spectra recorded at 295−500 nm. All experiments were performed with a temperature control using a Nova Ética model 521/3D (São Paulo, Brazil) thermostatic bath.

Aqueous stock solutions of βLG were prepared at concentrations of 1×10^{-5} or 1×10^{-4} mol L^{-1} in 5 mM phosphate buffer at pH 7.4 or 6.4 and in HCl solution of 1×10^{-2} mol L^{-1}, all with $\mu = 0.32$ (NaCl). Aqueous solutions of vanillin in a concentration of 1×10^{-3} mol L^{-1} were prepared in 5 mM phosphate buffer at pH 7.4 or 6.4 and in HCl solution of 1×10^{-2} mol L^{-1}, all with 20% ethanol and $\mu = 0.32$ (NaCl). The analytical concentrations of stock solutions were determined by ultraviolet−visible (UV−vis) spectrophotometry using the molar absorptivities reported in the literature or determined experimentally: βLG, $\varepsilon_{280\ nm} = 1.76 \times 10^4$ L mol^{-1} cm^{-1}; vanillin, $\varepsilon_{340\ nm,\ pH\ 2.0} = 5.5 \times 10^3$ L mol^{-1} cm^{-1}; and vanillin, $\varepsilon_{340\ nm,\ pH\ 7.4} = 1.4 \times 10^4$ L mol^{-1} cm^{-1}.[21] Solutions for fluorescence spectroscopy were prepared with vanillin varying between 0 and 200 μmol L^{-1} and keeping the protein concentration constant at 1 μmol L^{-1}.

Isothermal Titration Calorimetry (ITC). Titration calorimetric experiments were carried out with a MicroCal VP-ITC at the LNBio−LNLS facility (Campinas, São Paulo, Brazil). The experiments were conducted using aqueous phosphate buffer at 5.0 mmol L^{-1} with pH 7.4 and ionic strength of 0.32 mol L^{-1} adjusted with NaCl and containing 5% ethanol. A βLG solution of 179 μmol L^{-1} was prepared and submitted to exhaustive dialysis against the same buffer. The concentration of the βLG was determined spectrophotometrically.[21] A total of 1.49 mL of the dialyzed βLG solution was titrated with a 49.6 mmol L^{-1} vanillin solution using 35 injections, with a volume gradient from 1 to 15 μL. Dilution heat effects were corrected by separate experiments from the addition of vanillin solution into buffer and buffer into βLG solution. The apparent enthalpy change per mole of vanillin, for each injection, was calculated by integrating the area under the peak, taking the control titration as the baseline for heat of dilution correction. The apparent binding enthalpy change (ΔH_{app}), binding stoichiometry (n), and association constant (K_A) were estimated from the curve-fitting algorithm supplied by the Microcal VP-ITC instrument. The total ΔH_{app} was calculated from the sum of ΔH_{app} for each injection of vanillin. The apparent change in heat capacity ($\Delta C_{p,\ obs}$) for the binding of vanillin to βLG was obtained from the temperature dependence of the total ΔH_{app}.

Circular Dichroism Spectroscopy. All circular dichroism experiments were performed using a Jasco model J-810 (Jasco International Co.) spectropolarimeter at the LNBio−LNLS facility (Campinas, São Paulo, Brazil) using 1.0 mm quartz cells (Hellma GmbH and Co. KG, Müllheim, Germany). The data collection parameters were set to a scan rate of 50 nm/min, scan range of 190−260 at 0.5 nm intervals, response time of 4 s, sensitivity of 100 mdeg, accumulation of 6, and delay time for spectrum collection of 60 s. The CD data were recorded as mean residue ellipticity units (deg cm^2 $dmol^{-1}$ $residue^{-1}$).

Reaction Kinetics: Ferrylmyoglobin and Vanillin. A 0.2 mmol L^{-1} stock solution of metmyoglobin ($MbFe^{III}$) prepared in aqueous phosphate buffer at 5.0 mmol L^{-1} with pH 7.4 and ionic strength of 0.32 mol L^{-1} adjusted with NaCl was subject to purification on a 5.0 × 1.0 cm Sephadex G-25 column. After the purification process, the analytical concentration of $MbFe^{III}$ was determined spectrophotometrically ($\varepsilon_{525\ nm} = 7.70 \times 10^3$ L mol^{-1} cm^{-1}). $MbFe^{IV}{=}O$ was

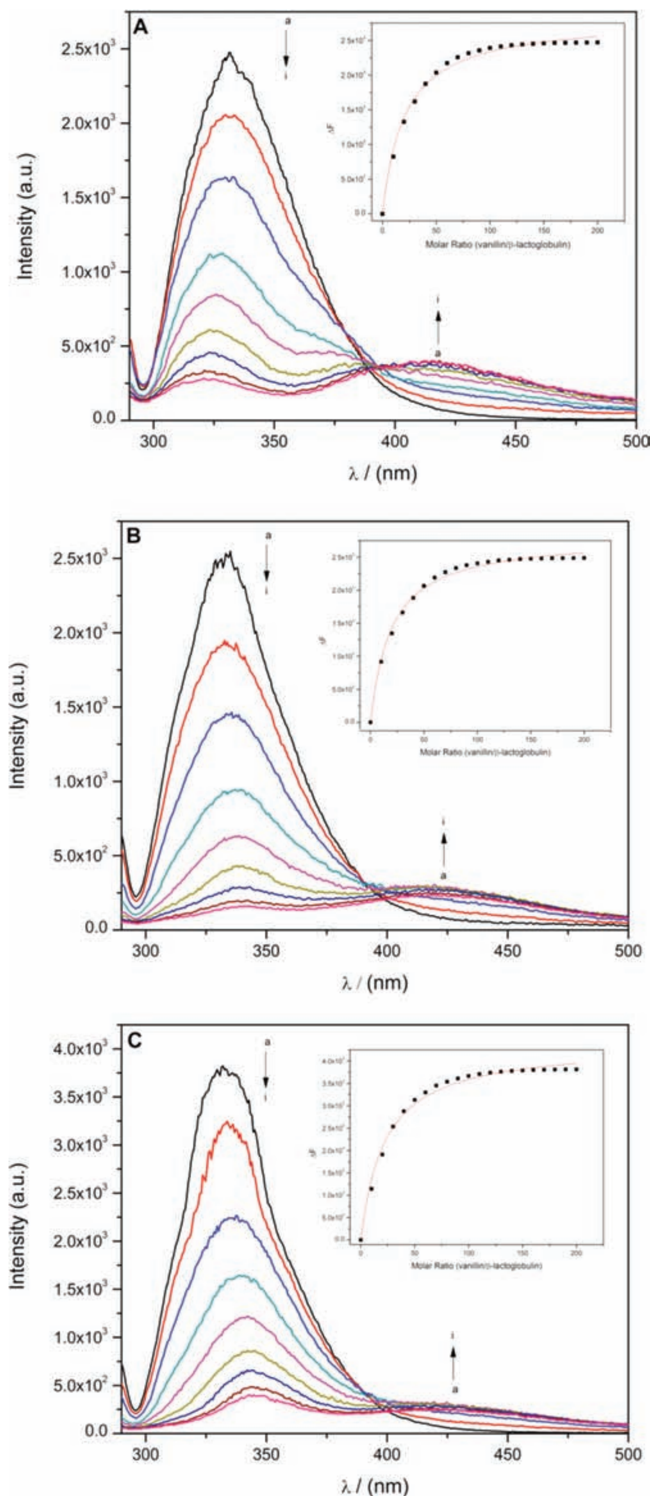

Figure 1. Emission spectra of βLG (1×10^{-6} mol L^{-1}) at (A) pH 7.4, (B) pH 6.4, and (C) pH 2.0 at $\mu = 0.32$ (NaCl) for 280 nm excitation, showing quenching by equidistant increasing concentrations of vanillin a−i ($0-2.0 \times 10^{-4}$ mol L^{-1}) at 25.0 ± 0.1 °C. (Inset) Plot of ΔF versus molar ratio [vanillin]/[βLG] for the determination of K_A according to the 1:1 binding model (eq 3).

generated by oxidation of $MbFe^{III}$ with H_2O_2 3-fold in excess for 3 min and further interrupted by the addition of 10 μL of aqueous solution of 47.4 mg mL^{-1} catalase. The concentration of $MbFe^{IV}{=}O$ was

Table 1. Association Constants and Number of Binding Sites Determined Experimentally by Fluorescence Titration (±SD)[a] for the Interaction between Vanillin and βLG at pH 2.0, 6.4, and 7.4 for 15, 25, and 45 °C, Together with the Derived Values for $\Delta H°$ and $\Delta S°$ for Binding of Vanillin to βLG at pH 7.4

pH	$K_{A,\ 25\ °C}$ ($\times 10^4$, L mol^{-1})	$K_{A,\ 15\ °C}$ ($\times 10^4$, L mol^{-1})	$K_{A,\ 45\ °C}$ ($\times 10^4$, L mol^{-1})	n[b]	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (J mol^{-1} K^{-1})
7.4	4.8 ± 0.1	5.8 ± 0.1	3.8 ± 0.2	1.4 ± 0.1	−10.2 ± 1	+55.5 ± 3
6.4	4.7 ± 0.1			1.3 ± 0.1		
2.0	3.3 ± 0.1			1.47 ± 0.06		

[a] SD = standard deviation from curve fitting. [b] n obtained at 25 °C using the logarithmic form of the Stern−Volmer equation (eq 2).

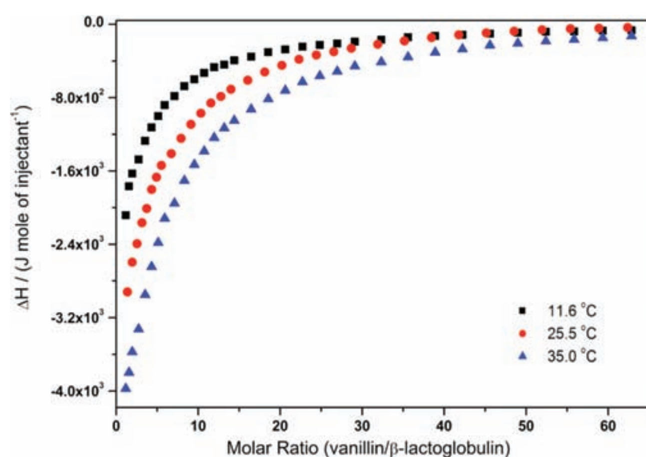

Figure 2. Isothermal microcalorimetric titration of a 1.79 × 10^{-4} mol L^{-1} βLG solution with a 5.0 × 10^{-2} mol L^{-1} solution of vanillin in phosphate buffer [pH 7.4, μ = 0.32 (NaCl)] at three different temperatures (35.0 ± 0.1, 25.5 ± 0.1, and 11.6 ± 0.1 °C).

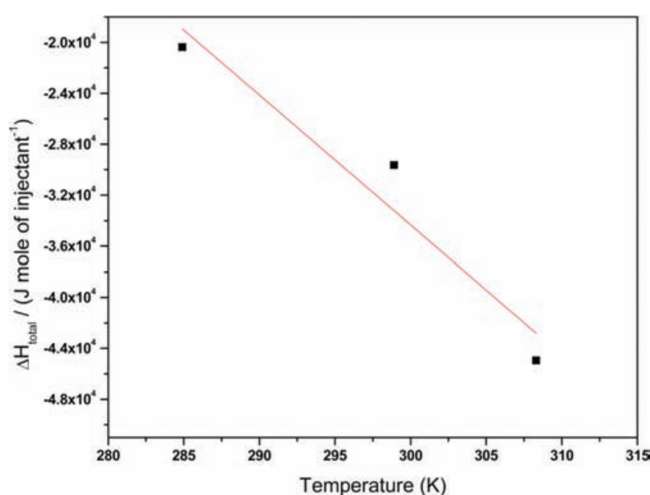

Figure 3. Variation of apparent ΔH_{total} versus temperature for titration a 1.79 × 10^{-4} mol L^{-1} βLG solution with a 5.0 × 10^{-2} mol L^{-1} solution of vanillin in phosphate buffer [pH 7.4, μ = 0.32 (NaCl)]. ΔC_p of eq 5 is determined according to $\Delta C_p = (\partial \Delta H_{total}/\partial T)_p$.

determined spectrophotometrically as previously described.[22]

$$[\mathrm{MbFe^{IV}{=}O}] = -62(A_{490\ nm} - A_{700\ nm}) + 242(A_{650\ nm} - A_{700\ nm}) - 123(A_{580\ nm} - A_{700\ nm})$$

The typical concentration of ferrylmyoglobin in kinetic experiments was 5.0 × 10^{-5} mol L^{-1}. βLG was 2.0 × 10^{-5} mol L^{-1} in all experiments, while the concentration of vanillin was ranging from 2 to 9 × 10^{-4} mol L^{-1} to provide pseudo-first-order conditions relative to MbFe^{IV}=O. The rate of decay of MbFe^{IV}=O was determined from absorption at 580 nm using a Hitachi U4100 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) and 1 cm quartz cells with two compartments, allowing for a mixing time of approximately 1 s (Hellma GmbH and Co. KG, Müllheim, Germany). All experiments were performed with the temperature controlled (±0.1 °C) by a Nova Ética model 521/3D (São Paulo, Brazil) thermostatic bath.

Table 2. Thermodynamic Parameters and K_A (±SD) from Isothermal Titration Microcalorimetric of a 1.79 × 10^{-4} mol L^{-1} βLG Solution with a 5.0 × 10^{-2} mol L^{-1} Vanillin Solution in Aqueous Phosphate Buffer at pH 7.4 [pH 7.4, μ = 0.32 (NaCl)] at Different Temperatures

temperature (°C)	K_A[a] ($\times 10^2$, L mol^{-1})	ΔH_{total} (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
25.6	5.6 ± 0.3	−29.7	−71.1
11.6	7.0 ± 1	−20.4	−20.9
35	4.8 ± 0.3	−45.0	−138

[a] n > 10, non-specific interaction.

RESULTS AND DISCUSSION

Binding of Vanillin to βLG. The interaction between vanillin and βLG was studied using two techniques: fluorescence spectroscopy based on βLG intrinsic tryptophan quenching by vanillin and ITC.

Bovine βLG contains two tryptophan residues (Trp19 and Trp61) in different environments, because Trp19 is buried inside the hydrophobic core of βLG, while Trp61 is in contact with the solvent at the βLG surface and with little contribution to fluorescence emission. βLG fluorescence is accordingly almost exclusively attributed to Trp19.[23]

For fluorescence quenching experiments, βLG solutions (1 × 10^{-6} mol L^{-1}) with pH 2.0, 6.4, or 7.4 were titrated with vanillin (1−200 mol L^{-1}) as a quencher and fluorescence spectra were recorded (panels A−C of Figure 1). For pH 2.0 (Figure 1C), the observed fluorescence was most intense, because the hydrophobic site of βLG is closed and Trp19 becomes less exposed to solvent, in effect increasing the fluorescence emission. For pH 6.4 (Figure 1B) and pH 7.4 (Figure 1A), the hydrophobic site becomes more open, exposing Trp19 to the solvent, resulting in comparable solvent quenching. Vanillin absorbs in the same wavelength region as Trp19 emits, and emission was corrected for inner-filter effects as proposed by Epps et al.[24] The number of vanillin binding sites (n) associated with βLG was determined using the Stern−Volmer equation for vanillin concentrations up

Table 3. Association Constants (K_A) for Binding of Vanillin to βLG as Obtained by Different Analytical Techniques

reference	experimental conditions	K_A (L mol^{-1})	technique
28	aqueous phosphate buffer [pH 7.2, μ = 0.3 (NaCl)]	3.2×10^2	affinity chromatography
29	water [μ = 0.2 (NaCl)]	5.8×10^3	UV−vis spectroscopy
	water	1.7×10^4	UV−vis spectroscopy
30	water (pH 6.0)	8×10^4	fluorimetry
this work	phosphate buffer [pH 7.4, μ = 0.32 (NaCl)]	4.8×10^4	fluorimetry
		5.6×10^2	microcalorimetry

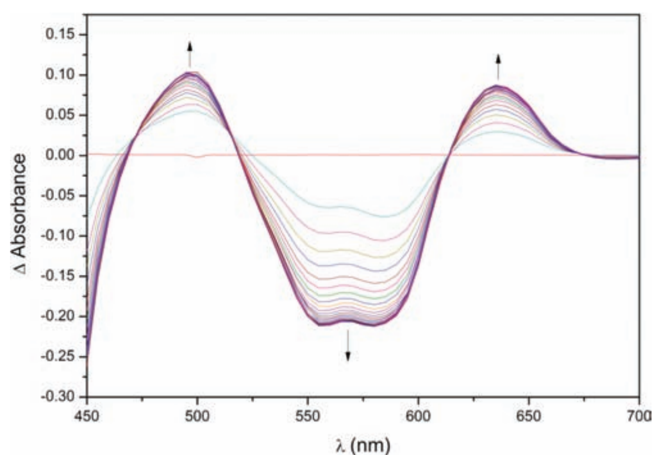

Figure 4. Difference absorption spectra obtained during the reaction of 6.65×10^{-5} mol L^{-1} MbFe^{IV}=O and vanillin at 2.0×10^{-4} mol L^{-1} in aqueous phosphate buffer [pH 7.4, μ = 0.32 (NaCl)] at 25 ± 0.1 °C. The time interval between spectra was 10 s.

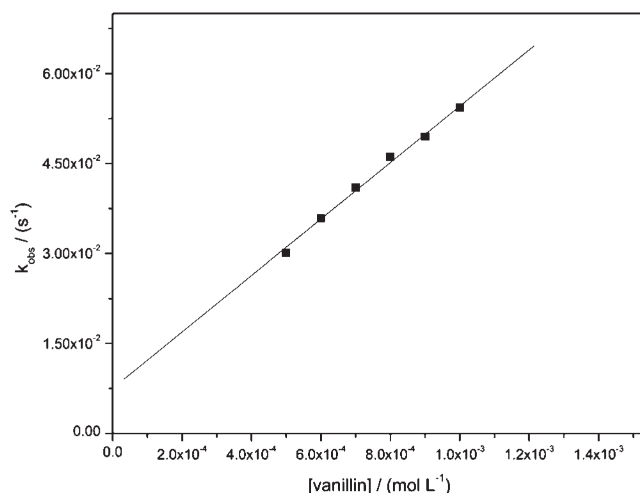

Figure 5. Observed rate constant for deactivation of MbFe^{IV}=O (3.1×10^{-5} mol L^{-1}) in aqueous phosphate buffer [pH 7.4, μ = 0.32 (NaCl)] and 25 ± 0.1 °C at varying excess concentrations of vanillin. The βLG concentration was constant at 2.0×10^{-5} mol L^{-1}.

to 8.0×10^{-5} mol L^{-1}

$$\frac{F_o}{F} = 1 + K_A[L]^n \qquad (1)$$

and in its logarithmic form

$$\log\left[\frac{(F_o - F)}{F}\right] = \log K_A + n\log[L] \qquad (2)$$

Because the determined value of n was close to unity, the association constant (K_A) for three temperatures at pH 7.4 and 2.0 at 25 °C was determined using a 1:1 binding model according to eq 3 as shown in Figure 1

$$\Delta F = \frac{(F_o - F_\infty)[L]}{K_D + [L]} \qquad (3)$$

where ΔF is the variation of the intrinsic βLG Trp19 fluorescence emission upon vanillin, L, binding as corrected for the inner-filter effect, F_o is the emission of the fluorescence probe in the absence of L, and F_∞ is the emission of the fluorescence probe with the binding sites saturated with vanillin.

The association constants and number of binding sites determined experimentally for the interaction between vanillin and proteins are reported in Table 1 for 15, 25, and 45 °C together with the derived values for $\Delta H°$ and $\Delta S°$ for binding of vanillin at βLG.

Binding of vanillin to βLG is clearly exothermic, and the positive binding entropy indicates that water is liberated from βLG upon vanillin binding. From an increase in the hydrogen ion concentration, an increase in the quenching of fluorescence was observed. This effect may be explained by better access of vanillin to Trp19 upon opening of the hydrophobic calyx.[25] βLG is fully negatively charged at pH 7.4 (pI = 4.8). Vanillin (pK_a = 7.4) is 50% on the anionic form, and electrostatic repulsion may decrease the access of vanillin to the protein. This difference could explain the blue shift in the fluorescence spectra upon vanillin binding to βLG at neutral pH in comparison to the red shift observed at acidic pH. The value for n is slightly higher than unity, and binding on the βLG surface near Trp61 may contribute to the overall quenching of βLG fluorescence. Stern−Volmer equation plots according to eq 1 show deviation from linearity for higher concentrations of vanillin (data not shown) in agreement with such excess binding, an effect studied in more details using ITC.

Binding of vanillin to βLG showed an exothermic character (Figure 2), in agreement with the temperature dependence of the fluorescence quenching of βLG by vanillin.

$$\beta\text{LG} + \text{vanillin} \rightarrow (\beta\text{LG/vanillin}) + \text{heat} \qquad (4)$$

Apparent ΔH of binding (eq 4) was found to vary with the temperature (Figure 3), indicating a significant difference in the observed heat capacity ($\Delta C_{p,\,obs}$) between products and reactants.

$$\Delta C_{p,\,obs} = C_{p(\text{vanillin}/\beta\text{LG})} - C_{p(\beta\text{LG})} - C_{p(\text{vanillin})} \qquad (5)$$

The enthalpy of association has high uncertainty, and an important source of uncertainty in the determination the value

Table 4. Second-Order Rate Constant and Activation Parameters (±SD) for the Reaction of $MbFe^{IV}{=}O$ with Vanillin and the Reaction of $MbFe^{IV}{=}O$ with Vanillin/*β*LG Complex in Aqueous Phosphate Buffer [pH 7.4, *μ* = 0.32 (NaCl)] for Varying Temperatures

	$k_{20\,°C}$ (L mol^{-1} s^{-1})	$k_{25\,°C}$ (L mol^{-1} s^{-1})	$k_{30\,°C}$ (L mol^{-1} s^{-1})	$k_{35\,°C}$ (L mol^{-1} s^{-1})	$k_{40\,°C}$ (L mol^{-1} s^{-1})	$\Delta H^‡$ (kJ mol^{-1})[a]	$\Delta S^‡$ (J mol^{-1} K^{-1})[a]
vanillin	32.9 ± 0.9	57 ± 1	81 ± 1	119 ± 7		58.8 ± 0.3	−14 ± 1
vanillin/*β*LG [b]	30.8 ± 0.6	48 ± 2	85 ± 6	123 ± 2	143 ± 7	68.6 ± 0.4	+17 ± 1

[a] For 25 °C, as derived from the Eyring equation. [b] *β*LG concentration was 2.0×10^{-5} mol L^{-1}.

Table 5. Bimolecular Rate Constant and Activation Parameters for the Reaction of $MbFe^{IV}{=}O$ with Selected Antioxidants and at 25 °C

	$k_{25\,°C}$ (L mol^{-1} s^{-1})	$\Delta H^‡$ (kJ mol^{-1})	$\Delta S^‡$ (J mol^{-1} K^{-1})	reference
chlorogenic acid	216	73	+41	31
ascorbate	2.9	51	−63	31
crocin	183	55	−17	32
rutin	105	69	+23	33
apigenin	125	65	+13	33
*β*LG	1.2	45	−93	34
iodide	0.34	43	−109	35
thiocyanate	0.12	45	−113	36

of apparent ΔH is the dissolution process of vanillin in the titration medium. Vanillin shows concentration-dependent self-association in aqueous solution, with a dissociation enthalpy of approximately 25 kJ mol^{-1} K^{-1} as demonstrated by nuclear magnetic resonance (NMR) experiments.[26,27] A negative value of $\Delta C_{p,\ obs}$, obtained from the slope of Figure 3, indicates increasing organization and more free rotation of the vanillin/*β*LG complexes compared to the reactants, in agreement with a strong association through hydrogen bonds rather than hydrophobic interactions.

Association of vanillin to *β*LG is concluded to occur at two different types of binding sites with different affinities. The stronger binding in the *β*LG calyx could be monitored by fluorescence quenching specific for the proximity of the Trp19 probe with a binding average of $n \approx 1$, while the microcalorimetric technique showed a global effect and an average binding with $n > 10$, as seen in Table 2. Using circular dichroism spectroscopy changes in the secondary structure of the protein could not be detected for *β*LG even for high concentrations of vanillin (data not shown), indicating that the interaction of *β*LG and vanillin is restricted to the tertiary structure of the protein. The two types of binding of vanillin to *β*LG are clearly evident from the different experiment techniques used, as seen in Table 3.

Deactivation of Ferrylmyoglobin by Vanillin. Ferrylmyoglobin was synthesized from metmyoglobin by the reaction with hydrogen peroxide, of which excess was removed by the addition of catalase. The purity of $MbFe^{IV}{=}O$ was controlled spectrophotometrically prior to the reaction with vanillin.

Deactivation of $MbFe^{IV}{=}O$ by vanillin was followed spectrophotometrically, as seen in Figure 4. The reaction is relatively fast and follows first-order kinetics for the excess of vanillin. The pseudo-first-order rate constant, k_{obs}, was calculated from absorption at 580 nm using nonlinear regression analysis. k_{obs} was found to depend linearly upon the concentration of the excess of vanillin, as seen in Figure 5

$$k_{obs} = k_1 + k_2[\text{vanillin}] \quad (6)$$

where k_1 is the rate constant for autoreduction of ferrylmyoglobin and k_2 is the second-order rate constant for deactivation of ferrylmyoglobin by vanillin. Similar results were obtained for the reduction of $MbFe^{IV}{=}O$ by vanillin in the presence of *β*LG. The second-order rate constant for different temperature conditions are collected in Table 4, together with the derived activation parameters. The deactivation of ferrylmyoglobin by vanillin is seen to be enthalpy-controlled for both free vanillin and vanillin bound to *β*LG. The value of k_2= 57 ± 1 L mol^{-1} s^{-1} obtained for the reduction of $MbFe^{IV}{=}O$ by vanillin is comparable to the value already know for the flavonoid hesperidin, also with an *ortho*-hydroxy/*ortho*-methoxy substitution pattern, but smaller than for flavan-3-ols, such as catechin and epicatechin, as seen in Table 5. Vanillin is, however, more efficient upon deactivation of $MbFe^{IV}{=}O$ than most other simple phenolic acids.[33]

The moderate low values of $\Delta H^‡$ combined with negative values of $\Delta S^‡$ reported for the reduction of $MbFe^{IV}{=}O$ by iodide ($\Delta H^‡$ = 43 kJ mol^{-1} and $\Delta S^‡$ = −109 J mol^{-1} K^{-1}) suggest a inner-sphere mechanism for electron transfer. For free vanillin and other phenolic compouds (Tables 4 and 5), the high enthalpy of activation and the positive values of entropy of activation rather indicate electron transfer by outer-sphere mechanism, with the reaction being enthalpy-controlled, in contrast to iodide with entropy control. Notably, binding of vanillin to *β*LG makes the entropy of activation more positive for deactivation of $MbFe^{IV}{=}O$. This seems to be in agreement with a long-range electron transfer by the outer-sphere reaction mediated by the Tyr or Trp residue between the two proteins, including a loss of organization in the transition state, suggesting a lower hydration in the outer-sphere transition state with a ternary complex, ferrylmyoglobin/*β*LG/vanillin.[37]

For ascorbate and the few plant phenolics for which activation parameters are known, the deactivation of ferrylmyoglobin, $\Delta H^‡$, linearly depends upon $\Delta S^‡$ (Figure 6). Such isokinetic behavior is indicative of a common reaction mechanism, which we ascribe as an outer-sphere electron transfer to the iron(IV) center. The other reductants investigated thus far deviate from this isokinetic behavior, and the mechanism with a significant contribution of inner-sphere electron transfer must be operating.

In summary, antioxidant properties of vanillin depend upon whether vanillin is free in solution or protein-bound, such as to *β*LG. Associations of vanillin and probably other phenolic compounds with globular whey proteins, such as *β*LG, create two pools of bound vanillin: one in the hydrophobic cavity and one on the surface of the protein with different types of association affecting their reactivity. The hydrophobic interaction in the calyx is of medium strength, specific, and exothermic,

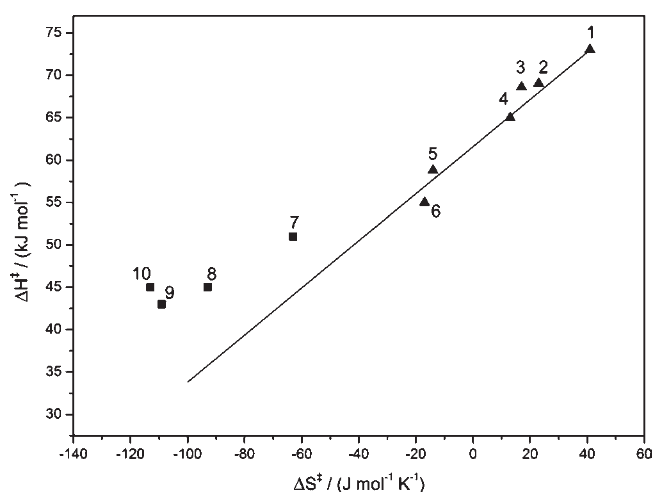

Figure 6. Isokinetic plots ($\Delta H^{\ddagger}$ versus $\Delta S^{\ddagger}$) for the reduction of $MbFe^{IV}=O$ to $MbFe^{III}$ by selected reductants in aqueous solution at neutral pH and 25 °C: (1) chlorogenic acid from ref 31, (2) rutin from ref 24, (3) βLG/vanillin complex from the present study, (4) apigenin from ref 33, (5) vanillin from the present study, (6) ascorbate from ref 31, (7) crocin from ref 32, (8) βLG from ref 34, (9) thiocyanate from ref 36, and (10) iodide from ref 35. The line was based on linear regression for reductants 1−6. Different mechanisms seem to be operating for the reductants (7−10) than for the plant phenols and ascorbate (1−6).

while the interaction at the surface is considered weak and nonspecific. The kinetic data for the reduction of ferrylmyoglobin to metmyoglobin by vanillin in the presence and absence of βLG demonstrate that the reactivity of vanillin is comparable to other phenolic compounds with an *ortho*-hydroxy/*ortho*-methoxy substitution pattern and, clearly, the deactivation of ferrylmyoglobin occurs with outer-sphere electron transfer. Finally, association of vanillin to βLG reduces the efficiency in deactivation of $MbFe^{IV}=O$ at low temperatures, but because of kinetic entropy/enthalpy compensation, vanillin becomes more efficient as a deactivator of $MbFe^{IV}=O$ at higher temperatures as a result of long-range electron transfer by the complexed vanillin and iron(IV) located in the hydrophobic heme pocket of myoglobin.

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