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Flavonoids as reductants of ferryl hemoglobin★

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The ferryl derivatives of hemoglobin are products of the reactions of oxy- and methemoglobin with hydrogen peroxide. Ferryl hemoglobins, either with or without a radical site on the protein moiety, are oxidizing species. Plant polyphenols, flavonoids, have been shown to act as antioxidants *in vivo* and *in vitro*. Reactions of met- and oxyhemoglobin with hydrogen peroxide in the presence of catechin, quercetin and rutin were studied. These flavonoids accelerated reduction of ferryl hemoglobin to methemoglobin. The rate constants of the reactions of ferryl hemoglobin with catechin, quercetin and rutin were in the order of 10^2 M^{-1} s^{-1}, i.e. similar to the rate constants of ferryl hemoglobin with intracellular reducing compounds like urate or ascorbate. The beneficial effect of flavonoids against oxidative damage of hemoglobin caused by hydroperoxides, reported in the literature, is probably, at least in part, connected with the ability of flavonoids to scavenge ferryl hemoglobin.

Keywords: ferryl hemoglobin, flavonoids, methemoglobin, oxyhemoglobin

INTRODUCTION

Hemoglobin (Hb) is the major heme protein of red blood cells. Fe(II)Hb is responsible for the transport of oxygen to tissues. *In vitro,* Fe(II)Hb undergoes slow autooxidation to give Fe(III)Hb (methemoglobin, metHb) and superoxide (Misra & Fridovich, 1972). The fraction of metHb, which cannot transport oxygen, in normal blood cells does not exceed 3% (Carrel *et al.,* 1975), but it can increase in the presence of certain drugs. The superoxide formed during Fe(II)Hb autooxidation dismutates to hydrogen peroxide. Hydrogen peroxide is a reactive oxygen species involved in the propagation of cellular injury in various pathophysiological conditions. The reaction of hydrogen peroxide with Fe(II)Hb (deoxyHb and oxyHb) and Fe(III)Hb (metHb) results in the formation of ferryl hemoglobin (ferrylHb) and ferrylHb with a globin-based radical, respectively (Winterbourn, 1990; Giulivi & Davies, 1994). *In vivo*, these reactions are of physiological relevance under ischemic conditions (Patel *et al.,* 1996*)*. Ferryl hemoglobin is able to oxidize proteins, nucleic acids and lipids (Kanner & Harel, 1985; Everse *et al.,* 1994; Goldman *et al.,* 1998; Kowalczyk *et al.,* 2007). The globin radical in ferrylHb is very unstable and rapidly decays to a longer-lived radical-free ferryl form (McArthur & Davies, 1993). FerrylHb undergoes slow acid-catalyzed autoreduction to the ferric (met) form (Tresoriere *et al.,* 2001), but the regenerated metHb is not identical with the initial met state: some hemichrome and dimers of Hb subunits are also formed (Kowalczyk *et al.,* 2007). The reduction of the ferryl form of hemoglobin is facilitated by some reducing compounds, including ascorbate (Giulivi & Davies, 1990), rutin (Grinberg *et al.,* 1994), urate (Everse & Hsia, 1997), melatonin (Tresoriere *et al.,* 2001), trolox (Tresoriere *et al.,* 2001), and epigallocatechin (Jia & Alayash, 2008).

Flavonoids are plant polyphenols and are present as common components in human diet. Their ability to act as antioxidants has been extensively

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Abbreviations: deoxyHb, deoxyhemoglobin; DMSO, dimethysulfoxide; ferrylHb, ferryl hemoglobin (Fe(IV)=O); Hb, hemoglobin; metHb, methemoglobin (Fe(III)); oxyHb, oxyhemoglobin (Fe(II)-O_2).

studied (Heim *et al.*, 2002, and references therein). On the other hand, their prooxidant activity has also been reported (Cotelle, 2001, and references therein). It has recently been shown that the flavonoid (-)epigallocatechin gallate, one of the main catechins isolated from green tea, efficiently reduces ferrylHb, but also enhances the autooxidation of human hemoglobin (Jia & Alayash, 2008).

In the present study we investigated the kinetics of the reduction of ferrylHb by three flavonoids: quercetin, rutin and catechin.

MATERIALS AND METHODS

Hemoglobin from bovine blood was obtained from Sigma. Methemoglobin was prepared by oxidation of Hb with a 2-fold excess of potassium ferricyanide and passage through a Sephadex G-25 column using 10 mM phosphate buffer (pH 7.0) as an eluent. Oxyhemoglobin was obtained by reduction of Hb with a 10-fold excess of sodium dithionite. The solution was then bubbled with O_2 and purified on a Sephadex G-25 column. Concentrations of metHb and oxyHb were determined spectrophotometrically using $\varepsilon_{405} = 1.79\times10^5$ M^{-1} cm^{-1} and $\varepsilon_{500} = 1.00\times10^4$ M^{-1} cm^{-1} for metHb, $\varepsilon_{415} = 1.25\times10^5$ M^{-1} cm^{-1} and $\varepsilon_{577} = 1.38\times10^4$ M^{-1} cm^{-1} for oxyHb, expressed per heme (Antonini & Brunori, 1971). FerrylHb was prepared by incubation 1.5×10^{-5} M MetHb with an equimolar amount of H_2O_2 in relation to the heme group in 10 mM phosphate buffer at room temperature for 5 min (Sztiller *et al.*, 2006).

Quercetin, rutin and catechin were obtained from Sigma. Stock solutions of quercetin and rutin were prepared in DMSO. Stock solution of catechin was prepared in water.

Absorbance spectra of oxy-, met- and ferryl hemoglobin were recorded in the range of 350–600 nm. Absorbance maxima of selected haemoglobin species are shown in Table 1. All spectrophotometric measurements were carried out at ambient temperature (23±1°C) using a Hewlett-Packard 8452A diode-array spectrophotometer. Water from MilliQ Plus was used throughout.

RESULTS AND DISCUSSION

We found that quercetin, rutin and catechin added to oxyHb at a molar ratio [flavonoid]/[heme] in the range of 1–15 did not significantly change the rate of autooxidation of oxyhemoglobin during the first 0.5 h. Our results differ from those published by Jia and Alayash (2008) who have observed an enhanced rate of autooxidation of cross-linked Hb in the presence of epigallocatechin gallate (EGCG).

Table 1. Absorbance maxima of deoxy-, oxy-, met- and ferrylhemoglobin in the Soret region

Compound	Absorbance maximum	Source
deoxyHb	430 nm	Antonini & Brunori, 1971
oxyHb	415 nm	Antonini & Brunori, 1971
metHb	405 nm	Antonini & Brunori, 1971
ferrylHb	418 nm	Herold & Rehmann, 2003

They explained the effect of EGCG by the formation of H_2O_2 during EGCG autooxidation and its subsequent reaction with oxyhemoglobin. Under our experimental conditions the rate of flavonoid autooxidation was probably significantly lower than the rate of autooxidation of oxyhemoglobin. It has been shown that the rate of H_2O_2 formation during autooxidation of epigallocatechins is relatively high in comparison to that measured during autooxidation of catechin (Mochizuki *et al.*, 2002; Munoz-Munoz *et al.*, 2008)

The absorbance spectra obtained after mixing oxyHb or metHb with H_2O_2 were characteristic for ferryl derivative of hemoglobin (ferrylHb with and without protein radical are spectroscopically indistinguishable). The values of the rate constant of the reaction of bovine metHb with H_2O_2 found in the literature vary from 13 M^{-1} s^{-1} (Nagababu *et al.*, 2002) to 3.7×10^3 M^{-1} s^{-1} (Patel *et al.*, 1996). We measured the rate of this reaction under pseudo-first order conditions by following the absorbance changes at 406 nm (near a maximum of MetHb) and found the second-order rate constant equal to 98 M^{-1} s^{-1} at pH 7.0. The reaction product, ferrylHb with a globin-based radical, also reacts with H_2O_2 to restore metHb (Nagababu & Rifkind, 2000), but this reaction is much slower. After mixing 3×10^{-5} M MetHb with 1.5×10^{-4} M H_2O_2 an absorbance spectrum characteristic for ferrylHb appeared after several minutes (Fig. 1). This spectrum was stable during the next several minutes and then slowly disappeared. When metHb was mixed with H_2O_2 in the presence of catechin, quercetin or rutin, a rapid decrease of the absorbance maximum of metHb, without any shift and then an increase of the absorbance maximum was observed (Fig. 2). Such absorbance changes were not observed when H_2O_2 was absent in the reaction mixture. It means that the investigated flavonoids reduce ferryl derivatives of hemoglobin in reactions characteristic for heme peroxidases:

$$\text{Fe(III)(peroxidase)} + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (1)$$

$$\text{Compound I} + SH \rightarrow \text{Compound II} + S^{\cdot} \quad (2)$$

$$\text{Compound II} + SH \rightarrow \text{Fe(III)(peroxidase)} + S^{\cdot} + H_2O \quad (3)$$

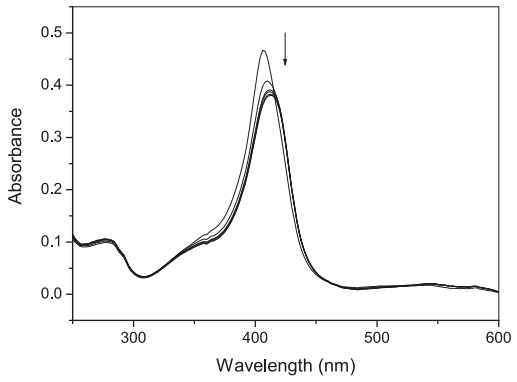

Figure 1. Reaction of MetHb with H_2O_2.
Absorbance spectra taken every 1 min after mixing 3×10^{-5} M MetHb with 1.5×10^{-4} M H_2O_2, l = 1 mm.

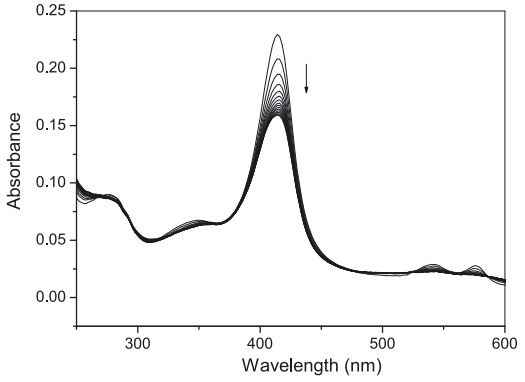

Figure 3. Reaction of oxyHb with H_2O_2.
Absorbance spectra taken every 1 min after mixing 2.2×10^{-5} M oxyHb with 1.5×10^{-4} M H_2O_2, l = 1 mm.

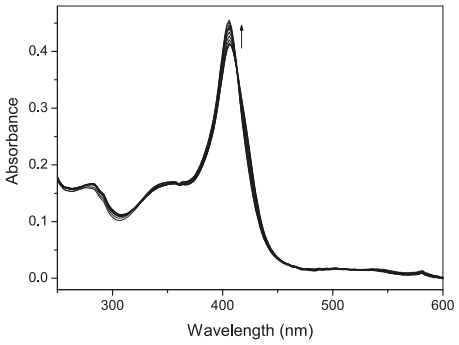

Figure 2. Reaction of MetHb with H_2O_2 and quercetin.
Absorbance spectra taken every 1 min after mixing 3×10^{-5} M MetHb, 1.5×10^{-4} M H_2O_2 and 1.5×10^{-4} M quercetin, l = 1 mm.

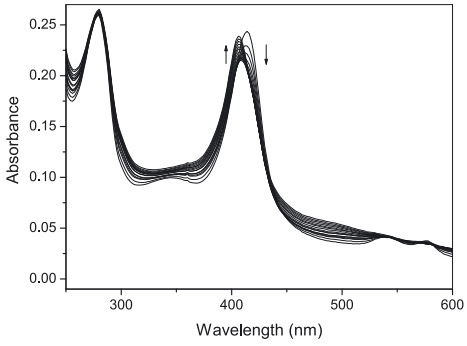

Figure 4. Reaction of oxyHb with H_2O_2 and catechin.
Absorbance spectra taken every 1 min after mixing 2.2×10^{-5} M oxyHb, 1.5×10^{-4} M H_2O_2 and 3.1×10^{-4} M catechin, l = 1 mm.

where compound I is a ferryl derivative (Fe(IV)=O) with a radical site on porphyrin or protein, compound II is a ferryl derivative without a radical site, and SH is a substrate to be oxidized. In the case of hemoglobin, ferrylHb with and without a globin-based radical are analogs of peroxidase compounds I and II, respectively. The lack of an absorbance maximum (or shoulder) characteristic for ferryl derivatives (at 414 nm) means that the rates of disappearance of ferryl derivatives in the reactions with flavonoids are comparable or higher than the rates of their formation. It is worth noting that under our experimental conditions the flavonoids did not react directly with H_2O_2 (we did not detect absorbance spectra of oxidized catechin, quercetin or rutin).

After mixing 2.2×10^{-5} M oxyHb with 1.5×10^{-4} M H_2O_2 a decrease of the absorbance maximum at 414 nm was observed during 15 min of the reaction (Fig. 3). When the investigated flavonoids were also added to this mixture, the decrease of absorbance at 414 nm was followed by a build-up of a spectrum with a maximum at 406 nm, characteristic for metHb (Fig. 4). Under such reaction conditions, ferrylHb which was formed in the reaction of oxyHb with H_2O_2 underwent reduction to metHb by the flavonoid.

In order to determine the rate constants of the reactions of the investigated flavonoids with ferrylHb, we preformed ferrylHb, mixed it with the flavonoids and observed the increase of absorbance at 406 nm, characteristic for metHb (Fig. 5). We assumed that the preformed ferryl species was an anolog of peroxidase compound II (ferrylHb with a protein radical is unstable with $t_{1/2}$ = 50 s (McArthur & Davies, 1993). We were unable to study this reaction under pseudo-first order conditions as catechin, quercetin and rutin, and their oxidation products absorb in the same wavelength range as hemoglobin. Thus, second-order rate constants were estimated from the initial reaction rates between ferrylHb and the flavonoids, measured for several flavonoid concentrations and taking $\Delta\varepsilon_{406}$(metHb – ferrylHb) = 0.64×10^5 M^{-1} cm^{-1} determined by us. The rate of autoreduction of ferrylHb was included into the calculation. It

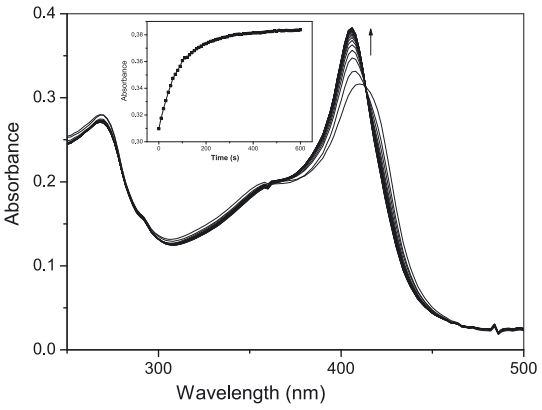

Figure 5. Reaction of ferrylHb with rutin.
Absorbance spectra taken every 30 s after mixing 2.5×10^{-6} M ferrylHb with 1×10^{-5} M rutin, l = 1 cm. *Inset:* Kinetic traces measured at 406 nm.

was at least one order of magnitude lower than the rate of the reduction of ferrylHb by the investigated flavonoids. The estimated rate constants of the reactions of ferrylHb with quercetin, rutin and catechin are collected in Table 2. They are of the order of 10^2 M^{-1} s^{-1}, i.e are similar to the rate constants of the reactions of ferrylHb with such reducing compounds like ascorbate, urate, nitrite and epigallocatechin gallate (Table 3).

A beneficial effect of flavonoids against oxidative damage of hemoglobin and red blood cells caused by hydroperoxides has been reported (Cesquini *et al.,* 2003; Pereira *et al.,* 2003). This effect seems to be, at least in part, connected with the ability of flavonoids to scavenge ferrylHb which is formed in the reaction of oxyHb with hydrogen peroxide or organic hydroperoxides. FerrylHb reacts with oxyHb to form metHb (comproportionation reaction (Giulivi & Davies, 1990). Thus, if we take into account the following reaction schemes:
a) in the absence of flavonoids (or other reducing agents):

$$\text{oxyHb} + H_2O_2 \rightarrow \text{ferrylHb} + O_2 + H_2O \quad (4)$$
$$\text{ferrylHb} + \text{oxyHb} + H_2O + \rightarrow 2\ \text{metHb} + O_2 \quad (5)$$
$$2\ \text{oxyHb} + H_2O_2 \rightarrow 2\ \text{metHb} + 2\ O_2 \quad (6)$$

Table 2. Estimated rate constants for reaction of some flavonoids with ferryl hemoglobin at pH 7.0 and 23°C

Flavonoid	Rate constant, M^{-1} s^{-1}
Catechin	0.87×10^2
Quercetin	3.6×10^2
Rutin	2.0×10^2

b) in the presence of flavonoids (or other reducing agents):

$$\text{oxyHb} + H_2O_2 \rightarrow \text{ferrylHb} + O_2 + H_2O \quad (4)$$
$$\text{ferrylHb} + \text{flavonoid} \rightarrow \text{metHb} + \text{oxidized flavonoid} \quad (7)$$
$$\text{oxyHb} + H_2O_2 + \text{flavonoid} \rightarrow \text{metHb} + \text{oxidized flavonoid} + O_2 + H_2O \quad (8)$$

we can see that the presence of flavonoids under conditions where ferrylHb is formed may prevent half of the oxyHb molecules from conversion to metHb (Grinberg *et al.,* 1994).

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Table 3. Rate constants of reduction of ferryl hemoglobin by different reducing compounds

Compound	Rate constant, M^{-1} s^{-1}	References
Ascorbate, 25°C, pH 7.4	15	Giulivi & Davies, 1990
Ascorbate, 37°C	4×10^2	Cooper *et al.*, 2008
Urate, 25°C, pH 7.4	1.53×10^2	Giulivi & Davies, 1990
Urate, 37°C	2×10^2	Cooper *et al.*, 2008
(-)Epigallocatechin gallate, 25°C, pH 7.4,	3.4×10^2	Jia & Alayash, 2008
Human oxyHb, 25°C, pH 7.4	23	Giulivi & Davies, 1990
Melatonin, 37°C, pH 7.4	40 (calculated from an initial reduction rate)	Tesoriere *et al.,* 2001
Nitrite, 20°C, pH 7	7.5×10^2	Herold & Rehmann, 2003
Trolox, 37°C, pH 7.4	30 (calculated from an initial reduction rate)	Tesoriere *et al.,* 2001

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