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Flavonol–serum albumin complexation. Two-electron oxidation of flavonols and their complexes with serum albumin

Olivier Dangles,*[a] Claire Dufour[b] and Stephan Bret[a]

[a] *Université Claude Bernard-Lyon I, UPRES A - CNRS 5078, Bât. 303, 43, bld du 11 Novembre 1918, 69622 Villeurbanne, France. E-mail: dangles@univ-lyon1.fr*
[b] *Institut National de la Recherche Agronomique, Unité des Arômes et Substances Naturelles, 2, place Viala, 34060 Montpellier, France*

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Quercetin (3,3′,4′,5,7-pentahydroxyflavone) and quercetin derivatives (3-methylquercetin, isoquercitrin, rutin) are strong polyphenolic antioxidants abundant in plants and in the human diet. Recent investigations have shown that significant concentrations of albumin-bound quercetin conjugates are present in the plasma of humans fed a quercetin-rich diet.

In this work, binding of quercetin and quercetin glycosides to bovine serum albumin (BSA) is quantitatively investigated by fluorescence spectroscopy. The strong fluorescence enhancement of quercetin upon binding points to the fact that a significant fraction of quercetin adopts a pyrylium-like structure in the complex. On the other hand, the observation of a very efficient quenching of tryptophan fluorescence by quercetin is consistent with a binding occurring in the IIA domain.

Flavonoid-derived quinones may be formed upon quenching of reactive oxygen species by flavonoids (antioxidant activity). In this work, the quinones are conveniently formed upon periodate oxidation of the selected flavonoids in methanol and in aqueous buffers with and without BSA. A kinetic investigation by UV–visible spectroscopy shows that albumin-bound flavonoids are oxidized as quickly as free flavonoids. Interestingly, the quercetin quinone, which is merely detectable in the absence of BSA because of fast solvent addition, is efficiently stabilized in the complex by charge transfer interactions (pH 9). No evidence for quercetin–BSA conjugates could be found, thus showing that water addition (and subsequent degradation) remains the sole significant pathway of quinone transformation in the complex.

Introduction

Flavonoids are widely distributed plant polyphenols involved in several biologically important mechanisms such as pigmentation, nitrogen fixation and chemical defense.[1] Many flavonoids also possess anti-cancer, anti-viral and anti-inflammatory properties which may be the consequence of their ability to inhibit a broad range of enzymes and to act as powerful antioxidants.[2] In particular, polyhydroxylated flavones and flavonols (3-hydroxyflavones) display electron-rich highly conjugated nuclei allowing them to quickly react with toxic reactive oxygen species (hydroxy, alkoxy and alkylperoxy radicals, singlet dioxygen, *etc.*)[3] and efficiently inhibit lipid peroxidation.[4] Being relatively abundant in the human diet, flavonoids could play an important role in the protective mechanisms against cardiovascular diseases and other pathologies in which reactive oxygen species are involved, as suggested by recent epidemiological studies.[5]

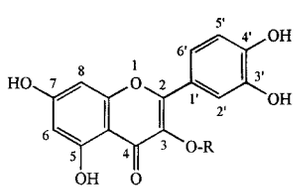
Human serum albumin (HSA), the well-known carrier of fatty acids in blood, has been shown to strongly bind quercetin (3,3′,4′,5,7-pentahydroxyflavone) and other structurally related flavonoids *in vitro*.[6] In addition, significant concentrations of quercetin conjugates were found in the plasma of rats fed a quercetin-rich diet, a result recently confirmed in man.[7] Such conjugates (glucuronides and sulfates of quercetin and 3′-methylquercetin) were essentially bound to albumin and retained significant antioxidant properties. Hence, the recognized protective effect of flavonoids on the vascular wall may be due to their albumin complexes rather than their free forms.

In this work, binding of quercetin and quercetin glycosides to bovine serum albumin (BSA) is quantitatively investigated by fluorescence spectroscopy (determination of the stoichiometries and binding constants). In the case of quercetin, the possible binding site and structural changes occurring in the flavonol nucleus upon binding are discussed.

Flavonoid quinones and quinonoid compounds could be formed in significant concentrations upon quenching of reactive oxygen species by flavonoids (antioxidant activity). Since quinones and quinonoid compounds may cause biopolymer modifications,[8] a kinetic investigation of flavonol oxidation by sodium periodate in the absence and in the presence of BSA is carried out in order to outline the possible consequences of flavonol–albumin binding on the formation and reactivity of flavonol quinones.

Results and discussion

Quercetin has been selected in this work because of its abundance in plants and because structure–activity relationships consistently rank it as one of the most powerful antioxidants in the flavonoid family.[2–4] Rutin, isoquercitrin and 3-methylquercetin (Scheme 1) are also considered in order to outline the influence



R = H (quercetin), Me (3-methylquercetin), β-D-Glc (isoquercitrin), α-L-Rham-1,6-β-D-Glc (rutin)

Scheme 1

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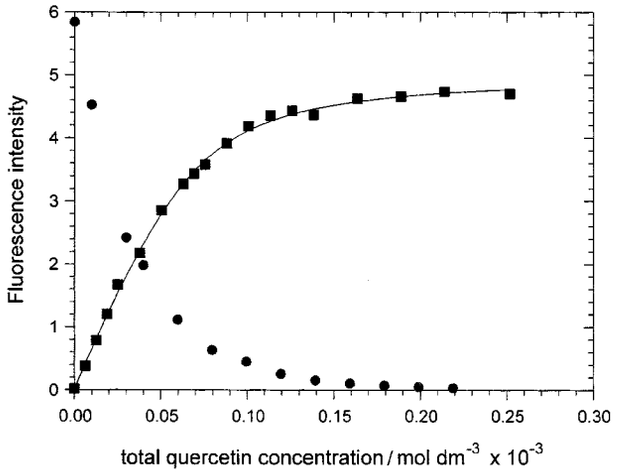

Fig. 1 Plot of the fluorescence intensity as a function of the total quercetin concentration in a pH 7.4 buffer containing BSA (7.52×10^{-5} mol dm^{-3}) (25 °C, ionic strength: 0.2 mol dm^{-3}). *Squares*: emission at 530 nm (excitation wavelength: 450 nm). The solid line is the result of the curve-fitting procedure (Scatchard analysis). *Circles*: emission at 340 nm (excitation wavelength: 295 nm).

of common substituents (methyl and glycosyl groups) on flavonoid oxidation and flavonoid–protein complexation.

Binding of quercetin and quercetin glycosides to BSA

The recent finding that significant concentrations of albumin-bound quercetin conjugates are present in the plasma of humans fed a quercetin-rich diet makes the quercetin–albumin complexation a biologically relevant model for investigating flavonoid–protein interactions. In this work, binding of quercetin to bovine serum albumin (BSA) is investigated by fluorescence spectroscopy. At pH 7.4, BSA was found to promote a strong saturable enhancement of quercetin fluorescence emission around 530 nm (excitation at 450 nm) (Fig. 1) as reported in the literature for HSA.[6a] Assuming n identical binding sites with a microscopic binding constant K (Scatchard analysis[9]), one obtains: $n = 0.95$ (±0.04), $K = 103$ (±16) $\times 10^3$ dm^3 mol^{-1} at 25 °C. Hence, the stoichiometry of the quercetin–BSA complex is 1 : 1.

In the same conditions, the BSA-induced fluorescence enhancements of the quercetin-3-glycosides, rutin and isoquercitrin (at saturation of the BSA binding sites) are respectively *ca*. 10 and 4 times as weak as for quercetin. Scatchard analysis of the quercetin-3-glycoside–BSA complexation yields $n = 2.66$ (±0.21), $K = 8.6$ (±1.1) $\times 10^3$ dm^3 mol^{-1} in the case of rutin and $n = 3.18$ (±0.15), $K = 14.5$ (±2.8) $\times 10^3$ dm^3 mol^{-1} in the case of isoquercitrin. Hence, the presence of a sugar moiety on the 3-position markedly weakens the flavonoid–BSA complexation and results in the rather loose binding of 2–3 flavonoid molecules to BSA.

Quercetin promotes a strong quenching of the BSA emission at 340 nm (excitation at 295 nm) (Fig. 1). For quercetin concentrations ensuring complete complexation of BSA (quercetin–BSA molar ratios higher than 2), the fluorescence emission of BSA is virtually suppressed. This efficient quenching is accompanied by a weak increase in the fluorescence emission at 530 nm typical of quercetin. Since excitation at 295 nm is expected to specifically involve tryptophan residues in proteins, this observation bears evidence of energy transfer occurring within the quercetin–BSA complex from an excited Trp residue to the flavonoid chromophore.

Among the two Trp residues present in the BSA structure, one (Trp 134) could be solvent-exposed whereas the other (Trp 212, Trp 214 in HSA) is buried inside the protein.[10] However, NMR investigations failed to detect signals characteristic of Trp residues, thus indicating that both lack mobility.[11] Trp 212 is part of the so-called IIA domain which is known to bind in its hydrophobic cavity a variety of ligands[10] including warfarin, a coumarin structurally related to flavonoids. X-ray crystallographic data confirm that Trp 214 in HSA is strategically located in the IIA domain to interact with bound ligands.[12]

The location of HSA-bound quercetin in the IIA domain was demonstrated from competitive binding studies between quercetin and warfarin.[6c] In this work, the observation of a very efficient quenching of tryptophan fluorescence by quercetin is consistent with this view.

3-Hydroxyflavone and its anion were recently shown to bind HSA to low affinity and high affinity sites, respectively.[13] The efficient energy transfer from BSA to neutral 3-hydroxyflavone was taken as evidence that the low affinity binding site was the IIA domain. Alternatively, the much slower energy transfer from BSA to the 3-hydroxyflavone anion suggested that the high affinity binding site was the IIIA domain.

Similar analysis cannot be carried out in the case of the quercetin–BSA complex because no spectral decomposition between the different acid–base forms of quercetin occurs. However, a spectroscopic titration of quercetin (5×10^{-5} mol dm^{-3}) between pH 5.0 and 8.2 in the presence of a constant concentration of BSA (7.52×10^{-5} mol dm^{-3}) yielded an absorbance *vs*. pH curve that could be fitted assuming a single proton transfer with $pK_a = 7.36$ (±0.03) at 25 °C and 0.2 mol dm^{-3} ionic strength. Hence, at physiological pH, BSA-bound quercetin is a mixture of two acid–base forms, probably neutral quercetin and its monoanion. The quercetin monoanion itself may be a mixture of tautomers formed upon deprotonation of the most acidic 4′-OH and 7-OH groups.[14] Since Scatchard analysis applies to the quercetin–BSA complex with an occupation number of *ca*. 1, it may be concluded that both neutral quercetin and its anion bind to the same site.

The quercetin–BSA complexation can also be investigated by fluorescence at pH 5.6 in spite of a much weaker BSA-induced fluorescence enhancement of quercetin. Scatchard analysis of the fluorescence titration curve yields: $n = 1.09$ (±0.04), $K = 81$ (±14) $\times 10^3$ dm^3 mol^{-1} at 25 °C. Hence, neutral quercetin and its anions roughly display the same affinity for BSA.

It must be emphasized that binding to BSA may help investigate the fluorescence properties of quercetin. Indeed, as a 5-hydroxyflavone, free quercetin fluoresces very weakly in both aqueous and non aqueous solvents.[15] For instance, we did not observe any significant fluorescence of quercetin in aqueous buffers, acetone or acetic acid. By contrast, quercetin displays a very strong fluorescence emission band around 520 nm (excitation at 450 nm) when dissolved in a 1 mol dm^{-3} HBr solution in acetic acid. Similar observations have already been made with flavones and flavonols[15,16] and interpreted by the protonation of O-4 in strongly acidic conditions and subsequent conversion of the neutral flavonoids into highly fluorescent pyrylium cations.

Binding to BSA in aqueous buffers is an alternative way to enhance quercetin fluorescence. A comparison between both experiments leads to the following observations:

(1) In spite of the very different conditions, the emission spectra of quercetin in HBr–acetic acid and in a pH 7.4 phosphate buffer containing BSA (excitation at 450 nm) are remarkably similar and display a maximum in the range 520–530 nm (Fig. 2, upper part).

(2) The excitation spectra of quercetin in HBr–acetic acid and in the phosphate–BSA buffer (emission at 530 nm) are also very similar and display a maximum in the range 450–460 nm.

(3) Whereas the excitation spectrum of quercetin coincides with its absorption spectrum in HBr–acetic acid, striking differences are observed in the phosphate–BSA buffer. Indeed, whereas BSA-bound quercetin displays an absorption maximum at 388 nm in the pH 7.4 phosphate buffer, it absorbs only very weakly in the range 450–460 nm where the excitation spectrum reaches its maximum (Fig. 2, lower part). Consist-

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Table 1 Relative total energies of quercetin monoanions (A) deduced from semi-empirical quantum mechanics calculations *in vacuum* (HyperChem program, AM1 parametrization). The figures refer to the positions of the negatively charged oxygen centers in the pyrylium mesomeric forms (see Scheme 2)

Form	$A_{4,7}$	$A_{4,4'}$	$A_{5,7}$	$A_{4',5}$	$A_{3,7}$	$A_{3,4'}$	$A_{4',7}$
E/kcal mol^{-1}	0	+5.6	+16.4	+10.9	+10.3	+11.9	+14.6

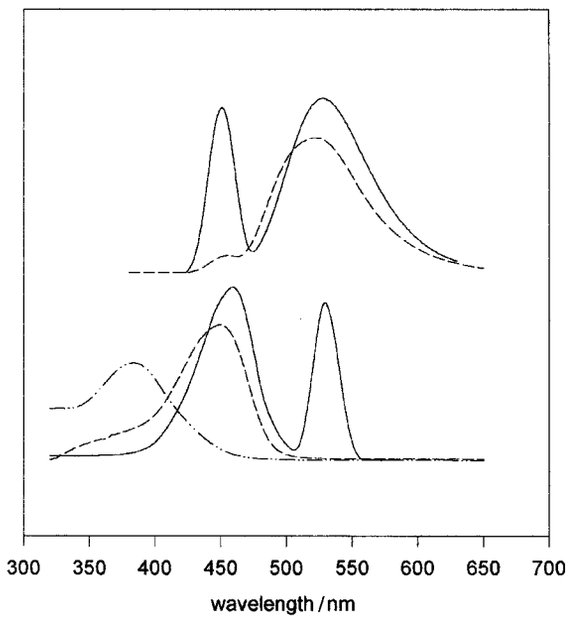
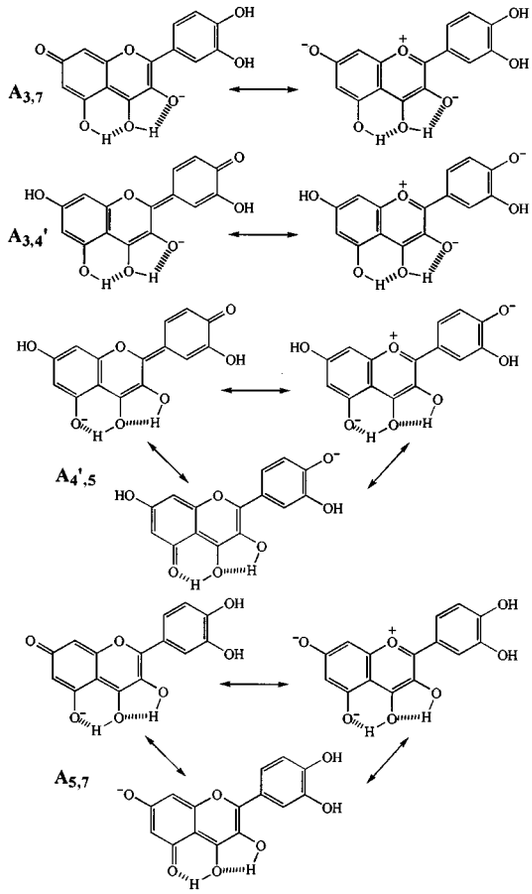


Fig. 2 *Upper part*: emission spectra of quercetin bound to BSA (*solid line*, quercetin–BSA molar ratio = 2, pH 7.4 phosphate buffer) and in HBr (1 mol dm^{-3})–acetic acid (*broken line*). Excitation wavelength: 450 nm. *Lower part*: excitation spectrum of BSA-bound quercetin (*solid line*, emission wavelength: 530 nm, quercetin–BSA molar ratio = 2, pH 7.4 phosphate buffer), absorption spectra of quercetin bound to BSA (*dotted line*, quercetin–BSA molar ratio = 2, pH 7.4 phosphate buffer) and in HBr (1 mol dm^{-3})–acetic acid (*broken line*).

ently, the fluorescence intensity at 530 nm is very weak when the sample is excited at 388 nm.

The latter observation suggests that bound quercetin in the ground state is actually a mixture of different forms with very different fluorescence properties. In weakly acidic and neutral aqueous buffers, free quercetin is essentially a mixture of non fluorescent 4-oxo forms. Binding to BSA may favour highly fluorescent pyrylium-like forms displaying a OH group on C-4 (4-hydroxy forms). This is consistent with the 11 nm bathochromic shift in the quercetin absorption band observed upon binding. Such tautomers could form upon intramolecular proton transfer along hydrogen bonds between OH-5 or OH-3 (donor) and O-4 (acceptor). In Table 1 are reported the calculated energies of tautomeric quercetin monoanions (Scheme 2) including the normal 4-oxo tautomers ($A_{4,7}$, $A_{4,4'}$) and the most probable 4-hydroxy tautomers ($A_{3,7}$, $A_{3,4'}$, $A_{5,7}$, $A_{4',5}$, $A_{4',7}$). Although much less stable (in their free form) than the normal 4-oxo tautomers, some highly fluorescent 4-hydroxy tautomers could be specifically stabilized upon binding to BSA, for instance, by hydrogen bonding between BSA and OH-4 (donor) and/or the 3- or 5-oxyanions (acceptor).

The close similarity between the excitation and emission spectra of BSA-bound quercetin with those of its free pyrylium cation points to a strong pyrylium character in the fluorescent tautomers of bound quercetin. Intramolecular proton transfer from OH-3 to O-4 leads to 4-hydroxy tautomers $A_{3,7}$ and $A_{3,4'}$ which display a stronger pyrylium character (one pyrylium form out of 2 mesomeric forms, see Scheme 2) than $A_{5,7}$ and $A_{4',5}$ formed upon intramolecular proton transfer from OH-5 to



Scheme 2

O-4 (one pyrylium form out of 3 mesomeric forms). The charge density on O-1 may be taken as a measure of the pyrylium character of the central ring. Values derived from semi-empirical quantum mechanics calculations are −0.14 for $A_{3,7}$ and $A_{3,4'}$ and −0.16 for $A_{5,7}$ and $A_{4',5}$. For comparison, values calculated for quercetin and its pyrylium cation are −0.13 and −0.09, respectively. We thus assume that $A_{3,4'}$ and $A_{3,7}$ are the major fluorescent tautomers of bound quercetin. Consistently, no fluorescence enhancement could be observed upon binding to HSA of luteolin (3′,4′,5,7-tetrahydroxyflavone), the 3-deoxy analog of quercetin.[6a]

It must be noted that tautomerization may also occur in the excited state through a fast intramolecular proton transfer from a highly acidic OH group (*e.g.*, OH-3 and OH-7) to the 4-keto group as demonstrated, for instance, in the case of 3-hydroxyflavone and 7-hydroxyflavone.[16,17] Such a phenomenon must also take place in the case of quercetin to account for the intensity of the emission band at 530 nm which is characteristic of pyrylium tautomers. However, it cannot explain the differences observed between the absorption and excitation spectra. Clearly, BSA allows the formation of significant, although probably weak, concentrations of pyrylium-like tautomers in the ground state.

Oxidation of quercetin and quercetin derivatives

Antioxidants are compounds which, at low concentrations, can protect biomolecules (proteins, nucleic acids, polyunsaturated lipids, sugars) from oxidative degradations.[18] Flavonoids may act in a variety of ways including direct quenching of the reactive oxygen species and inhibition of enzymes involved in the production of the reactive oxygen species and chelation of low valent metal ions (Fe^{2+}, Cu^{+}) able to promote radical formation through Fenton-type reactions.[2–4]

Upon quenching of one-electron oxidants such as oxygen-

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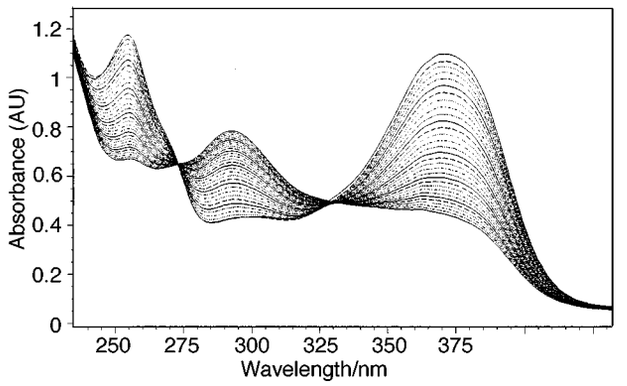


Fig. 3 Changes in the UV–visible spectrum of quercetin (5×10^{-5} mol dm^{-3}) after addition of sodium periodate (1 equiv) in methanol at 25 °C. Time interval: 0–2 h.

centered radicals, flavonoids are converted into aryloxy radicals. From pulse radiolysis experiments, such radicals were characterized by their UV–visible spectra and shown to quickly decay through second-order kinetics ($2k$ in the range 10^6–10^7 dm^3 mol^{-1} s^{-1}).[3] We have recently demonstrated that the second-order decay of quercetin radicals actually leads to quinones and quinonoid compounds which quickly form water adducts.[19] Hence, flavonoid-derived quinones and quinonoid compounds could be produced in significant concentrations upon flavonoid oxidation, especially in media of relatively low water content *e.g.*, in hydrophobic protein cavities and at the surface of biological membranes. Since quinones and quinonoid compounds have well-documented oxidizing and electrophilic properties which may eventually cause biopolymer modifications through oxidation and/or covalent coupling,[8] investigating the formation and reactivity of flavonoid quinones in the presence of biopolymers may be a biologically relevant issue.

Periodate oxidation in protic solvents is a very convenient way to generate quinones from the parent flavonoids. Kinetic investigations of flavonoid oxidation by sodium periodate have been carried out in methanol and in aqueous buffers with and without BSA.

Oxidation in methanol

Oxidation of quercetin with sodium periodate in methanol results in a decrease of the UV absorption bands characteristic of quercetin (λ_{max} = 370, 255 nm) with a simultaneous increase of a band with an absorption maximum at 293 nm (Fig. 3). In addition, NMR analysis in CD_3OD shows that all five aromatic protons of the chromophore are strongly shielded upon oxidation. Both UV–visible and NMR spectra point to restricted electronic delocalization in the oxidized product in comparison to quercetin.

The absorbance *vs.* time traces can be satisfactorily fitted assuming a bimolecular oxidation of quercetin (QH_2) leading to a quinone (Q) (rate constant k_1) that quickly adds two solvent molecules (S) on positions 2 and 3 to give QS_2 (rate constant k_2) (Scheme 3). Constant k_1 essentially refers to the breakdown of the quercetin–periodate adduct. As demonstrated for catechol,[20] fast kinetic techniques are required for detecting the step of adduct formation.

The following equations (1)–(3) were used in the curve-fitting

$$-\mathrm{d}[QH_2]/\mathrm{d}t = k_1[QH_2][IO_4] = -\mathrm{d}[IO_4]/\mathrm{d}t \quad (1)$$

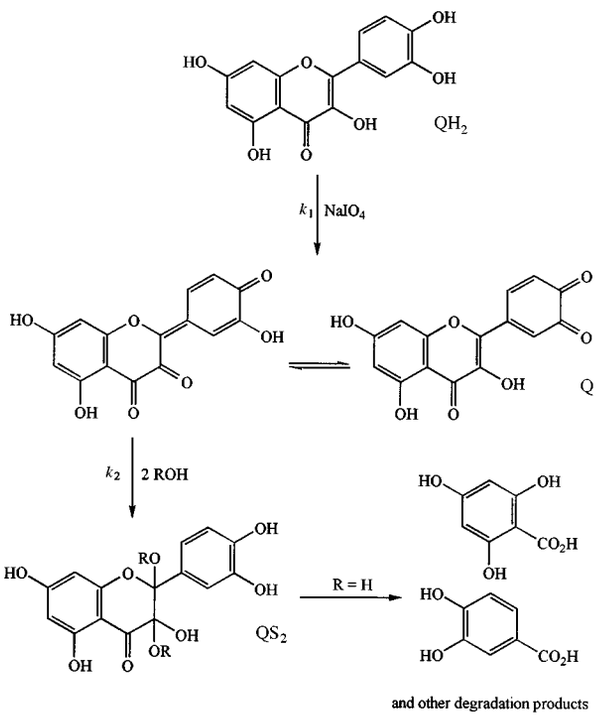
$$\mathrm{d}[Q]/\mathrm{d}t = k_1[QH_2][IO_4] - k_2[Q] \quad (2)$$

$$A = A_0[QH_2]/c + \varepsilon_1[Q] + \varepsilon_2[QS_2] \quad (3)$$

Table 2 Flavonol oxidation by $NaIO_4$ in MeOH at 25 °C. Flavonol concentration: 5×10^{-5} mol dm^{-3}. $NaIO_4$–flavonol molar ratio = 1 (quercetin) or 10 (3-*O*-methylquercetin). QH_2: flavonol, Q: flavonol quinone

Flavonol	Quercetin	3-*O*-Methylquercetin
k_1/dm^3 mol^{-1} s^{-1}	6.0 (±0.2)[a]	1.98 (±0.02)[a]
k_2/s^{-1}	4.4 (±0.2) × 10^{-4} [a]	[b]
$\lambda_{max}(QH_2)$/nm	372	359
ε(Q) at $\lambda_{max}(QH_2)$/ dm^3 mol^{-1} cm^{-1}	14500 (±130)	5450 (±80)

[a] Determined at $\lambda_{max}(QH_2)$. [b] No detectable solvent addition.



Scheme 3

procedure, where A: absorbance at time t, A_0: initial absorbance, c: total quercetin concentration, ε_1: molar absorption coefficient of Q, ε_2: molar absorption coefficient of QS_2.

Values for k_1 and k_2 are reported in Table 2. The structure of the quinone–methanol adduct is consistent with earlier reports in the literature.[21] In addition, CI mass analysis allowed detection of a quinone–methanol adduct (m/z = 333 (MH^+)). The quercetin quinone may be depicted as a mixture of two tautomeric *o*-quinone and *p*-quinonoid forms (Scheme 3).

3-Methylquercetin is oxidized by periodate 3 times as slowly as quercetin. By contrast, the 3-methylquercetin quinone does not undergo solvent addition and steadily accumulates as evidenced by the raising of a weak broad absorption in the range 420–550 nm. Such differences in reactivity can be interpreted as follows:

(1) The 3-hydroxy-4-keto group of quercetin is a suitable site for reaction with periodate. Indeed, even flavonols (3-hydroxyflavones) devoid of the catechol group quickly react with periodate in alcohols. Thus, a privileged route in periodate oxidation of quercetin may be periodate coordination with the 3-hydroxy-4-keto group and subsequent formation of the *p*-quinonoid form. With 3-methylquercetin however, the only possible, and probably less favourable, route is periodate coordination with the catechol nucleus and subsequent formation of the *o*-quinone.

(2) The quercetin quinone reacts with methanol through its *p*-quinonoid form and adds solvent molecules on C-2 and C-3. Methylation of OH-3 prevented the *o*-quinone–*p*-

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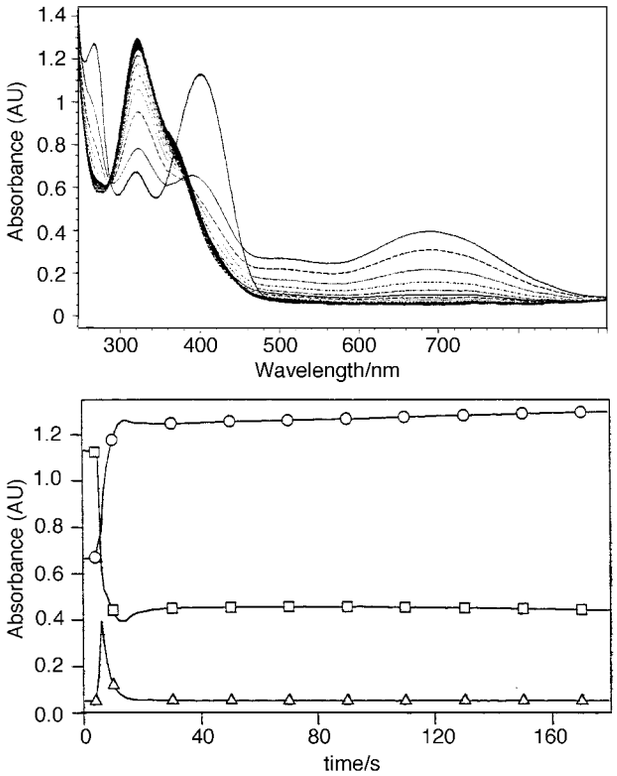

Fig. 4 *Upper part*: changes in the UV–visible spectrum of quercetin (5×10^{-5} mol dm^{-3}) after addition of sodium periodate (1 equiv) in a pH 9.0 buffer (25 °C, ionic strength: 0.2 mol dm^{-3}). *Lower part*: kinetic traces at 690 (△), 402 (□) and 324 nm (○).

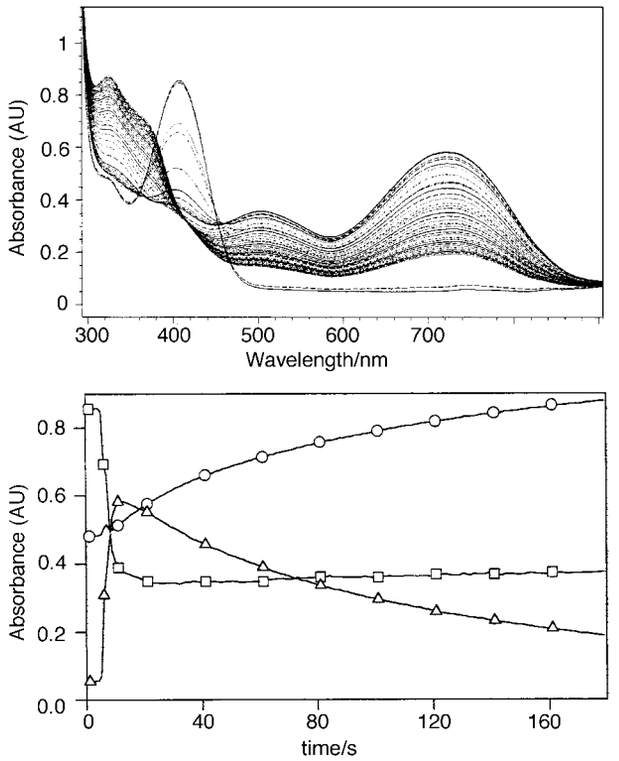

Fig. 5 *Upper part*: changes in the UV–visible spectrum of quercetin (5×10^{-5} mol dm^{-3}) after addition of sodium periodate (1 equiv) in a pH 9.0 buffer containing BSA (7.52×10^{-5} mol dm^{-3}) (25 °C, ionic strength: 0.2 mol dm^{-3}). *Lower part*: kinetic traces at 723 (△), 406 (□) and 324 nm (○).

quinonoid tautomerism leaving as the only possibility for the 3-methylquercetin quinone to react with the solvent through a Michael addition on the C-2′, C-5′ and/or C-6′ positions of its *o*-quinone nucleus. Such a reaction is probably very slow in methanol and is not detected in the course of the experiment.

Rutin was not significantly oxidized by periodate in methanol over a period of half an hour.

Oxidation in aqueous buffers

In the absence of BSA. Oxidation is much faster in aqueous buffers (pH 5.5 to 9.0) than in methanol. The wavelengths of absorption maximum for quercetin and the quinone–water adduct gradually shift to higher values when the pH is increased. This is consistent with deprotonation of the most acidic phenolic OH groups. At pH 5.5, quercetin is a mixture of neutral and monoanionic forms[22] (λ_{max} = 368 nm). At physiological pH values (pH 7.4), it is essentially dianionic (λ_{max} = 377 nm) but not significantly more prone to periodate oxidation. By contrast, at pH 9.0, quercetin is essentially trianionic (λ_{max} = 402 nm) and its periodate oxidation is much faster.

Remarkably, a weak, rapidly decaying absorption band around 690 nm is observed when oxidation is carried out at pH 7.4 and 9.0 (Fig. 4). This transient absorption (ΔE *ca.* 1.8 eV) is attributed to a quinone–quercetin charge transfer complex. Charge transfer must occur primarily from the HOMO of the donor (quercetin) to the LUMO of the acceptor (quinone). The calculated energy differences between the HOMO of the quercetin dianion (deprotonation of OH-4′ and OH-7) and the LUMO of the *o*-quinone and *p*-quinonoid 7-oxyanions are respectively 1.64 and 1.85 eV and thus fall in the correct range to account for a charge transfer process from the flavonoid to its parent quinone.

The first-order decay of the charge transfer band at 690 nm gives access to the rate constant of water addition on the quinone (rate constant k_2). At pH 7.4 and 9.0, this value was then used in the curve-fitting of the kinetic trace at λ_{max} of the water–quinone adduct (*ca.* 330 nm) which yielded the bimolecular rate constant of quinone formation (rate constant k_1). Values for k_1 and k_2 are reported in Table 3. At pH 9.0, periodate oxidation is so fast that only a rough estimate of k_1 can be given (1–3 × 10^4 dm^3 mol^{-1} s^{-1}).

No attempt was made to isolate oxidized products because of their well-known instability. Indeed, water addition on the quinone is followed by opening of the pyran ring and subsequent cleavage of the carbon chain.[23]

Periodate oxidation in the pH 9 phosphate buffer is slower for rutin and 3-methylquercetin than for quercetin (Table 4) in agreement with the investigation in methanol (Table 2). However, under those conditions, the rutin and 3-methylquercetin quinones decay with first-order rate constants of the same order of magnitude than that of the quercetin quinone.

In the presence of BSA. Periodate oxidation of quercetin in BSA containing aqueous buffers at pH 5.5 to 9.0 results in the following changes in the UV–visible spectrum of the flavonoid (Fig. 5):

(1) A fast decrease in the absorption band characteristic of quercetin (λ_{max} ranging from 375 nm at pH 5.5 to 408 nm at pH 9) followed, at pH higher than 7, by a slow increase.

(2) A monotonous increase in the absorption band characteristic of quinone–water adducts (λ_{max} in the range 320–340 nm, band masked by protein absorption at low pH).

(3) Above pH 7, a fast building-up of a new absorption band with λ_{max} ranging from 697 nm at pH 7.4 to 723 nm at pH 9. At pH 7.4, this band remains very weak and disappears within 10 seconds whereas, at pH 9, it grows very intense and persists for *ca.* 2 minutes.

The previous mechanism can be kept for fitting the absorbance *vs.* time traces, *i.e.* a second-order step of quercetin oxidation (rate constant k_1) leading to low concentrations of quinone which rapidly adds two solvent molecules (rate constant k_2). The values for k_1 and k_2 (Table 3) show that the kinetics of both steps are weakly altered by the presence of BSA

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Table 3 Quercetin oxidation by $NaIO_4$ in aqueous buffers (25 °C, ionic strength: 0.2 mol dm^{-3}). Quercetin concentration: 5×10^{-5} mol dm^{-3}. $NaIO_4$–quercetin molar ratio = 1

pH	5.5	7.4	9.0
k_1/dm^3 mol^{-1} s^{-1}	33.4 (±0.4) × 10^2 [b]	20.7 (±0.8) × 10^2 [c]	[e]
k_2/s^{-1}	0.060 (±0.003) [b]	0.63 (±0.02) [d]	0.379 (±0.003) [c]
k_1 [a]/dm^3 mol^{-1} s^{-1}	67.1 (±1.5) × 10^2 [b]	20.0 (±0.8) × 10^2 [c]	[e]
k_2 [a]/s^{-1}	0.111 (±0.005) [b]	0.087 (±0.007) [d]	11.83 (±0.09) × 10^{-3} [c]

[a] In the presence of BSA (7.52×10^{-5} mol dm^{-3}). [b] Determined at λ_{max}(quercetin). [c] Determined at λ_{max}(quinone–water adduct). [d] Determined from the first-order decay of the charge transfer band. [e] In the range 1–3 × 10^4 dm^3 mol^{-1} s^{-1}.

Table 4 Flavonol oxidation by $NaIO_4$ in a pH 9.0 phosphate buffer (25 °C, ionic strength: 0.2 mol dm^{-3}). Flavonol concentration: 5×10^{-5} mol dm^{-3} for quercetin and 3-methylquercetin, 3.7×10^{-5} mol dm^{-3} for rutin

Flavonol	Quercetin	3-Methylquercetin	Rutin
k_1/dm^3 mol^{-1} s^{-1}	1–3 × 10^4	30.4 (±0.8) × 10^2	40.6 (±0.6) × 10^2
k_2/s^{-1}	0.379 (±0.003)	0.22 (±0.01)	0.167 (±0.004)
k_1 [a]/dm^3 mol^{-1} s^{-1}	1–3 × 10^4	556 (±10)	86 (±8) × 10^2
k_2 [a]/s^{-1}	11.83 (±0.09) × 10^{-3}	0.197 (±0.006)	0.22 (±0.01)

[a] In the presence of BSA (7.52×10^{-5} mol dm^{-3}).

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except at pH 9.0 where BSA (7.52×10^{-5} mol dm^{-3}) slows down the addition of water on the quinone by a factor *ca.* 30. Remarkably, the oxidation of bound quercetin is as fast as, if not faster than, the oxidation of free quercetin (in the absence of BSA). Thus, the 3′,4′-dihydroxy group and/or the 3-hydroxy-4-keto group of bound quercetin seem to remain accessible to oxidizing agents such as the periodate anion.

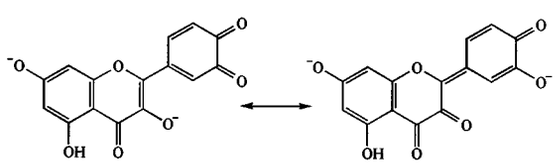
At pH 5.5 and 7.4, the quinone remains a very minor species that does not significantly accumulate during the kinetic runs. By contrast, at pH 9.0, the quinone reaches much higher concentrations as evidenced by the formation of an intense absorption band at 723 nm which is attributed to a quinone–BSA charge transfer complex (half-life *ca.* 1 min). In addition, a weaker absorption band at 504 nm also appears which may be characteristic of the free quinone.

The strong enhancement in charge transfer and quinone stability when the pH is raised from 7.4 to 9.0 may be a combination of effects:

(1) The deprotonation of residues (Lys, Cys, Tyr) that would enhance the donor character of the protein. For instance, charge transfer interactions between the thiolate group of a cysteine residue and the oxidized form of a flavine coenzyme have been demonstrated in the catalytic mechanism of glutathione reductase.[24] If quercetin is assumed to remain in the IIA domain upon oxidation, no free Cys residue is available for charge transfer interaction. By contrast, in addition to Tyr 261, up to 12 Lys residues[10] (from Lys 203 to Lys 284) may lose a proton on their side chain and act as donor centers.

(2) A pH-dependent conformational change in the protein allowing more favourable quinone–donor molecular contacts in the complex. Deprotonation of Lys residues is actually known to promote a conformational change in BSA from the N form to the B form.[10] In the B form which prevails at pH 9, the helical content is slightly lower than in the N form which prevails around neutrality. The conformational change between the N and B forms could place the quinone nucleus in tight contact with Trp 212 in the IIA domain, thus favouring charge transfer interactions. Hence, Trp 212 may act as a donor not only in the energy transfer process occurring in the quercetin–BSA complex but also in the charge transfer process occurring in the quinone–BSA complex. Whereas the former process can take place over large distances extending up to 6–8 nm,[25] the latter process requires significant HOMO (donor)–LUMO (acceptor) overlapping, typically occurring for donor–acceptor distances lower than 0.8 nm. Hence, the strong quinone–BSA charge transfer band observed at pH 9 may be evidence of a close contact between the quinone nucleus and Trp 212.

(3) The deprotonation of the quinone. Deprotonation of the *o*-quinone 7-oxyanion on OH-3 and deprotonation of the *p*-quinonoid 7-oxyanion on OH-3′ both lead to two mesomeric structures which outline the large electron delocalization in the quinone dianion (Scheme 4). Hence, the second deprotonation



Scheme 4

of the quercetin quinone may be primarily responsible for the much higher stability of the quinone at pH 9 as shown by its much slower reaction with water (Table 3).

Size exclusion chromatography on a G100 Sephadex gel resulted in the complete recovery of unmodified BSA. No trace of stable flavonoid–protein adducts could be detected. This is in contrast to previous works reporting the formation of stable covalent adducts between BSA and the *o*-quinone of caffeic acid (generated by polyphenol oxidase catalyzed oxidation of caffeic acid).[26]

Periodate oxidation of rutin and 3-methylquercetin in a pH 9.0 phosphate buffer was only slightly affected by the presence of BSA (Table 4). In particular, BSA has no influence on the kinetics of water addition on the quinones and no quinone–protein charge transfer complex could be detected.

Substitution of OH-3 in flavonols has the following consequences:

(1) It may markedly weaken the binding to BSA as demonstrated in this work in the case of the quercetin-3-glycosides rutin and isoquercitrin. Because of the large structural similarity between 3-substituted flavonols and their quinones, loose binding to BSA may be postulated for the quinones themselves in a way that does not allow suitable donor–acceptor molecular contacts in the complexes.

(2) It holds the corresponding quinones in an *o*-quinone structure that could be much less prone to charge transfer interactions than the *p*-quinonoid nucleus of the quercetin quinone.

Conclusion

The large changes induced by the quercetin–BSA complexation in the fluorescence properties of both partners are consistent with a 1:1 binding occurring in the IIA domain and allowing

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the formation of significant concentrations of highly fluorescent pyrylium-like quercetin tautomers. Quercetin-3-glycosides display lower affinities for BSA than quercetin.

Periodate oxidation in pH 5.5–9.0 aqueous buffers is as fast for the flavonol–BSA complexes as for the free flavonols and leads to quinones which quickly add water molecules and are then degraded. At pH 9, water addition on the quinone is much slower and large concentrations of quinone in charge transfer interaction with BSA can be detected in the first step of the reaction. However, even under such conditions, no evidence of BSA–quinone covalent adducts could be found, thus showing that water addition remains the sole significant pathway of quinone deactivation. This observation suggests that flavonoid quinones that may form upon quenching of reactive oxygen species by flavonoids (antioxidant activity) are innocuous, rapidly degraded compounds rather than potentially damaging electrophiles or oxidizing agents.

Experimental

Materials

Quercetin, rutin and BSA (fraction V, MW = 66500 g mol^{-1}) were from Sigma-Aldrich. Isoquercitrin was from Extrasynthese (Genay, France). 3-Methylquercetin was a gift from Professors B. Voirin and M. Jay (Université Claude Bernard-Lyon I). The following buffers were prepared: 0.2 mol dm^{-3} acetate buffer (pH 5.5), 0.02 mol dm^{-3} Na_2HPO_4 (pH 7.4), 0.05 mol dm^{-3} Na_2HPO_4–0.05 mol dm^{-3} NH_4Cl buffer (pH 9.0). In all buffers, the ionic strength is 0.2 mol dm^{-3} (adjusted by NaCl addition when needed).

Absorption spectra

Spectra were recorded on a Hewlett-Packard 8453 diode-array spectrometer equipped with a magnetically stirred quartz cell (optical pathlength: 1 cm). The temperature in the cell was kept at 25 °C by means of a water-thermostated bath.

Fluorescence spectra

Fluorescence spectra were recorded on a BioLogic spectrometer using a quartz cell thermostated at 25 °C.

Quercetin–BSA complexation

Two parts of a solution of BSA (7.25×10^{-5} mol dm^{-3}) in a pH 7.4 phosphate buffer were respectively added with methanol and a solution of quercetin in methanol (quercetin concentration after dilution in the buffer = 2.5×10^{-4} mol dm^{-3}). The final methanol concentration was 4% in both solutions. Both solutions were then mixed in different ratios in order to cover the quercetin concentration range 0–2.5×10^{-4} mol dm^{-3}. The binding constant and number of binding sites on BSA were calculated from the plot of the fluorescence intensity at 530 nm (excitation at 450 nm) as a function of the total quercetin concentration according to the Scatchard method.[9]

Periodate oxidation

In a typical experiment, 50 mm^3 of a freshly prepared 2×10^{-3} mol dm^{-3} solution of flavonoid in methanol was diluted into 1.9 cm^3 of buffer (with or without BSA) placed in the spectrometer cell and thermostated at 25 °C. At time zero, 50 mm^3 of a 2×10^{-3} mol dm^{-3} solution of sodium periodate in water was added.

Size exclusion chromatography

To 50 mg of BSA dissolved in 10 cm^3 of a pH 9.0 phosphate buffer were successively added 200 mm^3 of a freshly prepared 2.5×10^{-3} mol dm^{-3} solution of quercetin in methanol and 100 mm^3 of a 5×10^{-3} mol dm^{-3} solution of sodium periodate in water. After a few minutes, a part of the reaction mixture (5 cm^3) was laid on the top of a G100 Sephadex columm (mass of gel: 10 g) conditioned with a pH 5.5 phosphate buffer. After elution with the same buffer, the samples containing unmodified BSA (based on its UV spectrum) were gathered. The amount of BSA was determined spectroscopically and found to be the same (within experimental error) as the initial amount.

Methanol–quercetin quinone adduct

A 10^{-2} mol dm^{-3} solution of quercetin in CD_3OD was added with an equivalent amount of sodium periodate. After stirring for a few hours, the ^{1}H-NMR spectrum was recorded (300 MHz, 27 °C): δ (ppm) = 7.14 (d, J = 2.2 Hz, H-2′), 7.02 (dd, J = 8.5, 2.2 Hz, H-6′), 6.79 (d, J = 8.5 Hz, H-5′), 5.99 (d, J = 1.8 Hz, H-8), 5.95 (d, J = 1.8 Hz, H-6). Mass (CI, sample in CH_3OH): m/z = 333 (MH^+, mono-adduct).

Data analysis

The curve-fittings of absorbance *vs*. time plots were carried out on a Pentium 120 PC using the Scientist program (MicroMath, Salt Lake City, Utah, USA). Beer's law and sets of differential kinetic equations with initial conditions on concentrations were input in the model. Curve-fittings were achieved through least square regression and yielded optimized values for the parameters (kinetic rate constants, molar absorption coefficients). Standard deviations are reported.

Calculations

Semi-empirical quantum mechanics calculations were run at zero kelvin *in vacuum* on a Pentium 90 PC using the HyperChem program (Autodesk, Sausalito, California, USA) with the AM1 parametrization.

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