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Reactivity of Bovine Whey Proteins, Peptides, and Amino Acids toward Triplet Riboflavin as Studied by Laser Flash Photolysis

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The reaction between the triplet excited state of riboflavin and amino acids, peptides, and bovine whey proteins was investigated in aqueous solution in the pH range from 4 to 9 at 24 °C using nanosecond laser flash photolysis. Only tyrosine and tryptophan (and their peptides) were found to compete with oxygen in quenching the triplet state of riboflavin in aqueous solution, with second-order rate constants close to the diffusion limit, 1.75×10^9 and 1.40×10^9 L mol^{-1} s^{-1} for tyrosine and tryptophan, respectively, with β-lactoglobulin and bovine serum albumin having comparable rate constants of 3.62×10^8 and 2.25×10^8 L mol^{-1} s^{-1}, respectively. Tyrosine, tryptophan, and their peptides react with the photoexcited triplet state of riboflavin by electron transfer from the tyrosine and tryptophan moieties followed by a fast protonation of the resulting riboflavin anion rather than by direct H-atom abstraction, which could be monitored by time-resolved transient absorption spectroscopy as a decay of triplet riboflavin followed by a rise in riboflavin anion radical absorption. For cysteine- and thiol-containing peptides, second-order rate constants depend strongly on pH, for cysteine corresponding to $pK_{aRSH} = 8.35$. H-atom abstraction seems to operate at low pH, which with rising pH gradually is replaced by electron transfer from the thiol anion. From the pH dependence of the second-order rate constant, the respective values for the H-atom abstraction ($k = 1.64 \times 10^6$ L mol^{-1} s^{-1}) and for the electron transfer ($k = 1.20 \times 10^9$ L mol^{-1} s^{-1}) were determined.

KEYWORDS: Amino acids; laser flash photolysis; photooxidation; riboflavin; bovine whey proteins

INTRODUCTION

Riboflavin (vitamin B2) is widely present in foods and has been shown to act as a photosensitizer of many biological substrates (*1*−*5*). Milk is an important source of riboflavin, and because riboflavin may transform ground state oxygen into the very aggressive singlet oxygen or directly initiate free radical processes, light-induced changes in milk often become critical for product quality (*6*−*9*). Photooxidation reactions in milk not only affect the sensory quality but may also lead to loss of nutrients and formation of potentially toxic oxidation products (*5*). The off-flavors generated by light-induced oxidation in dairy products fall into two groups: the off-flavors initially formed derive from amino acids, peptides, and proteins after short-tem light exposure, while long-term exposure to light initiates reactions that involve lipids (*10*). Despite several studies of the photochemical oxidation of proteins and lipids in milk and dairy products (*3*, *9*−*13*), relatively little information is available about the mechanism of the initial step in the formation of light-induced off-flavors and the kinetics of light-induced protein oxidation in milk.

The aim of the present work is to contribute to a better understanding of the mechanism and the kinetics of the early events in the light-induced oxidation of milk proteins sensitized by riboflavin. We have determined the bimolecular rate constant for the reaction of the riboflavin triplet state with bovine β-lactoglobulin, bovine serum albumin (BSA), and small peptides present in milk, which may act as quenchers of the riboflavin triplet state. Also, the rate constant for the reaction with free amino acids was determined in order to identify possible sites of attack of the riboflavin triplet state on milk proteins.

MATERIALS AND METHODS

Materials. The pH of the aqueous solutions was buffered with analytical grade KH_2PO_4 (Merck, Darmstadt, Germany), pH 4, and Na_2HPO_4/KH_2PO_4 (Merck) for pH values from 4.5 to 9. The ionic strength = 0.2 M was adjusted with NaCl (Merck). The amino acids (cysteine, cystine, methionine, tryptophan, tyrosine, phenylalanine, and histidine) and the peptides (HCysGlyOH and HCys(CysGly)GlyOH) from Sigma-Aldrich (Steinheim, Germany) and the peptides (HTyrGlyOH, HGly-

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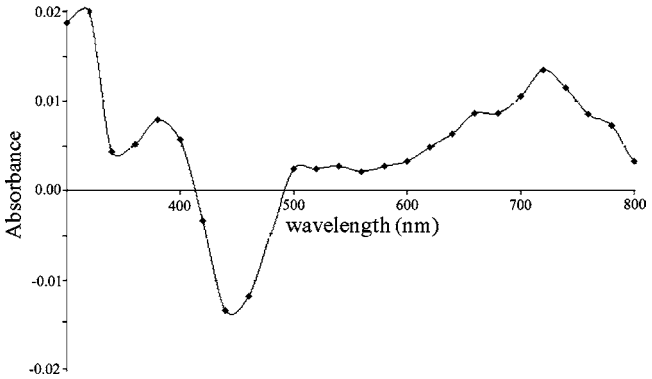

Figure 1. Transient absorption (excited state minus ground state) spectrum of 6.4 10^{-7} M riboflavin in aqueous solution deoxygenated with N_2 at pH 6.4 and 24.0 ± 0.5 °C recorded 0.2 μs after the 440 nm laser pulse.

TyrOH, and HGlyTyrGlyOH) from Bachem Feinchemikalien AG (Bubendorf, Switzerland) were all used as received. The β-lactoglobulin (MW 18300 g mol^{-1}) from bovine milk was obtained from Arla Foods Ingredients (Videbaek, Denmark). BSA (MW 66 kDa) from bovine milk (99% purity) was obtained from Sigma-Aldrich. The distilled water was purified using a Milli-Q Plus system (Millipore Corp., Bedford, MA). The nitrogen gas used for anaerobic experiments was of high purity (99.99%). Samples were purged with high purity nitrogen in cuvettes closed by rubber septa for 30 min prior to experiments, and a flow of nitrogen was maintained in the closed cuvettes during the laser flash photolysis. Similar results were obtained for a solution for which freeze−pump−thaw cycles were used to remove oxygen.

Laser Flash Photolysis Kinetic Experiments. Laser flash photolysis experiments were carried out with an LKS.50 spectrometer from Applied Photophysics Ltd. (Leatherhead, United Kingdom). The third harmonic at 355 nm of a pulsed Q-switched Nd:YAG laser was used to pump a dye laser (Spectron Laser System, Rugby, United Kingdom) using Coumarin 120, which has a emission peak at 440 nm. The intensity of the 10 ns laser pulse was approximately 2.3 mJ cm^{-2}. A R928 photomultiplier tube from Hamamatsu (Hamamatsu, Japan) was used to detect the transient absorption (300−800 nm). Appropriate UV cutoff filters were used to minimize the sample degradation by the monitoring light. The samples were excited in 0.5 cm × 1.0 cm fluorescence cuvettes or, for the protein solutions, using a four-window flow cell 0.5 cm × 0.5 cm from Hellma (Mulheim, Germany). All measurements were made with fresh solutions thermostated at 24.0 ± 0.5 °C and purged with N_2 before the experiment. The rate constants are reported together with standard deviations.

RESULTS AND DISCUSSION

Irradiation of riboflavin solutions at 440 nm generates the lowest energy riboflavin singlet excited state, which by efficient intersystem crossing yields the very reactive riboflavin triplet state implicated in a wide range of photobiological processes (*1*, *2*, *4*, *14*−*18*). **Figure 1** shows the transient absorption spectrum of the riboflavin triplet state in N_2-saturated aqueous solution at pH 6.4, which is characterized by absorption maxima at 320, 380, 660, 720, and 780 nm and a bleaching centered at 445 nm assigned to the depletion of the ground state. This transient absorption of the reactive triplet state, which is used to monitor the reaction of the triplet state with amino acids, peptides, and proteins in real time, is similar to the transient spectra reported for riboflavin and flavin in the literature (*4*). The triplet lifetime was estimated to be 13.4 μs from a monoexponential fit to the decay curve (see **Figure 2**). **Figure 2** shows the time profile observed at 720 nm used to monitor the triplet−triplet (T−T) transition in the kinetic experiments.

Quenching of Triplet Riboflavin by Amino Acids and Peptides. The triplet decay of triplet riboflavin followed first-order kinetics in the absence and in the presence of certain amino

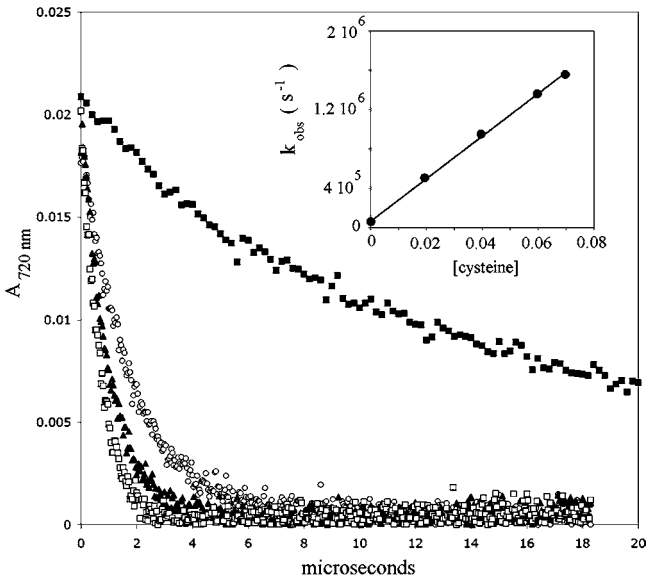

Figure 2. Transient time profile observed at 720 nm (24.0 ± 0.5 °C) in the absence (■) and presence of different concentrations of cysteine as quencher (other symbols). Insert: Dependence for the observed decay constant for triplet riboflavin on the cysteine concentration in mol L^{-1}.

Table 1. Second-Order Rate Constant for Quenching of Riboflavin Triplet State by Amino Acids, at pH 6.4 (Raw Milk pH), 24.0 ± 0.5 °C, and N_2-Saturated Solutions

amino acid	k (L mol^{-1} s^{-1})
cysteine	$2.16 \pm 0.03\ 10^7$
tyrosine	$1.40 \pm 0.10\ 10^9$
tryptophan	$1.75 \pm 0.09\ 10^9$
cystine	quenching not observed
methionine	$6.36 \pm 0.29\ 10^7$
histidine	$5.22 \pm 0.13\ 10^7$
phenylalanine	quenching not observed

acids, which can transfer electrons or donate hydrogen to the riboflavin triplet state. The decay of the triplet state was found to be accelerated by the presence of certain amino acids with decay rates proportional to the concentration of the amino acids, as may be seen for cysteine in **Figure 2**, and similar results were obtained for other amino acids. From the pseudo-first-order decay kinetics monitored at 720 nm, the second-order reaction rate constants were determined from the pseudo-first-order rate constants for different amino acids concentrations, as can be seen in **Table 1**.

The results show that a broad range of rate constants were obtained for the amino acids in question, except for tyrosine and tryptophan, which both exhibit a second-order rate constant corresponding to a diffusion-controlled bimolecular reaction. The results obtained for tyrosine and tryptophan and the less efficient histidine are qualitatively in agreement with results reported for the reaction with triplet flavin mononucleotide (*4*, *19*). The cysteine and phenylalanine did not exhibit any quenching effect of the triplet state of riboflavin, observations, which suggest that a group with good H-donation or electron donation ability should be present in the side chain of the amino acid in order to be an efficient quencher. Tyrosine and tryptophan did not show any pH dependence, providing strong evidence for an electron transfer mechanism operating for these two amino acids in agreement with other reports in the literature (*17*, *19*).

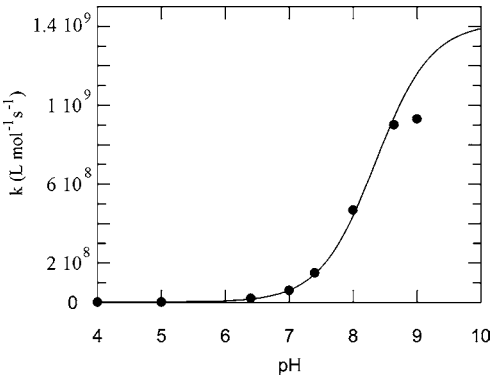

Figure 3. pH dependence of the second-order rate constant for the riboflavin triplet state quenching by cysteine at 24.0 ± 0.5 °C in anaerobic aqueous solution.

An interesting behavior was observed for cysteine- and thiol-containing peptides, for which rate constants were found to show a strong pH dependence that follows the deprotonation curve of the thiol group corresponding to the pK_a value (**Figure 3**). The pH dependence of the second-order rate constant for cysteine in the pH range from 4 to 9 could be described by a single acid−base equilibrium corresponding to the following pH dependence for the observed second-order rate constant, k_{obs}:

$$k_{obs} = k_{acid}\,[10^{-pH}/(10^{-pH} + 10^{-pK_a})] + k_{base}\,[10^{-pK_a}/(10^{-pH} + 10^{-pK_a})] \quad (1)$$

Using eq 1, in which k_{acid} and k_{base} denote the rate constant for the thiol (RSH) and the thiol anion (RS^-), respectively, and pK_a = 8.35 (*20*) for cysteine (RSH), the parameters k_{acid} and k_{base} were fitted to the rate constant data from **Figure 3** to yield the solid line in **Figure 3** and the second-order rate constant for the H-atom abstraction, $k_{acid} = 1.64 \times 10^6$ L mol^{-1} s^{-1}, and for the direct electron transfer, $k_{base} = 1.2 \times 10^9$ L mol^{-1} s^{-1}. These results for the two rate constants are in accordance with data obtained experimentally for low pH and high pH, $k_{RSH} = 1.65 \times 10^6$ mol L mol^{-1} s^{-1} (pH 2.0) and $k_{RS^-} = 9.30 \times 10^8$ L mol^{-1} s^{-1} (pH 9.0), indicating that the increase in the second-order rate constant for cysteine at increasing pH can be attributed to the rise in the concentration of the thiolate ion and a switch in the mechanism to electron transfer at a high pH. At the pH of raw milk pH (~6.4), a small amount of RS^- is present and the observed second-order rate constant, 2.16×10^7 L mol^{-1} s^{-1}, depends on both mechanisms. The pH dependence of the rate constant for the reaction between triplet riboflavin and cysteine was previously reported to follow a sigmoid type curve that, however, was not assigned to a specific mechanism (*1*, *15*, *18*). H-atom abstraction from hydrogen donors has previously been demonstrated for aromatic amino acids (*19*). The different pH profile observed in the present study between aromatic amino acids and cysteine adds strong support to a shift from electron transfer to hydrogen atom transfer at a low pH for cysteine.

The rate constants obtained for the quenching of triplet riboflavin by the amino acids suggest that only tyrosine and tryptophan can act as a quencher for the riboflavin triplet state in milk and under physiological conditions in an aerobic medium, since the rate constant for the bimolecular reaction between the oxygen and the riboflavin triplet state is 9.8×10^8 L mol^{-1} s^{-1}. To evaluate whether the amino acid side chains in the protein can exhibit the same behavior as observed for the free amino acids, we carried out experiments using small peptides to mimic the behavior of the amino acid moieties in the protein. H-Cys-Gly-OH and its oxidized form, H-Cys-(CysGly)-Gly-OH, recently identified in milk as a potential antioxidant (*21*), were also included in the investigation. The second-order rate constants determined for these peptides using the same approach for the amino acids are presented in **Table 2**.

Table 2. Second-Order Rate for Quenching of Riboflavin Triplet State by Small Peptides, at pH 6.4 (Raw Milk pH), 24.0 ± 0.5 °C, and N_2-Saturated Solutions

peptide	k (L mol^{-1} s^{-1})
H-Cys-Tyr-Cys-Tyr-OH	$1.85 \pm 0.25\ 10^9$
H-Cys-Gly-OH	$6.73 \pm 0.10\ 10^7$
H-Cys(CysGly)Gly-OH	quenching not observed
H-Tyr-Gly-OH	$1.25 \pm 0.13\ 10^9$
H-Gly-Tyr-OH	$1.56 \pm 0.27\ 10^9$
H-Gly-Tyr-Gly-OH	$2.08 \pm 0.16\ 10^9$

Table 3. Second-Order Rate Constant for Quenching of Triplet Riboflavin by Whey Proteins in Aqueous N_2-Saturated Solution at pH 6.4 (Raw Milk pH) and at pH 9, 24.0 ± 0.5 °C

	k (L mol^{-1} s^{-1})	
whey protein	pH 6.4	pH 9.0
β-lactoglobulin	$3.62 \pm 0.31\ 10^8$	$4.98 \pm 0.36\ 10^8$
BSA	$2.25 \pm 0.12\ 10^8$	$3.82 \pm 0.25\ 10^8$

The results obtained for the small peptides that contain tyrosine showed that the kinetics of deactivation by amino acids in the peptides was similar to what was observed for the free amino acids. The peptides, which contain cysteine, showed a small second-order rate constant at pH 6.4, which we assign mainly to an H-atom abstraction mechanism. Furthermore, a significant pH dependency similar to cysteine was observed. However, the role of the sulfur moiety in the antioxidant activity of milk toward light-induced oxidation is not to act as a direct quencher of triplet riboflavin but rather to act as a scavenger of radicals formed after the reaction of triplet riboflavin with other molecules. From our present findings, it is however expected that the amino acid moiety in peptides and in protein chains has a similar behavior as observed for the corresponding free amino acids.

Electron Transfer from Bovine Whey Proteins to Riboflavin Triplet State. The results obtained for amino acids and peptides indicate that only the aromatic amino acids can compete with oxygen in quenching of the triplet state of riboflavin at raw milk pH. Consequently, these two amino acids are expected to react with triplet riboflavin under physiological conditions, because they are present in the major whey proteins in bovine milk, β-lactoglobulin, and BSA, which were also included in the investigation.

As can be seen in **Table 3**, the rate constants obtained for the reaction of triplet riboflavin with these bovine whey proteins are also close to the diffusion control and competitive with oxygen in quenching of the triplet riboflavin under physiological conditions. No pH dependency of the reaction between triplet riboflavin and the whey proteins was observed as only a small increase in rate was noted with rising pH, which may be assigned to conformational changes or unfolding of the protein with rising pH. All together, the above results are in accordance with an electron transfer mechanism since electron transfer will show no or little pH dependence. H-abstraction from thiol groups is accordingly competitive with neither the reaction of oxygen

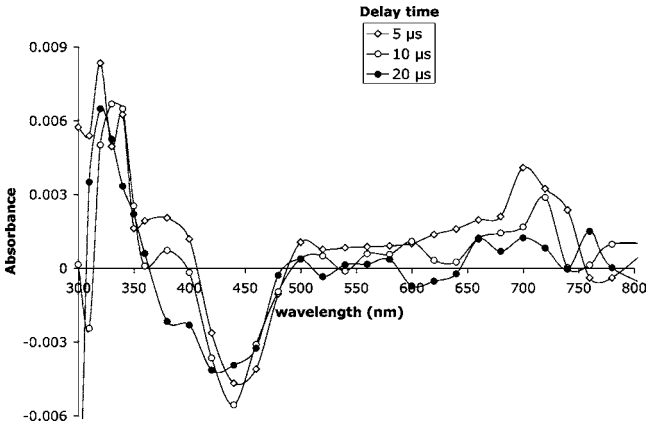

Figure 4. Transient absorption spectra (excited state minus ground state absorption) recorded 5, 10, and 20 μs after the laser pulse into an anaerobic solution containing riboflavin (34 mM) and β-lactoglobulin (0.16 mM) at 24.0 ± 0.5 °C.

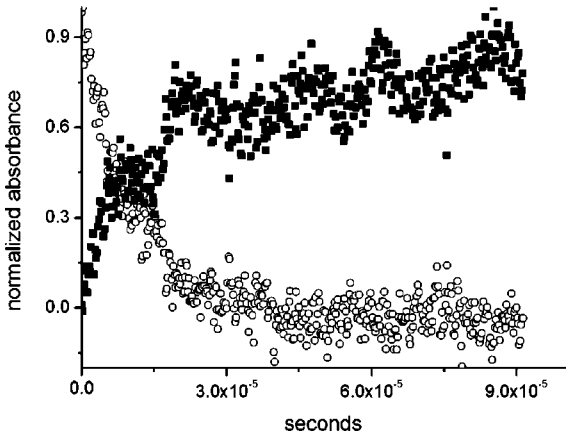

Figure 5. Transient time profile observed for the radical (D–D transition) $RfH^{\bullet}$ at 340 nm (growth, ■) and decay (○) profile for the triplet ^3Rf (T–T transition) at 720 nm of a solution containing riboflavin (34 mM) and β-lactoglobulin (0.16 mM) at 24.0 ± 0.5 °C.

nor the electron transfer from aromatic amino acids to the excited triplet state of riboflavin.

Additional evidence for the electron transfer mechanism was obtained from the transient absorption spectra of a solution containing riboflavin and β-lactoglobulin at different delay times: 5, 10, and 20 μs after the 10 ns laser pulse (**Figure 4**). The spectrum at 5 μs corresponds to the anion radical, $Rf^{\bullet-}$ (red shifted, band centered at 340 nm), while the spectra at later time correspond to the neutral radical $RfH^{\bullet}$ (blue shifted, band at 320 nm) formed by a fast protonation of the anion radical at physiological pH, given the riboflavin anion radical $pK_a = 8.3$ (*4*). The transient absorption spectra obtained in the presence of β-lactoglobulin show similar absorption bands to those reported for ^3Rf, $Rf^{\bullet-}$, and $RfH^{\bullet}$ suggesting that the principal mechanism of triplet deactivation is an initial electron transfer from the protein moiety to the triplet riboflavin (*19*). **Figure 5** shows the kinetic time trace for the growth of the signal at 340 nm corresponding to $Rf^{\bullet-}$ and the decay of the triplet excited state of riboflavin at 720 nm.

Conclusion. Riboflavin-sensitized light oxidation of whey proteins in bovine milk can be attribute to a so-called type I mechanism that proceeds via direct electron transfer from the tyrosine and/or tryptophan side chains in the protein to the triplet riboflavin yielding a protein radical and a reduced riboflavin radical, which subsequently can generate reactive oxygen molecules that can attack biological targets and then trigger the oxidative process:

$$^1Rf + h\nu \rightarrow {}^1Rf^* \rightarrow {}^3Rf \quad (2)$$

$$\text{protein} + {}^3Rf \rightarrow {}^2\text{protein}^{\bullet+} + {}^2Rf^{\bullet-} \quad (3)$$

$$^2Rf^{\bullet-} + H^+ \rightarrow {}^2RfH^{\bullet} \quad (4)$$

$$2\ {}^2RfH^{\bullet} \rightarrow \{RfH_2{-}RF\} \rightarrow RFH_2 + Rf \quad (5)$$

$$RfH_2 + O_2 \rightarrow \{Rf{-}\text{radical} + O_2^{\bullet-}\} \rightarrow Rf + H_2O_2 \quad (6)$$

$$^2Rf^{\bullet-} + O_2 \rightarrow Rf + O_2^{\bullet-} \quad (7)$$

$$^2\text{protein}^{\bullet}/{}^2RfH^{\bullet}/O_2^{\bullet-} + \text{biomolecule} \rightarrow \text{oxidative products} \quad (8)$$

Direct deactivation of the triplet state riboflavin by oxygen to form reactive oxygen species should also be considered (type II mechanism). However, for the actual concentrations in milk, quenching by proteins and peptides will dominate kinetically favoring the type I mechanism. Further studies should be carried out to evaluate the role of the radicals produced during photooxidation of whey proteins, and the riboflavin radical needs special attention since the reactivity of this radicals has not been studied in any detail. Such studies should also include the mechanism by which the initially formed riboflavin radical and protein radical are transformed into the photoproduct, dimethyl disulfide, known as the main component of the "burnt feather" off-flavor of light-exposed dairy product (*8*). Furthermore, the coupling between protein oxidation and lipid oxidation in dairy products should be studied also on a mechanistic level (*22*).

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