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Dityrosine, 3,4-Dihydroxyphenylalanine (DOPA), and Radical Formation from Tyrosine Residues on Milk Proteins with Globular and Flexible Structures as a Result of Riboflavin-Mediated Photo-oxidation

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ABSTRACT: Riboflavin-mediated photo-oxidative damage to protein Tyr residues has been examined to determine whether protein structure influences competing protein oxidation pathways in single proteins and protein mixtures. EPR studies resulted in the detection of Tyr-derived *o*-semiquione radicals, with this species suggested to arise from oxidation of 3,4-dihydroxyphenylalanine (DOPA). The yield of this radical was lower in samples containing β-casein than in samples containing only globular proteins. Consistent with this observation, the yield of DOPA detected on β-casein was lower than that on two globular proteins, BSA and β-lactoglobulin. In contrast, samples with β-casein gave higher yields of dityrosine than samples containing BSA and β-lactoglobulin. These results indicate that the flexible structure of β-casein favors radical−radical termination of tyrosyl radicals to give dityrosine, whereas the less flexible structure of globular proteins decreases the propensity for tyrosyl radicals to dimerize, with this resulting in higher yields of DOPA and its secondary radical.

KEYWORDS: tyrosine oxidation, riboflavin, milk proteins, termination reaction, secondary radical

INTRODUCTION

Photo-oxidation is believed to play an important role in damage to foods that contain a high content of photosensitizers, of which dairy products are excellent examples.[1] The mechanism by which sensitizer-mediated photo-oxidation takes place was initially outlined by Foote.[2,3] Two processes appear to play major roles: formation of substrate-derived radicals or excited states as a result of energy or electron transfer reactions (type I photoreactions) and energy transfer from an excited triplet state of the sensitizer to O_2 yielding singlet oxygen (O_2 in its $^1\Delta_g$ state; type II photoreactions).[2,3] Singlet oxygen is a highly reactive oxidant, which is known to damage proteins.[4,5] Although there has been considerable discussion as to the targets of sensitizer-mediated oxidation, recent studies indicate that tryptophan (Trp) and tyrosine (Tyr) residues are the only amino acids that are able to compete with unsaturated fatty acids[6] or oxygen[7] in the quenching of triplet state riboflavin. Riboflavin is found in high concentration in dairy products,[8,9] and protein Trp and Tyr residues may therefore be important targets of photo-oxidation in milk and in other food products in which proteins that contain these residues are present at high concentrations. Whether such species contribute to oxidative damage by propagating damage or acting as "sinks" for oxidizing species remains to be established.

The primary (amino acid composition), secondary, and tertiary structures of proteins can each influence the extent and nature of protein oxidation.[10−12] Free amino acids have been reported to be superior to peptides in quenching singlet oxygen,[13] whereas native globular proteins showed lower quenching ability toward singlet oxygen than the unfolded species.[14] Peptides and proteins have been reported to have the same quenching activity as the corresponding free amino acids when they were completely unfolded.[13,14] Whereas physical quenching of excited states, a common process with Trp residues, results in no chemical change to the target molecule, chemical quenching results in oxidation of the target amino acids/protein, and recent studies using milk proteins have shown that the relative contents of protein carbonyls (a generic marker of protein oxidation) and dityrosine (a product of Tyr oxidation) were lower in globular proteins than in less well-ordered structures such as casein proteins (α-, β-, or κ-casein) subjected to riboflavin-mediated photo-oxidation.[11,15]

Both Tyr phenoxyl radicals and the dimeric oxidation product dityrosine arising from these radicals have been detected on lactoperoxidase-mediated oxidation of milk,[16] indicating that this residue is readily oxidized. Both dityrosine[17−19] and the alternative Tyr oxidation product 3,4-dihydroxy-L-phenylalanine (DOPA)[17,18,20] have been used as markers of protein oxidation, but the role of protein structure in determining the yields of both Tyr-derived radicals and these two Tyr-derived oxidation products has not been examined in detail. In the present study riboflavin-mediated photo-oxidation, a process reported to be of major importance in milk exposed to light, was investigated in relation to protein structure using model systems. It was hypothesized that the accumulation of tyrosyl radicals and secondary oxidation products of Tyr may depend on the flexibility of the protein structure. It has been established that the formation of dityrosine requires the reaction of two Tyr phenoxyl radicals; such reactions therefore require the two radical centers to be able to come in close proximity. In contrast, other Tyr oxidation products such as DOPA do not require radical−radical reactions.

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As such, the structure of a protein might be expected to modulate the yield of different Tyr oxidation products, but this has not previously been investigated in detail. In this study, this facet has been examined with both single proteins and protein mixtures subjected to riboflavin-induced photo-oxidation. The structural requirements for these two competing processes may be of particular significance for milk proteins, as milk contains both globular whey proteins and caseins that have more flexible secondary structural elements.

MATERIALS AND METHODS

Riboflavin (>98%), boric acid (>99.5%), potassium iodide (99.9%), β-casein (90%), β-lactoglobulin (>90%), bovine serum albumin (BSA) (>96%), α-lactalbumin type III (>85%), DOPA (>98%), and zinc sulfate heptahydrate were purchased from Sigma-Aldrich (St. Louis, MO). Iodide (>99.5%) was from Fluka (Buchs, Switzerland), and sodium hydrogen carbonate (99.5%) was from AnalaR Merck Pty Limited (Kilsyth, VIC, Australia). All other used chemicals were of analytical grade.

Photosensitized Reaction. Protein samples were illuminated in the presence of 13 μmol/L riboflavin using visible light (Kodak AV 2050 slide projector) fitted with a 250 W halogen lamp passed through a 345 nm cutoff filter for 60 min under normal atmospheric conditions at 21 °C in 50 mM phosphate buffer, pH 6.8.

Iodination of Tyr Residues. Iodination of the Tyr residues in the BSA and β-lactoglobulin was performed according to the method of Østdal et al.[18] Solutions of BSA or β-lactoglobulin (10 mg/mL, 25 mL) were cooled on ice, and 2.98 mL of ice-cold 0.05 mol/L I_2/0.24 mol/L KI was added. After 15 min at 4 °C, excess reagent was removed by centrifugation of the samples through a 30K or 10K molecular mass cutoff centrifugal filter from Amicon Ultra (Danvers, MA) at 4 °C. The samples were then washed twice with 10 mL of 0.1 M borate/0.01 M carbonate buffer using the same procedure. The resultant recovered protein samples were then resuspended in 1 mL of 50 mM phosphate buffer, pH 6.8, before being loaded onto a PD10 column (Amersham Bioscience) equilibrated and eluted with 50 mM phosphate buffer, pH 6.8. Protein concentrations were determined using Bradford Protein Assay Kit I from Bio-Rad in 96-well plates.

Detection of Radicals by EPR Spectroscopy. Radicals formed as a result of photo-oxidation were detected at room temperature or detected and quantified using low-temperature electron paramagnetic resonance (EPR) experiments.[18] For low-temperature experiments, samples were inserted in EPR tubes (Wilmad Suprasil EPR tubes, catalog no. 727-SQ-250 M or 706-PQ-9.50), frozen in liquid nitrogen, and inserted into a liquid nitrogen Dewar within the EPR spectrometer cavity (EMX X-band spectrometer; Bruker Biospin GmbH, Rheinstetten, Germany) equipped with 100 kHz modulation and a cylindrical ER 4103TM cavity. Modulation amplitude was 2 G, microwave power was 2.0 mW, and receiver gain was 1.0×10^5. Each spectrum was acquired as a mean of 16 accumulated scans. For room-temperature experiments, the detection of radicals was performed using a flat cell. The spectra were accumulations of 10 scans. Modulation amplitude was 1 G, microwave power was 2.0 mW, and receiver gain was 2.0×10^5. All EPR measurements were performed at least twice.

Quantification of the Tyr Oxidation Products Dityrosine and DOPA by HPLC. Analysis of dityrosine and DOPA formed on the photo-oxidized and control proteins was performed according to the method of Hawkins et al.[12] The samples were reduced with 397 μmol/L $NaBH_4$ before acid precipitation on ice with 10% (w/v) of TCA followed by centrifugation for 2 min at 10000*g*. The samples were washed once with 5% (w/v) TCA and twice with ice-cold acetone, with the protein pellets recovered by centrifugation after each treatment (as described above). The pellets were subsequently air-dried, placed in digestion tubes, and subjected to multiple cycles of vacuum evacuation and gassing with N_2, before the final evacuation and 18 h gas phase acid hydrolysis of the protein to its constituent free amino acids using 6 mol/L HCl and 20 μg/mL thioglycolic acid. After subsequent removal of the acids by vacuum centrifugation, the samples were redissolved in buffer A (10 mmol/L phosphoric acid with 100 mmol/L perchlorate, pH 2) and filtered before injection onto a C18 column (Pelliguard). The samples were eluted from the column using a gradient elution system consisting of isocratic elution with 97% solvent A (10 mmol/L phosphoric acid with 100 mM perchlorate, pH 2) and 3% solvent B (80% methanol) for the first 22 min, then to 20% solvent B over the next 20 min, before changing to 50% in 1 min and holding there for another 10 min. The column was equilibrated for 10 min between samples. The flow rate was 1 mL/min, and the column was maintained at 30 °C. Elution of parent amino acid (Tyr) was monitored by its UV absorbance at 280 nm, whereas the oxidation products were measured using a fluorescence detector with DOPA detected using λ_{ex} = 280 nm and λ_{em} = 320 nm (first 22 min of the run) and dityrosine detected using λ_{ex} = 280 nm and λ_{em} = 410 nm for the remainder of the run. Compounds were identified on the basis of their spectroscopic properties and elution times and quantified by use of standard curves generated using authentic standards (DOPA and dityrosine). A previous study has reported that the absolute recovery of these materials under these conditions is >98%.[21] Dityrosine was prepared using horseradish peroxidase and H_2O_2 as described by Amando et al.[22]

Detection of Protein Changes Using SDS-PAGE. SDS-PAGE was utilized to examine protein structural differences between control and photo-oxidized proteins. Both reduced and nonreduced samples were examined. Equal volumes were mixed of diluted protein sample (200-fold) and sample-loading buffer containing 2% (w/v) SDS-PAGE, 10% (v/v) glycerol, 2.5% (v/v) saturated bromophenol blue, and 5% (v/v) 2-mercaptoethanol in 300 mM, pH 6.8, Tris-HCl. The samples were heated at 100 °C for 5 min. Nonreduced samples were mixed with sample loading buffer containing no 2-mercaptoethanol and were not heated. Samples (20 μL, ca. 3 μg of protein) were loaded onto 4−15% precast SDS−polyacrylamide gels and run at 150 V for 50 min using SDS Tris−glycine buffer in a Bio-Rad (Reagents Park, NSW, Australia) system. Bands were visualized by silver staining.[23]

RESULTS

Radicals Formed in Milk Proteins after Riboflavin Mediated Oxidation. Solutions of BSA or β-lactoglobulin (0.45 mmol/L, illuminated with light with λ > 345 nm for 60 min in the presence of 13 μmol/L riboflavin) were rapidly frozen and examined by low-temperature EPR spectroscopy. Intense signals were detected in each case (Figure 1), but not in control materials (protein solutions not exposed to light, or illuminated in the absence of riboflavin). A similar EPR spectrum was detected with β-casein, but the intensity of this signal, as indicated by the peak area, was much lower than with BSA or β-lactoglobulin. The peak-to-peak line width of this signal was in the range of 9−13 G for all proteins. Exposure of riboflavin solutions to light under identical conditions in the absence of protein did not give rise to detectable EPR signals under the conditions employed.

To confirm whether or not the amino acid residue responsible for the observed EPR signals was a Tyr residue, samples of BSA and β-lactoglobulin were iodinated, a process that modifies Tyr residues,[24] and identical photo-oxidation experiments were carried out with subsequent detection of radicals by EPR. This treatment resulted in the loss of the EPR signal detected on both globular photo-oxidized proteins (Figure 1), which is consistent with the assignment of the observed signal to a Tyr-derived radical (spectra for β-lactoglobulin not shown). Similar experiments were not

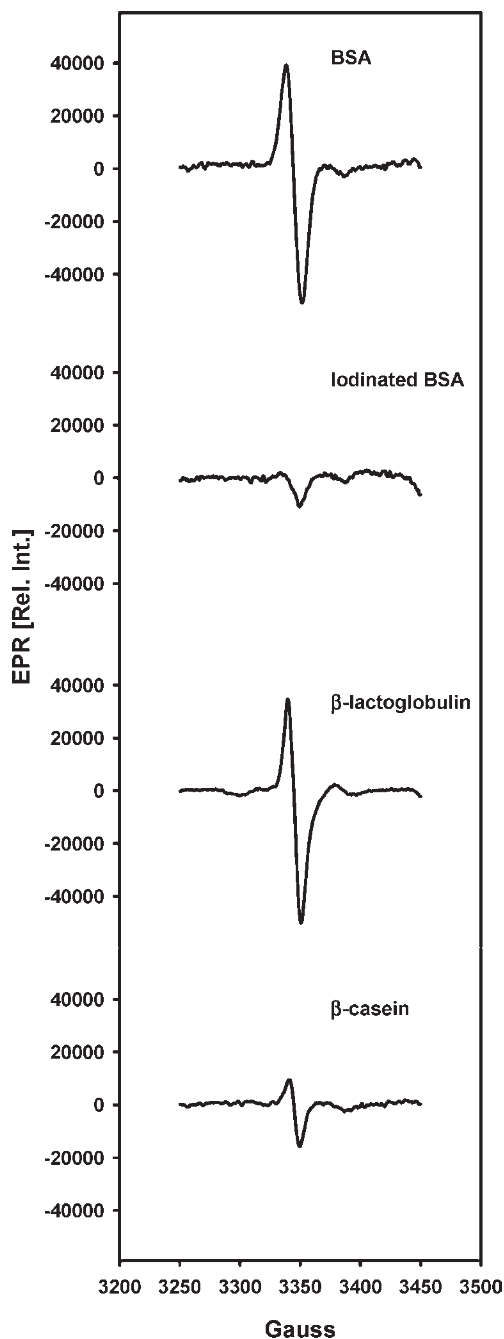

Figure 1. EPR spectra detected at low temperature of radicals formed on BSA (top spectrum), iodinated BSA (second spectrum), β-lactoglobulin (third spectrum), and β-casein (bottom spectrum), respectively, after riboflavin (13 μmol/L) mediated photo-oxidation (λ > 345 nm) at pH 6.8 and subsequent freeze quenching. The protein concentration was 0.45 mmol/L in each sample. Spectra for BSA and β-lactoglobulin were recorded after 60 min of photo-oxidation; the spectrum for β-casein was recorded after 35 min of oxidation. Modulation amplitude was 2 G; microwave power, 2.0 mW; and receiver gain, 1.0×10^5. Each spectrum shown is an accumulation of 16 scans.

attempted with β-casein due to the lower intensity of the EPR signals detected on this protein.

An identical radical signal was also detected by EPR spectroscopy carried out at room temperature for BSA (Figure 2A). To obtain further information on the identity of the species that gives rise to this signal, experiments were also carried out in the presence of high levels of Zn^{2+} ions, which are known to stabilize semiquinone radicals.[25] However, to keep the metal ions in solution, these experiments had to be conducted at a slightly acidic pH. The radical detected with BSA at pH 5.5 was lower in intensity than that detected at pH 6.8 in the absence of Zn^{2+} ions, but in the presence of Zn^{2+} the intensity of the signal detected at pH 5.5 increased approximately 2-fold, which is consistent with the presence of a semiquinone radical. For β-lactoglobulin at pH 5.5, addition of Zn^{2+} also markedly increased the intensity of the radical signal (Figure 2B).

The time course of accumulation of the Tyr-derived radical on the target proteins was examined using a range of light exposure times (0−60 min) with identical solutions of the three native proteins (0.45 mmol/L BSA, β-lactoglobulin, or β-casein with 13 μmol/L riboflavin). The intensity of the EPR signal detected from BSA increased steadily over the first 40 min of light exposure (Figure 3A). After this time, a steady state concentration of radicals was detected. With β-lactoglobulin, only low levels of radicals were detected over the first 20 min of light exposure (i.e., there was a lag phase in radical accumulation), followed by a more rapid increase in signal intensity. After 40 min of light exposure, the concentration of radicals detected with this protein also reached a plateau value. With β-casein, only weak EPR signals were detected during light exposure, indicating that radicals do not accumulate significantly on this protein with increasing light exposure. This may result from either a slow rate of radical generation or a more rapid decay rate of these species once formed.

The lifetime of the Tyr-derived radicals was examined in experiments in which proteins were photo-oxidized for 60 min and subsequently incubated for various periods in the dark before freeze quenching of the solutions and examination by EPR spectroscopy. The radicals generated on BSA and β-lactoglobulin decayed at similar rates with $t_{1/2} \sim 10$ min (Figure 3B). For β-casein, an accurate determination of the lifetime was not possible due to the low level of radicals accumulated on the protein; it was, however, clear that the radicals present on this protein were short-lived compared to the radicals on the other proteins.

The accumulation of radicals was also examined in mixtures of proteins to determine whether the presence of additional species modulated either the extent of radical formation or its decay kinetics once generated. The buildup of radicals was examined as described above in mixtures containing 0.33 mmol/L BSA with 0.33 mmol/L of β-casein, or 0.33 mmol/L BSA with 0.43 mmol/L β-lactoglobulin, respectively (Figure 4A). These concentrations of added β-casein and β-lactoglobulin result in equal masses of protein (7.8 mg/mL) being present in each case and 30 mg/mL protein in total in each sample. The data obtained with these two systems were then compared to the system containing 0.45 mmol/L BSA (i.e., the same total mass of protein in each system). In the mixtures containing both BSA and β-lactoglobulin, the accumulation of radicals showed a lag phase (as observed with β-lactoglobulin alone) over the first 10 min of light exposure; after this period, a steady accumulation of protein radical was detected, reaching a plateau value after ~50 min of light exposure. The plateau level of radicals detected in this mixed system was similar to or higher than that detected with BSA alone. With a mixture of BSA and β-casein, only low levels of radicals were detected by EPR throughout the entire period of illumination. This is in stark contrast to the system containing BSA alone.

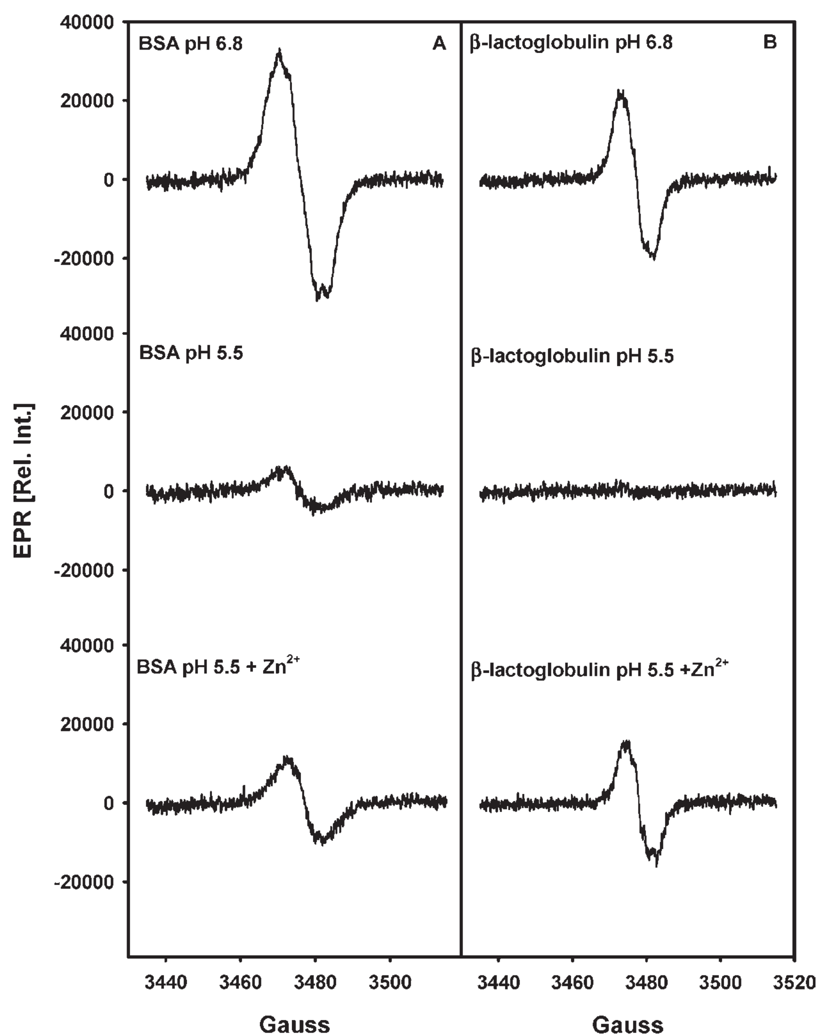

Figure 2. EPR spectra detected at room temperature of radicals formed on (A) BSA at pH 6.8 (top spectrum), at pH 5.5 (second spectrum), and at pH 5.5 with 100 mmol/L $ZnSO_4$ (bottom spectrum) or (B) β-lactoglobulin at pH 6.8 (top spectrum), at pH 5.5 (second spectrum), and at pH 5.5 with 100 mmol/L $ZnSO_4$ (bottom spectrum), respectively, after riboflavin (13 μmol/L) mediated photo-oxidation (λ > 345 nm) for 60 min. The protein concentration was 0.45 mmol/L in all samples. Modulation amplitude was 1 G; microwave power, 2.0 mW; and receiver gain, 2.0×10^5. Each spectrum shown is an accumulation of 10 scans.

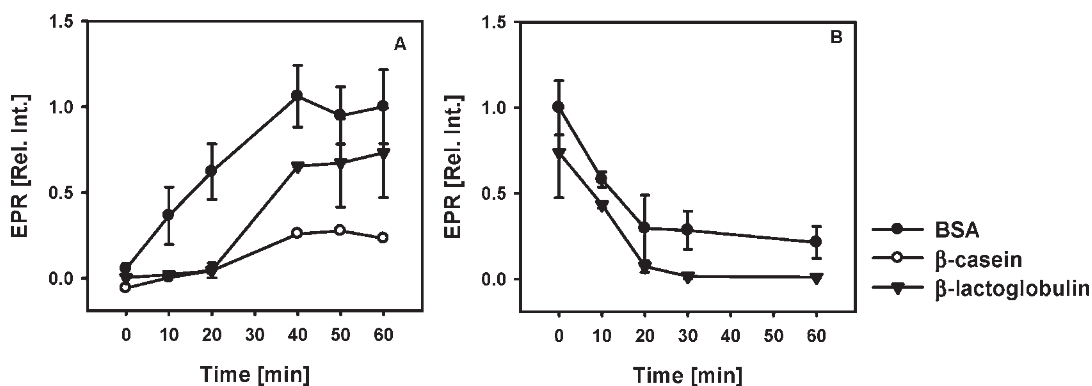

Figure 3. (A) Free radical accumulation on BSA (soild circles), β-casein (open circles), and β-lactoglobulin (inverted solid triangles), respectively, after riboflavin (13 μmol/L) mediated photo-oxidation (λ > 345 nm) at pH 6.8, subsequent freeze-quenching, and examination by low -temperature EPR spectroscopy. (B) Lifetime of radicals formed in BSA and β-lactoglobulin after photo-oxidation (60 min). The protein concentration was 0.45 mmol/L in all samples. Modulation amplitude was 2 G; microwave power, 2.0 mW; and receiver gain, 1.0×10^5. Each spectrum was acquired as an accumulation of 16 scans. Bars indicate mean and range of duplicate determinations.

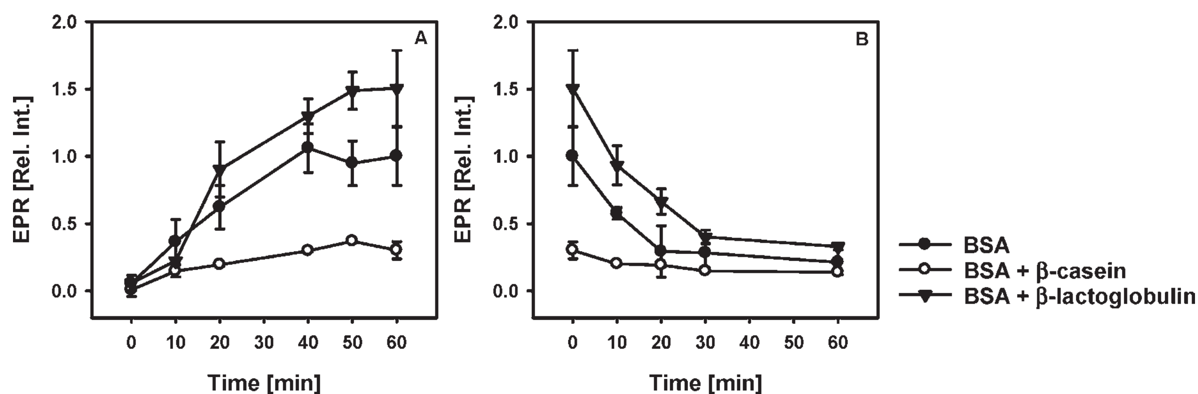

Figure 4. (A) Free radical accumulation detected by low-temperature EPR spectroscopy on 0.45 mmol/L BSA (solid circles) and in mixtures of 0.33 mmol/L BSA with 0.33 mmol/L β-casein (open circles) or 0.33 mmol/L BSA with 0.43 mmol/L β-lactoglobulin (inverted solid triangles) during riboflavin (13 μmol/L) mediated photo-oxidation (λ > 345 nm) at pH 6.8. The used protein concentrations yield an equal total mass of 30 mg of protein/mL in all samples. (B) Lifetime of radicals formed in the protein mixtures after photo-oxidation (60 min). Modulation amplitude was 2 G; microwave power, 2.0 mW; and receiver gain, 1.0×10^5. Each spectrum shown is an accumulation of 16 scans. Bars indicate mean and range of duplicate samples.

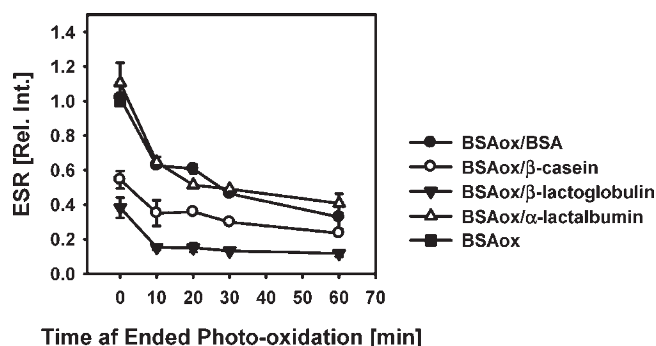

Figure 5. Decay of radicals formed on photo-oxidized BSA after addition of the nonoxidized proteins: BSA (solid circles), β-casein (open circles), β-lactoglobulin (inverted solid triangles), and α-lactalbumin (open triangles). The level of radicals after 60 min of photo-oxidation of BSA is used as control (solid squares). The quantity of added (nonoxidized) protein was equal in mass (7.8 mg/mL protein) in each case corresponding to a final concentration of oxidized BSA of 0.33 mmol/L with the addition of nonoxidized protein: 0.12 mmol/L BSA, 0.33 mmol/L β-casein, 0.43 mmol/L β-lactoglobulin, and 0.55 mmol/L α-lactalbumin, respectively. BSA (0.33 mmol/L) was photo-oxidized (λ > 345 nm) with riboflavin (13 μmol/L) at pH 6.8 before addition of the nonoxidized protein, freeze quenched, and subsequently examined by low-temperature EPR spectroscopy. Modulation amplitude was 2 G; microwave power, 2.0 mW; and receiver gain, 1.0×10^5. Each spectrum shown is an accumulation of 16 scans. Bars indicate mean and range of duplicate samples.

The decay of the radicals detected in these protein mixtures was examined after the cessation of light exposure, as described above. For the BSA/β-lactoglobulin system, the radicals detected had a half-life of >10 min (Figure 4B). In contrast, the lifetime of the (low levels of) radicals detected in the BSA/β-casein mixture was very short, and this could not be accurately quantified.

The effect of additional nonoxidized (native) protein on the postphotolysis lifetime of the radicals generated by light/riboflavin on 0.33 mmol/L BSA was also examined (Figure 5). The amount of added, nonoxidized protein was equal in mass (7.8 mg/mL) in each case. When nonoxidized β-casein (0.33 mmol/L) was added to the preoxidized BSA, an immediate marked reduction in the concentration of the BSA radicals was detected (of approximately 30% of the original value). When nonoxidized β-lactoglobulin (0.43 mmol/L) was added to the oxidized BSA, a reduction in the radical concentration of approximately 20% was detected. When native BSA (0.12 mmol/L) was added to 0.33 mmol/L preoxidized BSA (i.e., total BSA concentration = 0.45 mmol/L) of oxidized BSA, no reduction in the concentration of radicals in the samples was detected. The effect of an additional small whey protein α-lactalbumin, which has significant α-helical structure, was also examined in similar experiments. Addition of 0.55 mmol/L nonoxidized α-lactalbumin to the preoxidized BSA did not affect the concentration of radicals detected.

Quantification of Tyr Oxidation Products. As EPR signals assigned to Tyr-derived semiquinone radicals were detected with all of the proteins investigated, the concentrations of two well-characterized oxidation products of tyrosine, DOPA and dityrosine, were quantified by HPLC after photo-oxidation and acid hydrolysis of the proteins to their free amino acids. The former is a potential precursor of the postulated semiquinone species. In each case, the levels of the oxidized amino acid products were determined relative to the parent amino acid Tyr, to compensate for any losses during sample processing and analysis. Dityrosine and DOPA were detected on all of the photo-oxidized proteins but to different extents (Figure 6). Low or insignificant levels of the oxidized derivatives were detected in control protein samples not exposed to light (data not shown). With all three proteins, the concentrations of both dityrosine and DOPA detected by HPLC (expressed relative to the experimentally determined content of the parent amino acid, Tyr, which did not change significantly) increased over the first 10 min of light exposure, but reached a plateau value after this time. The plateau levels of these oxidation products varied between proteins, with the highest levels of dityrosine (3 μmol of dityrosine/mol of Tyr) and the lowest levels of DOPA (0.1 μmol of DOPA/mol of Tyr) detected on β-casein. For BSA, the converse was detected: the lowest levels of dityrosine (0.2 μmol of dityrosine/mol of Tyr) and the highest levels of DOPA (0.5 μmol of DOPA/mol of Tyr). β-Lactoglobulin yielded intermediate levels of both products (1 μmol of dityrosine/mol of Tyr and 0.3 μmol of DOPA/mol of Tyr).

Dityrosine and DOPA were also detected in the mixtures containing two proteins, but to different extents (Figure 7). The time course of product formation was, however, similar to those detected with the single proteins, that is, accumulation of both products over the first 10 min of light exposure, with plateau values detected at later times. Whereas the concentration of dityrosine detected on BSA

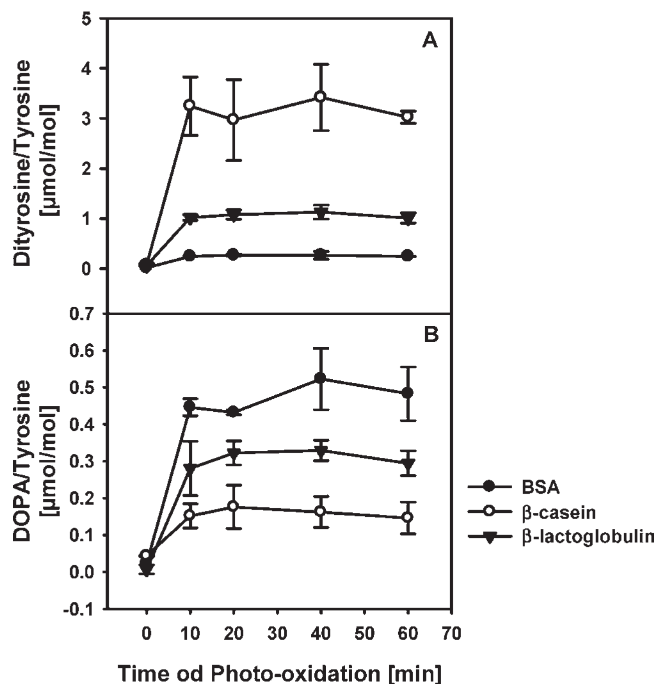

Figure 6. Time-dependent accumulation of (A) dityrosine and (B) DOPA during riboflavin (13 μmol/L) mediated photo-oxidation (λ > 345 nm) at pH 6.8 of BSA (solid circles), β-casein (open circles), or β-lactoglobulin (inverted solid triangles). The protein concentration in each case was 0.45 mmol/L. After photo-oxidation, samples were processed for HPLC analysis with fluorescence detection (DOPA was detected using λ_{ex} = 280 nm and λ_{em} = 320 nm and dityrosine using λ_{ex} = 280 nm and λ_{em} = 410 nm) and UV (λ_{ex} = 280 nm) for detection of tyrosine. Bars indicate mean and range of duplicate samples.

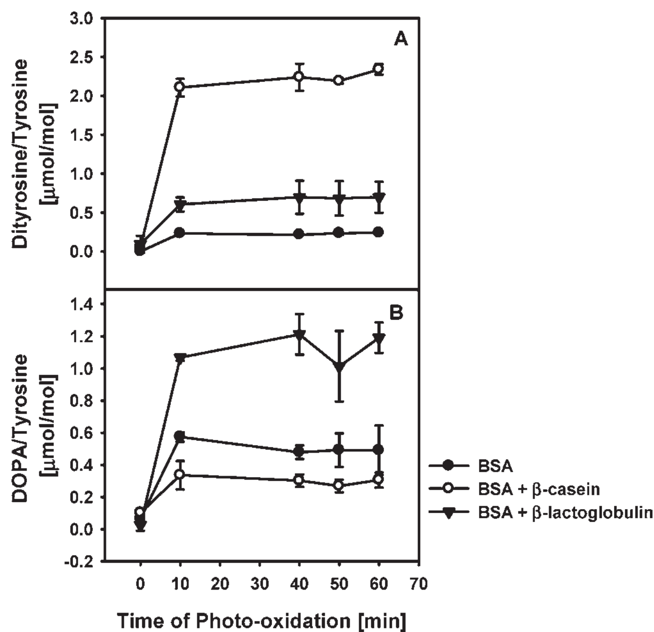

Figure 7. Time-dependent accumulation of (A) dityrosine and (B) DOPA upon exposure to light in the presence of 13 μmol/L riboflavin at pH 6.8. The mixtures contained 0.45 mmol/L BSA (solid circles), 0.33 mmol/L BSA and 0.33 mmol/L β-casein (open circles), or 0.33 mmol/L BSA and 0.43 mmol/L β-lactoglobulin (inverted solid triangles), respectively. The used protein concentrations yield an equal total mass of 30 mg of protein/mL in all samples. After photo-oxidation (λ > 345 nm), samples were processed for HPLC analysis with fluorescence detection (DOPA was detected using λ_{ex} = 280 nm and λ_{em} = 320 nm; dityrosine using λ_{ex} = 280 nm and λ_{em} = 410 nm) and UV (λ = 280 nm) for detection of tyrosine. Bars indicate range of duplicate determinations.

alone was 0.2 μmol/mol of Tyr (Figure 7A), the presence of β-lactoglobulin in the mixture (i.e., BSA + β-lactoglobulin) increased the concentration of dityrosine to 0.5 μmol/mol of Tyr (Figure 7B). Similarly, the presence of β-casein in addition to BSA increased the dityrosine concentration to 2 μmol/mol of Tyr. An inverse pattern was detected for DOPA, with the concentration of DOPA being 0.3 μmol/mol of Tyr in the sample with BSA/β-casein (Figure 7B), 0.5 μmol/mol of Tyr for BSA alone, and 1 μmol of DOPA/mol of Tyr for the BSA + β-lactoglobulin mixture.

Detection of Protein Changes Using SDS-PAGE. The structural consequences of riboflavin-mediated photo-oxidation of these proteins, both individually and in mixtures, were examined by SDS-PAGE (Figure 8). Oxidized samples and corresponding controls were examined under both reducing (Figure 8A) and nonreducing (Figure 8B) conditions. No significant formation of either polymers/aggregates or protein fragments was detected in the absence or presence of riboflavin alone (no light exposure) with any of the proteins. Exposure to light in the absence of riboflavin had no significant effect on BSA (lane 3) or β-lactoglobulin (data not shown), but weak bands from a dimer were detected with β-casein (lane 6). In the complete systems (protein, riboflavin, and light), no significant polymers or fragmentation were seen with BSA (lane 4) or β-lactoglobulin (data not shown), but marked formation of polymers (assigned to dimer, trimer, and tetramer species) was detected with β-casein (lane 7), together with a loss of the parent protein band. These polymers were detected on gels with both reduced and nonreduced β-casein, consistent with the formation of nonreducible covalent bond formation. Additional bands were detected at the top of the gels, and increased formation of dimer and higher polymers was observed in the complete β-casein system with nonreduced samples. The material at the top of the gels was not detected for the reduced samples, which is consistent with an assignment of these high molecular mass materials to reducible aggregates.

Extension of these studies to protein mixtures did not provide any evidence for interprotein cross-links, except in those systems that contained β-casein. Thus, mixtures of BSA with β-casein provided evidence for aggregate formation (lane 10), but the apparent mass of these bands was similar to those detected with β-casein alone (lane 7) or for BSA alone (lane 4), suggesting that there was limited, or no, formation of intermolecular cross-links between these proteins. No additional bands from either aggregates or fragments were detected when nonoxidized proteins were added postillumination to prephotolyzed samples (data not shown).

DISCUSSION

The data obtained in this study are consistent with the formation of long-lived radical species upon exposure of a number of protein solutions to light of wavelength >345 nm in the presence of riboflavin. Iodination experiments carried out with both BSA and β-lactoglobulin are consistent with an assignment of this species to a Tyr-derived radical. Although this species was the only radical detected by the methods employed (room- and low-temperature EPR spectroscopy), we cannot exclude the formation of other radicals as a result of riboflavin-mediated photo-oxidation. The exact mechanism of formation of these protein-derived radicals has not

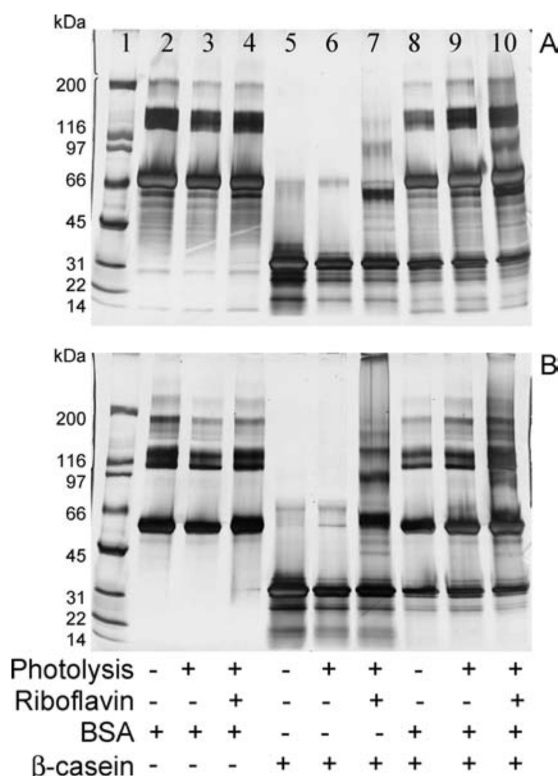

Figure 8. SDS-PAGE of (A) reduced and (B) nonreduced BSA (lanes 2−4), β-casein (lanes 5−7), or BSA and β-casein mixtures (lanes 8−10) after 60 min of riboflavin (13 μmol/L) mediated photo-oxidation (λ > 345 nm) at pH 6.8. The protein concentration in the reaction mixtures was 0.45 mmol/L in samples with single proteins (lanes 2−7), and in the samples with the protein mixture (lanes 8−10) the concentration of each protein was 0.33 mmol/L. Included on the gels are non-photo-oxidized proteins (samples stored in the dark at 4 °C) and proteins exposed to light for 60 min in the absence of riboflavin. Each of the two gels represents one of three experiments performed on individual independent samples. The molecular masses of the markers are shown in kDa.

been ascertained, but previous studies on riboflavin photochemistry are consistent with the occurrence of type I photochemistry involving excited states of the sensitizer and subsequent one-electron transfer from the protein Tyr residues to the sensitizer.[26,27] Subsequent rapid deprotonation of the oxidized Tyr residue would give rise to a phenoxyl radical. The narrow (peak-to-peak) line width of the observed signal of ∼13 G is lower than that reported in other studies for tyrosyl phenoxyl radicals (typically >20 G),[19,28−30] suggesting an alternative structure. This narrower line width and the increased radical concentration (as measured by signal intensity) in the presence of Zn^{2+} ions are consistent with (but do not prove) the presence of an *o*-semiquinone species. Previous studies have shown that Zn^{2+} ions stabilize such radicals by chelation, and it is well-established that this type of radical can be generated after oxidation of DOPA.[25] The absence of well-defined hyperfine couplings on the observed signal prevents easy assignment between these two possibilities. The lifetime of the Tyr-derived radical determined in the present study is similar to that reported for BSA and β-lactoglobulin after lactoperoxidase-catalyzed oxidation,[16] whereas the tyrosine *o*-semiquinone radical has been reported to have a much longer lifetime.[25] It should, however, be noted that these time course data reflect the difference between the rates of formation and decay of the species under study, and hence absolute rate constants for the formation and decay of these radicals cannot be determined. However, the marked effect of Zn^{2+} ions strongly supports an assignment of the observed radical as a semiquinone.[25] We therefore suggest that the detected radical is an *o*-semiquinone formed by oxidation of DOPA generated via initial protein oxidation. This assignment is consistent with the observed time course of detection of the protein oxidation products and the radical species, with the increase in concentration of dityrosine and DOPA by HPLC preceding that of radical detection by EPR.

Whether or not the primary photo-oxidation of Tyr residues occurs via a direct interaction of the excited state sensitizer with the target residue or via the mediation of other readily oxidized residues (such as Trp) cannot be established from the current data (cf. data for rapid radical transfer between Tyr and Trp residues).[27,31]

The accumulation of radicals upon riboflavin-mediated photo-oxidation was dependent on the protein structure, and the concentration of the radical detected by EPR was considerably lower in β-casein-containing samples than with the globular proteins. In contrast, the highest concentrations of dityrosine and lowest levels of DOPA were detected with β-casein. These data are consistent with the formation of tyrosyl radicals on this protein and rapid subsequent reaction to give dityrosine rather than formation of DOPA and secondary semiquinone radicals from further oxidation of this species. Lactoperoxidase-induced Tyr phenoxyl radicals have previously been shown to be more reactive when formed on casein than on the globular proteins β-lactoglobulin and BSA.[16] The less rigid structure of β-casein when compared to β-lactoglobulin and BSA therefore appears to favor rapid radical−radical reaction of Tyr phenoxyl radicals (and hence low steady state concentrations of these species) with this resulting in high yields of dityrosine rather than DOPA and its secondary radical. High concentrations of dityrosine were also detected in a mixture of BSA and β-casein, but whether this arises predominantly from cross-links between β-casein Tyr phenoxyl radicals or also involves BSA−β-casein cross-links cannot be determined from the HPLC data. However, the absence of noticeable BSA−β-casein cross-links on the SDS-PAGE gels suggests that these are mainly cross-links solely involving β-casein.

With the globular proteins BSA or β-lactoglobulin, or a mixture of these two proteins, the accumulation of DOPA was higher than with β-casein, and the levels of dityrosine were lower with the globular proteins. These results are consistent with these two products arising from competing pathways from a single species, the Tyr phenoxyl radical, with the relative fluxes between these pathways determined by the protein structure. Thus, for mobile/disordered structures, radical−radical termination to give dityrosine is favored, but in situations where steric or other factors decrease the rate of dimerization, DOPA is formed as an alternative product. Even though dityrosine and DOPA have been used extensively as markers of protein oxidation, the role of protein structure in determining the relative yields of these species has not, to our knowledge, been examined previously. This observation is of potential importance in understanding how milk proteins may limit oxidative damage[32,33] as phenoxyl radicals have been shown to act as initiators of lipid peroxidation[34] and such oxidation in milk is known to give rise to off-flavors and changes in palatability, nutritional content, and structure.[35,36] Enhanced formation of dityrosine may therefore function as a termination reaction avoiding such oxidation. Even though dityrosine has been detected in several dairy products[9,16,37] and it is well established that caseins form dityrosine more readily than globular proteins,[11,16]

DOPA has not so far been used as a marker for oxidation in milk. Therefore, it is still uncertain whether or not the same dependence on structure is present in the milk where the caseins are located in the micelles.

The lag phase observed in the accumulation of Tyr-derived radicals in the samples containing β-lactoglobulin may be due to a free thiol (Cys_{121}) residue present in β-lactoglobulin, which is believed to be (at least partially) responsible for the reported radical scavenging capacity of this protein.[38] Two mechanisms may be responsible for this lag phase: preferential oxidation of the Cys residue by the riboflavin-derived oxidants or repair of an initial Tyr phenoxyl radical by the Cys residue. Precedents for both processes have been reported in the literature.[39,40] The current data do not allow these two processes to be distinguished, but the enhanced decay of the BSA *o*-semiquinone radical on addition of nonoxidized β-lactoglobulin is consistent with a radical repair reaction. In metal ion-catalyzed oxidation of whey (high molecular mass) fractions, blocking of free thiols gave a significant decrease in scavenging capacity,[41] indicating that thiols are important protective agents in other oxidation scenarios, and not just with regard to photo-oxidation. This activity of the Cys residue on β-lactoglobulin, the most abundant whey protein in milk, may play a critical role in the antioxidant activity reported for whey protein preparations,[33,42] but further work is required to confirm this. The limited quenching of the radical on BSA by nonoxidized β-casein, which does not contain a free Cys residue, may arise from radical transfer from oxidized BSA to β-casein and subsequent termination of the radicals on β-casein. The greater overall apparent quenching capacity of β-casein compared to globular proteins other than β-lactoglobulin (species with a high α-helical content, such as α-lactalbumin) confirms that the tertiary structure of the proteins is of major importance for their antioxidant capacity.[19]

Overall, the data obtained in this study indicate that riboflavin-mediated photo-oxidation of multiple proteins generates the Tyr oxidation products dityrosine and DOPA as well as secondary radicals believed to be semiquinone species formed by further oxidation of DOPA. The extent of accumulation of these species varies with the protein examined, with the yields being dependent on the nature/structure of the target protein. Dityrosine formation appears to act as a radical sink, primarily in samples with β-casein, preventing high yield of DOPA and its secondary semiquinone radical, which in contrast appears to be the major pathway for globular proteins.

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