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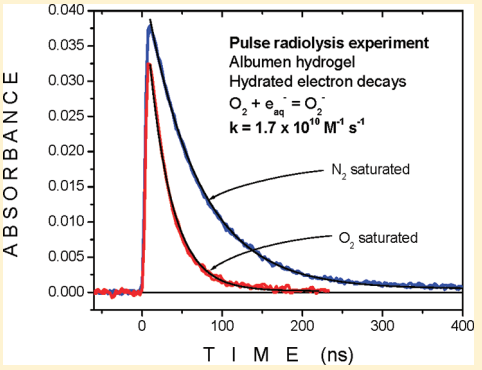
Effects of Microviscosity, Dry Electron Scavenging, and Protein Mobility on the Radiolysis of Albumen Hydrogel

Keith M. Krise and Bratoljub H. Milosavljevic*

Department of Chemistry, The Pennsylvania State University, 104 Chemistry Research Building, University Park, Pennsylvania 16802, United States

ABSTRACT: Nanosecond pulse radiolysis experiments performed on the oxygen and nitrogen saturated thick fraction of egg white (which has an immeasurably high macroviscosity) produced a rate constant for hydrated electron reaction with oxygen equal to 1.7×10^{10} M^{-1} s^{-1} at 18 °C, indicating that, due to the very low microviscosity, hydrated electrons are as mobile and reactive in the albumen hydrogel as in neat water. Also, the radiolytic yield for the hydrated electron (G-value) in the thick fraction of egg white (measured at the end of a 14 ns electron pulse) was found to be 86% of that determined in neat water, which can be attributed to the reaction of dry electrons with the protein constituents. Steady-state γ radiolysis studies on air-saturated thick and thin fraction (true solution) revealed that the immobility of egg white proteins disfavors reactions that result in molecular mass change.



INTRODUCTION

There is a great deal of work on proteins in aqueous solutions aimed at understanding their radiological damage and the associated biochemical impacts. Particular attention has been devoted to the mechanisms and kinetics of reactions involving free radicals such as the hydrated electron, hydroxyl radical, O_2^-, and its conjugate acid HO_2. These research efforts resulted in a large number of reaction schemes and related rate constants and activation energies.[1,2] However, how these data are used and interpreted when proteins (and other biomacromolecules) are organized in three-dimensional structures (as in real biological systems) has been explored to a much lesser extent, although in such structural arrangements important and unique physicochemical effects exist. For example, with this protein organization, the macroviscosity becomes infinitely high and the microviscosity controls reaction rates. Also, the immobility of protein molecules must be accounted for when modeling the radiation-induced processes in more structured systems because some intermolecular reactions observed in solutions may not occur, while some intramolecular reactions (with otherwise small probability) may become significant. Regarding protein reactivity, there is an important entity, bound water, which plays a role in many fundamental biochemical processes, including protein folding, enzymatic catalysis, and biomolecular recognition.[3] In spite of a relatively high amount of bound water (10% in the thick fraction of hen egg white used in this experiment) its role in the radiation chemistry of proteins has not been explored. In addition, albumen hydrogel is a rather concentrated microheterogeneous system, so the direct action of ionizing radiation and dry electron reactions may also be important (as in real biological systems).

Hen albumen is a convenient and inexpensive biological system for studying the aforementioned effects. It consists of a thin and thick fraction. Our previous research has shown that the thick fraction of egg white is a hydrated, protein gel composed of a protein network connected by disulfide bonds with ~90% water (comparable to mucous membranes water content). The thin fraction of hen egg white is a true protein solution with a protein composition similar to the thick fraction.[4,5] Despite the relatively high protein concentration (~12%), the viscosity of thin fraction is relatively low (~3 mPa·s) in comparison with that of a 10% poly(vinyl alcohol) (PVA) solution (~1000 mPa·s), which can be attributed to the globular protein conformation.[6] The thick fraction is similar to the mucous membrane that lines mammalian luminal surfaces as both contain highly glycosylated, hydrated, and cysteine-rich proteins, so the thick fraction could serve as a model system for the mucous membrane.[7−13] Hen albumen has previously attracted the attention of radiation chemists. Pulse radiolysis experiments were performed on homogenized hen egg white and unique results were reported, which are worth further investigation and clarification.[14] In particular, the decay of hydrated electron was found not to be affected by saturation of the egg white by pure oxygen or by deoxygenation with argon. On the other hand, in the same medium, the hydrated electron was found to readily react with another hydrated electron scavenger, nitrous oxide. Such selectivity toward the reaction with oxygen in this biological matrix (to the best of our knowledge) has not been observed in any other reaction medium. The effect of bound water was also mentioned, and it was suggested that it may cause a decrease in the hydrated electron yield, but no conclusions were drawn because the amount of bound water was unknown.

With the thick and thin fractions of hen egg white properties now much better characterized from a physicochemical perspective,

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in this work the radiolysis of the thick fraction of egg white (protein hydrogel, with immeasurably high macroviscosity but very low microviscosity) is compared with that of the thin (true protein solution). This allows a much deeper understanding of the early events in radiation chemistry observed in these proteinaceous matrixes and can aid in understanding the radiation chemistry of the mucous membrane.

EXPERIMENTAL SECTION

Materials. Chemicals for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) were of electrophoresis grade purity and were used as received from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Egg white thick and thin fractions were isolated from fresh hen eggs from a local farm.

Time-Resolved Pulse Radiolysis and Steady-State γ Irradiations. Pulse radiolysis experiments were performed using 4−15 ns pulses of 8 MeV electrons from the Notre Dame Radiation Laboratory linear accelerator (TB-8/16-1S linac; University of Notre Dame, South Bend, IN, USA). Details of the linac, the spectrophotometric detection setup, and the computer-controlled data acquisition and detection systems are described elsewhere.[15] All measurements were performed at 18 °C in a high-purity silica cell of 1 cm optical path length. The concentration of radicals generated was approximately $(1-5) \times 10^{-6}$ M/pulse as determined by the thiocyanate dosimeter. For steady-state experiments, egg white samples were sealed in glass ampules and irradiated at a cobalt-60 γ-source (Pennsylvania State University Radiation Science and Engineering Center, Breazeale Nuclear Reactor). Due to extensive foaming, thick and thin fractions of egg white samples cannot be deoxygenated by purging with inert gases. Our method to deoxygenate samples is described in detail elsewhere.[5,6]

SDS-PAGE. Analysis of thick and thin fractions of egg white was done using SDS-PAGE according to Laemmli.[16] Samples were boiled for 2 min at 100 °C in sample buffer with 5% β-mercaptoethanol. SDS-PAGE was done using a Bio-Rad Mini-Protein Tetra Cell System (model 165-8001) and Bio-Rad Power-Pac Basic (Bio-Rad Laboratories; Hercules, CA, USA). Proteins were visualized by Coomassie Blue staining, and UnScan It Gel (Silk Scientific, Inc., Orem, UT, USA) was used for gel analysis.

Differential Scanning Calorimetry. A TA Instruments, Inc. differential scanning calorimeter (model DSC Q200) was used to measure the enthalpy of melting. Deionized water and the thick fraction of hen egg white were hermetically sealed in Tzero aluminum sample pans (Thermal Analysis Instrument, Inc.). Samples were cooled from room temperature to −50 °C and subsequently heated to +50 °C at a heating rate of 10 °C/min.

RESULTS AND DISCUSSION

Hydrated Electron Absorption Spectrum. To determine the amount of bound water, a differential scanning calorimetry (DSC) experiment was performed on the sample used in the pulse radiolysis experiment, and it was found that it contained 90% freezable water.[17] From our previous characterization of the microscale properties of the thick fraction of egg white,[5] it was determined that the freezable water has rheological properties very similar to those of neat water; therefore, it is reasonable to assume that the hydrated electrons formed in that (*free*) water will have the same properties (including the optical absorption spectrum) as those formed in neat water. From this, the question arises as to how nonfreezable (*bound*) water affects the hydrated electron properties. An attempt was made to answer this question by performing a pulse radiolysis experiment. Figure 1 compares the normalized transient absorption spectra obtained using both deoxygenated neat water and thick fraction. Data are recorded at the end of the electron pulse. The absolute *G*-value for the hydrated electron at the end of the pulse in neat water is 2.6/(100 eV). Normalization was performed as follows. The spectrum corresponding to the (deoxygenated) thick fraction was normalized with respect to that to the (deoxygenated) neat water. Figure 2 shows that the absorbance of hydrated electrons produced in the thick fraction is 23% lower than that produced in neat water; therefore, the normalization was performed by dividing the hydrated electron spectrum in the thick fraction by 0.77. The absorption of the cysteine anion radical formed within the pulse is responsible for the higher absorbance in the thick fraction spectrum in the wavelength range of 330−480 nm.[18] Otherwise, at the end of a 14 ns electron pulse, in the wavelength interval of 480−750 nm, the spectra are the same within the experimental error, with the maxima coinciding.

Generally, how excess electrons will interact with water molecules depends on the state of water and there are at least three

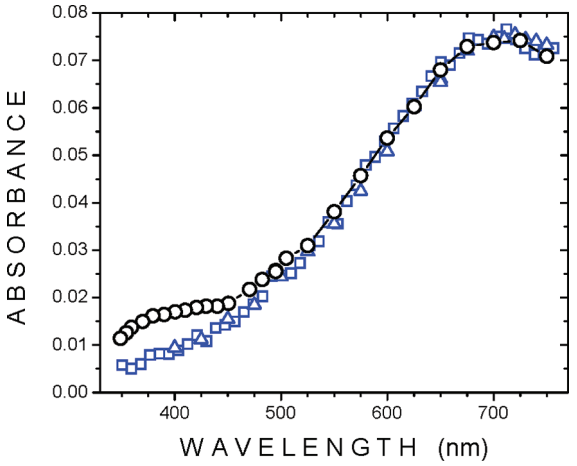

Figure 1. Pulse radiolysis data. Absorption spectra of the hydrated electron in water (blue) and the thick fraction of egg white (black).

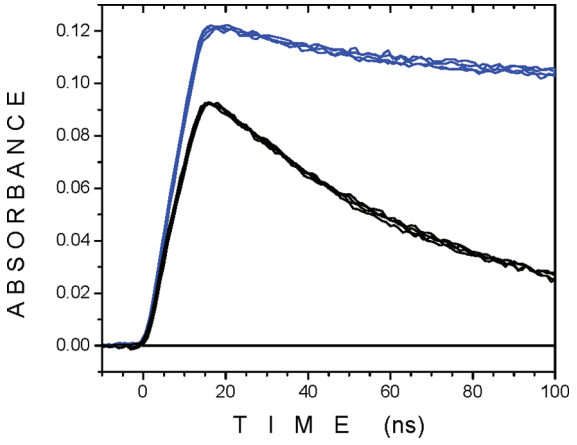

Figure 2. Pulse radiolysis data (λ = 700 nm; pulse width = 14 ns) comparing the initial yields of hydrated electrons in water (blue) and the thick fraction (black) at the same dose.

different situations: (1) excess electrons in clusters, (2) bulk solvated electrons, and (3) electrons trapped in bound water. The first two have been studied extensively,[19,20] and there is a common consensus about their spectral properties; however, little is known about the electrons trapped in bound water at room temperature. When performing the aforementioned experiment, the spectra of the hydrated electron in the region of 550−700 nm were measured several times to detect any small difference between the two; however, a hypsochromic shift in the hydrated electron spectral maximum corresponding to the thick fraction with respect to that obtained in neat water was not observed. This was done because we expected to observe the contribution of the electron localized in the bound water in that spectral region. Namely, previous studies on the radiolysis of PVA showed that the trapped electron absorption spectrum maximum is dependent on the number of water molecules in the trap.[21,22] A bathochromic shift in the trapped electron spectrum maximum (from 500 to 600 nm at room temperature in a pulse radiolysis experiment) was observed upon hydration of the PVA film (from none or almost none to 10% by mass). In addition, an increased thermal stability (lifetime from minutes at 77 K to weeks long below 120 K) was measured in a steady-state experiment. These two pieces of data provide evidence that the water is involved in electron trapping. Also, the trapped electron absorption spectrum maximum corresponding to the 10% water in the PVA sample is centered at 555 nm at 77 K, while that observed in the PE-*co*-PVA (44% PE) film containing 10% water has the maximum centered at 585 nm at 77 K. This means that increasing the average water content from 0.27 molecule per hydroxyl group in the PVA film to 0.45 molecule per hydroxyl group in the PE-*co*-PVA film produces a 30 nm bathochromic shift.[23] It can be concluded that the more water molecules in the trap, the lower the energy corresponding to the peak maximum (at least when the number of water molecules in water clusters is as low as in the aforementioned experiments). The strong temperature effect on the absorption maximum wavelength observed in the hydrated PVA film indicates that a lack of relaxation would cause a hypsochromic shift in the hydrated electron trapped in the water bound to protein molecules. Although the above-mentioned considerations are related to bound water in a solid-state polymer film, there could be an analogous situation with protein hydration in gels. Recent dielectric spectroscopy measurements on lysozyme (an egg white protein constituent) showed two different types of water surrounding protein molecules, the *tightly* bound water with the dielectric loss maximum centered around 100 MHz and *loosely* bound water centered around 3−5 GHz.[24] Although the relaxation times are close to the detection limit of the pulse radiolysis setup used, it was worthwhile to attempt to detect the difference in the hydrated electron absorption spectrum. No difference was noted, as can be seen in Figure 1; picosecond resolution may be needed to observe the effect.

Hydrated Electron Decay. Our previous characterization of the thick fraction indicated water pools of various sizes, which may cause heterogeneous kinetics; however, this was not observed in our results. The hydrated electron decay fit over six half-lifetimes was found to be perfectly single-exponential.

Hydrated Electron Yield. Figure 2 compares the decays of hydrated electrons monitored at 700 nm in both deionized water and the thick fraction of egg white irradiated with the same doses as those in the absence of oxygen (obtained in four separate pulse radiolysis experiments).

From a comparison of corresponding absorbances, a significant decrease in the hydrated electron yield in the thick fraction with respect to that of neat water was observed; however, the concentration of hydrated electrons that reacted within the pulse must be taken into account before the *G*-value can be reported. The current pulse profile was measured, and it was determined that it could be well-approximated as a rectangular pulse (which means that the increase in the dose with time is linear within the electron pulse), and, therefore, the fraction of hydrated electrons reacted in the pulse was determined using a previously detailed method that compares the concentration of hydrated electron in the presence and absence of hydrated electron reactions with protein.[14] The rate of hydrated electron formation can be described by the differential equation

$$\frac{d[e_{aq}^-]}{dt} = r - k[e_{aq}^-] \quad (1)$$

where r (linear, constant) is the rate of hydrated electron formation and k is the pseudo-first-order rate constant for the hydrated electron decay. Integration of the differential eq 1 from time zero to t and rearrangement enables the calculation of the percentage of hydrated electron that reacts within the pulse

$$\frac{[e_{aq}^-]}{[e_{aq}^-]_0} = \frac{1 - \exp(-kt)}{kt} \quad (2)$$

where t is the pulse width (in seconds). The validity of the methodology used was confirmed by measuring the hydrated electron yields in oxygen saturated water under experimental conditions identical to those when the yield was measured in the thick fraction of egg white. In the thick fraction, the amount of hydrated electrons reacting with the pulse is 10% of that when none of the protein reacts. Therefore, for the thick fraction of egg white, the relative *G*-value (yield) is 86% of that of neat water. The percentage of dry electron scavenged in radiolysis is significant and should be taken into account when modeling the radiolysis of mucous membrane.

An attempt was made to relate the amount of dry electron scavenged by protein molecules to that corresponding to individual, free amino acid constituents of the same concentration. Namely, it had been shown previously that amino acids readily react with precursors of the hydrated electron.[25,26] The yield was found to decrease exponentially with the amino acid concentration[26,27]

$$I/I_0 = \exp(-[C]/C_{37}) \quad (3)$$

where [C] is the concentration of the amino acid and C_{37} is concentration that reduces the initial yield of electrons to $1/e$. Applying eq 3, we calculated the contribution of the amino acids cysteine, histidine, glycine, and arginine contained in the ovalbumin and conalbumin to the dry electron scavenging. The C_{37} values for these amino acids were taken from refs 25 and 26. SDS-PAGE densitograms indicated that the ovalbumin concentration was 60% and that of conalbumin was 17% of the total protein concentration. In the 12% protein by mass sample, amino acids in ovalbumin, and conalbumin would decrease the hydrated electron yield 3.1 and 3.2%, respectively. If these were the only dry electron scavenging proteins, the hydrated electron yield should be 93.7%; however, we measured a value of 86%. The difference can be attributed to the following: (a) it is reasonable to assume that 23% of the proteins for which the amino acid content is not known also scavenge dry electrons (however, their contribution could not be calculated); or (b) the nonpolar amino acids, for which the C_{37} values are not

known, may also significantly contribute to the dry electron scavenging.

Our value for the hydrated electron yield in the thick fraction is significantly higher than the value previously reported.[14] To elucidate the reasons for the discrepancy observed, the experimental details of our work and previous work were analyzed and compared. (a) We worked with 12% protein samples; Cercek and Cercek[14] did not report their value. If they had worked with a sample with higher protein content that would have resulted in a smaller hydrated electron *G*-value than ours;[28] however, the protein concentration effect cannot account for the difference observed. (b) The difference in dose corrections applied is large and could be the cause of observed differences. Namely, to account for the chemical composition of the egg white, a 0.933 dose correction factor was used in the previous work. No details were given as to how the factor was calculated. In our experiments, we used the dose correction factor equal to 1.05. We measured the thick fraction mass density and found it to be equal to 1.050 g cm^{-3}. Assuming that the elemental content of other protein constituents is not different from that of ovalbumin (which is 60% by mass of the total protein content), we calculated the electronic density of the thick fraction by summing the electronic density of 12% ovalbumin and 88% of that of water in the solution, the mass density of which was 1.050 g cm^{-3}. The value obtained was 5% higher than that of neat water. The relative dosimetry (using a Faraday cup) showed that the thick fraction received 5% more energy than in the case of neat water under identical experimental conditions, which confirms the accuracy of our dose correction factor. The correction factors applied in our and their experiments can result in a 12% difference in the hydrated electron *G*-value. (c) The previous work was done using air saturated egg white samples, whereas in our studies the samples were saturated with nitrogen. The corresponding values of the rate constants of the hydrated electron decay are 2.22×10^7 and 1.45×10^7 s^{-1}, respectively. The difference between the two is significant; when calculating the amount of electrons reacting within a 10 ns pulse, a 30% difference is obtained.

Direct Effect of Radiation. Due to high protein concentration, the direct effect of radiation should be considered. The direct effect cannot be responsible for the decrease in the hydrated electron *G*-value. Namely, 12.5% of the energy is adsorbed by the proteins resulting in hole formation and impinged electrons (that can further ionize). When their energy becomes low enough, the impinged electrons can recombine with the holes produced in proteins; however, that percentage is low due to the low hole concentration (0.8 μM per 20 Gy absorbed which corresponds to 14 ns electron pulse used in our experiments). Because of the 90% water and ~1 M amino acid (contained in the proteins) concentration, it is more likely that they will get hydrated (and become observable at the end of the electron pulse) or react with the amino acids prior to the hydration. However, the direct effect should be taken into account in the analysis of products of protein steady-state radiolysis.

Effects of Microviscosity. To assess the mobility and reactivity of the hydrated electron in the thick fraction of egg white, the rate constant of the diffusion-controlled reaction of molecular oxygen with hydrated electron was measured using pulse radiolysis technique. Contrary to what was previously reported,[14] the presence of molecular oxygen does affect the decay of hydrated electrons in the thick fraction (Figure 3). The lifetime for the decay of hydrated electrons in the absence of oxygen at 18 °C was determined to be 6.90×10^{-8} s and in the presence of

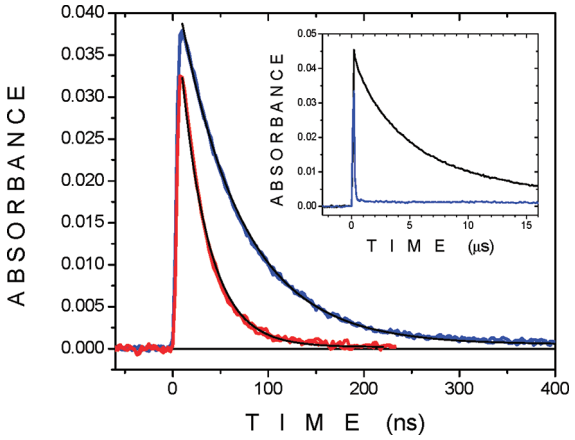

Figure 3. Pulse radiolysis data used to calculate the rate constant for the reaction of oxygen and hydrated electron in the thick fraction of egg white at 18 °C. Hydrated electron decays in the absence (blue) and presence (red) of molecular oxygen. Black lines correspond to first-order exponential fit. Insert shows the hydrated electron decay in deoxygenated neat water (black) and deoxygenated thick fraction (blue).

oxygen 2.75×10^{-8} s at the same temperature. Using pseudo-first-order kinetics, the rate constant for the reduction of molecular oxygen by hydrated electron was determined to be 1.7×10^{-10} M^{-1} s^{-1}.

Within the experimental error (5%), the second-order rate constant measured in the thick fraction of egg white is the same as the value in neat water (1.8×10^{-10} M^{-1} s^{-1}). Since our previous research[5] showed that the mobility of molecular oxygen in the thick fraction of egg white is the same as in neat water, it can be concluded that hydrated electrons are as mobile and reactive in the thick fraction as in neat water. This result stresses the importance of knowing the microviscosities when modeling the radiation chemical processes in biological systems.

The dose used in our experiments, ~20 Gy/pulse, resulted in the concentration ~ 12 μM of radicals, and under these conditions the decay of hydrated electron is complex and cannot be fit using a single-exponential decay (Figure 3 insert). As is well-known, the second-order reactions (such as $e_{aq}^- + OH$ and $e_{aq}^- + e_{aq}^-$) dominate in the first three or four half-lifetimes. The only difference can arise from the impurity concentration, and that affects the last 10% of the decay; our value for the rate constant is 7×10^4 s^{-1}. The (pseudo-first-order) rate constant for the hydrated electron reaction with proteins in the thick fraction was measured to be 1.45×10^7 s^{-1}. The insert in Figure 3 illustrates that the hydrated electron decay in neat water is too slow to affect the measurement of the rate constant for the reaction of hydrated electron with oxygen.

Effects of Protein Immobility. The majority of reactions important for radiation biochemistry have been studied in homogeneous solutions; however, in many biological media, including mucous membranes, cell membranes, and the cytoplasm, reactants are immobilized, which may alter the contributions of specific reactions to the overall reaction mechanism. The thick and thin fractions are convenient systems to study the effects of immobility on the radiation chemistry of proteins because the former is a protein hydrogel (in which proteins are immobile) while the latter is a true protein solution (in which proteins are mobile) and their chemical composition is very similar. Therefore, from a comparison of the results obtained by irradiating the two, one can deduce

the effects of protein mobility. In this work, we explored the radiation damage accompanied with a change in molar masses. Steady-state γ radiolysis experiments were performed, and the irradiated samples were analyzed using SDS-PAGE. The gels obtained were scanned to quantify the degree of protein scission and cross-linking. Figure 4 shows a representative electrophoretogram and corresponding densitograms of air-saturated thick and thin fractions, either nonirradiated or γ-irradiated at doses of 0.4 and 0.8 Mrad. Both protein scission and cross-linking occur in the thin and thick fractions as indicated by the appearance of new low and high molecular mass bands, as well as a decrease in the intensities of protein constituent bands. Quantitative data, summarized in Table 1, were obtained by integrating the normalized densitograms of nonirradiated and irradiated samples. From Figure 4B,C, it can be observed that irradiation results in a decrease in intact ovalbumin (45 kDa) in both thin and thick fractions; however, the decrease is more marked in the thin fraction than the thick fraction [the thin fraction *G*-values for the reaction of ovalbumin (Table 1) are 54 and 62% higher than the *G*-values for thick fraction at 0.4 and 0.8 Mrad, respectively].

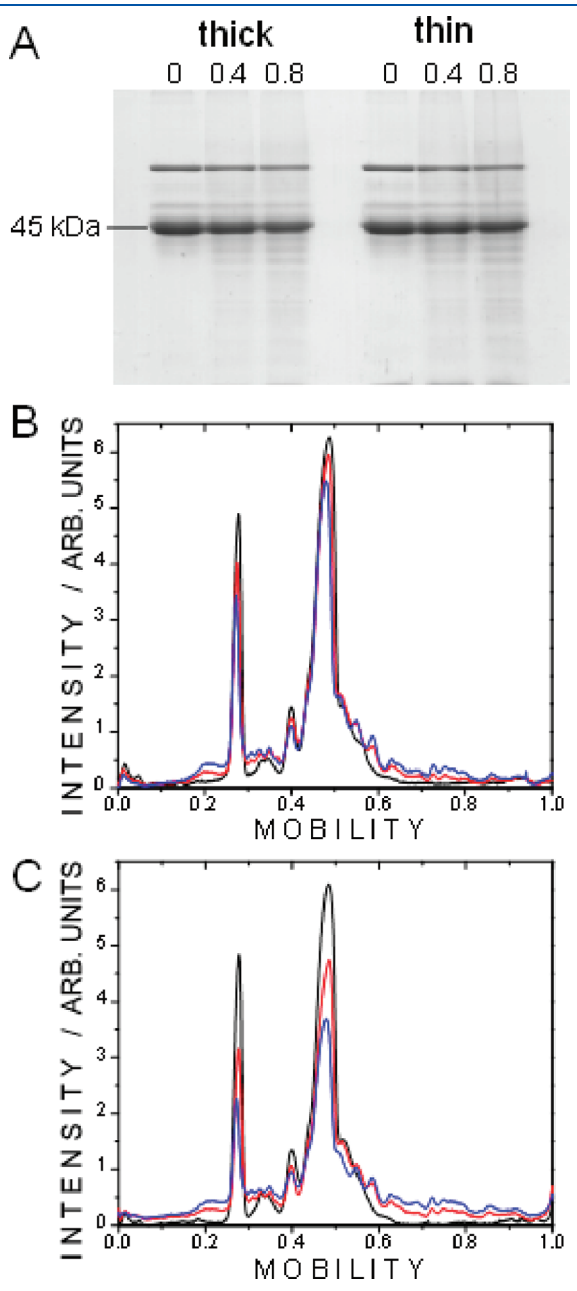

Figure 4. Representative SDS-PAGE electrophoretogram (A) and densitograms of air saturated thick (B) and thin fractions (C) of hen egg white. In densitograms, nonirradiated (black) and γ-irradiated at 0.4 (red) and 0.8 Mrad (blue).

Results from our previous research of radiation-induced agglomeration of ovalbumin in aqueous solution can be used to rationalize how the restricted mobility of proteins affects yields of radiation-induced radical reactions in the thick fraction. Namely, the study of radiation-induced agglomeration of ovalbumin in nitrous oxide saturated aqueous solution showed that the *G*-value increased upon increasing the protein concentration.[29] The effect was rationalized as a consequence of competition between parallel inter- and intramolecular radical reactions wherein intramolecular reactions generated products incapable of undergoing further agglomeration. The higher concentration of (mobile) proteins decreases the encounter time, thus, favoring protein molecule cross-linking. The mobility of radicals produced by the radiolysis of water in the pools and channels that surround protein molecules in the thick fraction are similar to that in neat water because of the very similar microviscosity (as mentioned above); however, when protein radicals are generated in immobile proteins comprising the three-dimensional network of the thick fraction, the probability of these protein radicals reacting with other radicals is minimized. The situation corresponding to the thick fraction is more complex because of the protein fragmentation due to the direct action of ionizing radiation and dry and hydrated electron scavenging that break S−S bridges, making protein molecules mobile. In addition, the presence of oxygen leads to the peroxy radical formation which in turn results in protein scission. Despite this, the cross-linking and scission are higher in the thin fraction than in the thick fraction, leading to the conclusion that immobility of proteins disfavors reactions that result in molecular mass changes.

CONCLUSIONS

G-value for the hydrated electron in the thick fraction of egg is 86% of that determined in neat water; the difference observed can be attributed to dry electron scavenging by proteins. The rate constant for the reduction of oxygen with the hydrated electron at 18 °C was found to be 1.7×10^{10} M^{-1} s^{-1}, a value very similar

Table 1. Summary of Data Obtained from Analysis of SDS-PAGE Densitograms[a]

	% scission		% cross-linking		*G*-value × 10^8 (mol/Gy)	
dose (Mrad)	thin	thick	thin	thick	thin	thick
0.4	11.2 ± 1.0	7.4 ± 1.4	4.7 ± 1.1	2.1 ± 0.8	−(9.7 ± 3.6)	− (4.5 ± 1.7)
0.8	18.7 ± 1.6	12.1 ± 2.6	7.6 ± 2.0	2.9 ± 1.9	−(14 ± 1.1)	− (5.3 ± 0.9)

[a] Errors indicate the standard deviation for $n = 6$ experiments.

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to that obtained in neat water. From this it can be concluded that the hydrated electron is as mobile and reactive as in neat water; this effect is due to the very low microviscosity of the thick fraction of egg white. Significantly smaller degree of radiation-induced cross-linking in the thick fraction of egg white compared to the thin fraction was observed. This can be attributed to the restricted mobility of proteins in the thick fraction which disfavors intermolecular radical-induced reactions in their competition with intramolecular reactions. The S−S bond reduction with hydrated and dry electrons makes the protein molecules mobile; that is why some cross-linking is observed in the thick fraction. Due to the presence of oxygen, scission is also observed, the extent of which is smaller in the thick fraction, again, due to the restricted mobility. No effects of the bound water on the hydrated electron spectrum in the thick fraction were observed on a nanosecond time scale; faster resolution may be required to detect an effect.

AUTHOR INFORMATION

Corresponding Author

*E-mail: bhm11@psu.edu. Fax: + (814) 863-6195.

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