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Research paper

Experimental evidence of the reciprocal oxidation of Bovine Serum Albumin and Linoleate in aqueous solution, initiated by HO• free radicals

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ABSTRACT

An investigation of radiation-induced oxidation of aqueous bovine serum albumin (BSA) in the presence of linoleate (LH) at pH 10.5 has been carried out in order to better understand the respective oxidative processes involved in both lipid and protein phases. Solutions containing BSA (15 μmol L^{-1}) and linoleate (15–600 μmol L^{-1}) below the critical micellar concentration (cmc = 2000 μmol L^{-1}), have been irradiated by γ-rays (^{137}Cs) at radiation doses ranging from 10 to 400 Gy (dose rate 9.5 Gy min^{-1}). It can be noticed that, in the absence of BSA, the main hydroperoxides formed from HO•-induced linoleate oxidation below the cmc, do not exhibit a conjugated dienic structure. This was also verified in the presence of BSA. Selected chemical markers of oxidation have been monitored: non-conjugated dienic hydroperoxides and conjugated dienes (without hydroperoxide function) for linoleate oxidation, and carbonyl groups for BSA oxidation. We have shown that for the lowest linoleate concentration (15 μmol L^{-1}) in the presence of BSA (15 μmol L^{-1}), the formation of conjugated dienes was not observed, meaning that LH was not exposed to HO• radicals attack. However, non-conjugated dienic lipid hydroperoxides were simultaneously detected, indicating that LH was secondarily oxidised by BSA oxidised species. Moreover, the oxidation of linoleate was found to be enhanced by the presence of BSA. For the highest linoleate concentration (600 μmol L^{-1}), the expected protection of BSA by LH was not observed, even if LH monomers were responsible for the total scavenging of HO• radicals. In this latter case, the formation of non-conjugated dienic lipid hydroperoxides was lower than expected. Those results showed that BSA was not oxidised by the direct action of HO• radicals but was undergoing a secondary oxidation by non-dienic lipid hydroperoxides and/or lipid radical intermediates, coming from the HO•-induced linoleate oxidation.

1. Introduction

Biological membranes and lipoproteins are constituted of lipids closely bound to proteins. Under oxidative stress conditions, reactive oxygen species (ROS) can initiate a process of oxidation of both lipidic and proteic fractions. Polyunsaturated fatty acids (PUFAs) are present in numerous biological systems such as membranes, phospholipid bilayers and lipoproteins. Their oxidation leads to hydroperoxides as primary products [1], and is usually responsible for breakdown or malfunction of membranes [2–5]. Model systems are often used to study the peroxidation of PUFAs, such as micelles in aqueous solution. The concentration of PUFAs in those systems has a crucial importance on the structure of the peroxides generated and the yields of peroxidation [6–8]. Detection methods for peroxidation products are usually based on the loss of lipid substrate, the formation of conjugated dienes [9], the measurement of oxygen uptake [10,11], the formation of thiobarbituric acid reactive species (TBARS) [12], or the quantification of hydroperoxides [7,8].

Serum albumin is an abundant protein found in blood plasma and interstitial fluids, and is generally considered as a multifunctional transport protein. Bovine serum albumin (BSA) contains 583 amino acid residues divided into three homologous α-helicoidal domains [13], and has properties similar to those of human serum albumin (HSA). A study on HSA complexed with mono and polyunsaturated fatty acids has revealed the existence of seven binding sites distributed across the protein [14].

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The radiation-induced oxidation of BSA has been the subject of numerous studies. Davies et al. [15–18] have shown that superoxide radicals ($O_2^{\bullet -}$) are bad initiators of BSA oxidation (in accordance with the low general reactivity of superoxide radicals towards amino acids from proteins [19]) and that the oxidation induced by hydroxyl radicals ($HO^{\bullet}$) leads to an increase in the global negative charge of the protein (indicating a degradation of NH_3^+ groups), a decreasing of tryptophane fluorescence, an increasing of bityrosine fluorescence, a generation of carbonyl groups and a fragmentation of albumin. Furthermore, BSA is susceptible to be oxidised by other oxidative substances, such as ozone [20,21], iron or copper [22], glucose or fructose [23–26]. As well, products from lipidic peroxidation, such as 13-HPODE (13-hydroperoxy-octadecadienoic acid), have been shown to react with BSA, leading to a loss of lysine residues, a generation of carbonyl groups and new amino acid related compounds, such as quaternary pyridine residues [27–29].

In the present work, radiolysis of water has been used to initiate the radical oxidation of a model system, constituted of BSA and linoleate monomers (pH 10.5) in aqueous solution. Each compound has been oxidised separately as a control and assessed for selected chemical markers: formation of conjugated dienes and hydroperoxides, and consumption of linoleate for the lipidic part; increase of bityrosine and decrease of tryptophane fluorescences, along with quantitative measurements of carbonyl groups for the proteic part. Then, mixed linoleate/BSA solutions containing a fixed concentration of BSA (15 μmol L^{-1}), and increasing linoleate concentrations (varying from 15 to 600 μmol L^{-1}) have been irradiated (from 10 to 400 Gy, with a dose rate of 9.5 Gy min^{-1}) and assessed for the same markers of lipid peroxidation and protein oxidation as the controls. Our goal was to find the concentration ratios $[linoleate]_0/[BSA]_0$ allowing to demonstrate non-ambiguously the interactions of lipid peroxidation products with the protein and conversely the interactions of the protein hydroperoxides with the lipid.

2. Materials and methods

2.1. Preparation of linoleate and BSA aqueous solutions

Sodium linoleate C18:2 (LH), purity 99%, and the bovine serum albumin (BSA), essentially fatty acid free, have been purchased from Sigma–Aldrich (St Louis, MO, USA). Both were stocked at 4 °C under argon, to avoid their auto-oxidation. Aqueous solutions of LH or BSA were prepared just before irradiations, in order to decrease at a minimum the influence of the oxygen-induced oxidation. Ultra pure water was employed for the dilutions (18 MΩ, Maxima Ultra Pure Water, ELGA) and the pH was adjusted to 10.5 by addition of sodium hydroxide (0.1 mol L^{-1}). This high pH is required to allow a complete solubilisation of fatty acids in water [30,31]. Preparation of BSA aqueous solution has been performed in the same way, in particular regarding the pH. To evaluate the denaturation of BSA at pH 10.5, a solution of BSA was prepared and pH was adjusted to 7.0 in order to compare the results obtained in terms of BSA oxidation (see Results section).

Aqueous solution containing both BSA and linoleate were prepared by mixing mother solutions of each compound. The concentration of BSA was fixed at 15 μmol L^{-1}, whereas the concentration of LH was ranging from 15 to 600 μmol L^{-1} (see Table 1). The ratio $r = [LH]_0/[BSA]_0$ was thus ranging between 1 and 40.

2.2. Oxidation of BSA and linoleate by water gamma radiolysis

Gamma irradiations were performed with an IBL 637 irradiator (CIS Biointernational, Gif-Sur-Yvette, France), using a Cesium 137 γ-ray source whose activity was approximately 222 TBq (6000 Ci). The radiation dose, expressed in Gy (1 Gy = 1 J kg^{-1}), was directly and linearly dependent on the exposure time to the ^{137}Cs source: the longer the exposure, the higher the radiation dose. The dosimetry was determined by the Fricke method [32,33], and the dose rate was found to be 9.5 Gy min^{-1} in our experiments.

Table 1
Composition of the aqueous solutions of Bovine Serum Albumin (BSA) and linoleate (LH) under focus. A and F correspond to "blank" solutions of BSA and LH respectively. pH of each solution is adjusted to 10.5 by adding sodium hydroxide (0.1 mol L^{-1}).

Solution	$[LH]_0$ μmol L^{-1}	$[BSA]_0$ μmol L^{-1}	$r = [LH]_0/[BSA]_0$
A	–	15	BSA only
B	15	15	1
C	150	15	10
D	300	15	20
E	600	15	40
F	15 to 600	–	LH only

Since all the solutions were irradiated under air, both hydroxyl ($HO^{\bullet}$) and superoxide ($O_2^{\bullet -}$) free radicals were produced, with formation yields respectively equal to 2.8×10^{-7} mol J^{-1} and 3.4×10^{-7} mol J^{-1} [33] respectively. However, knowing that $O_2^{\bullet -}$ radicals are not good initiators of fatty acid [34,19], or protein oxidation [15,19], only $HO^{\bullet}$ radicals were able to initiate the one-electron oxidation of linoleate and BSA, in our experimental conditions. At pH 10.5, $O^{\bullet -}$ free radical, the conjugated basic form of $HO^{\bullet}$ (pKa = 11.9 [35]) account for less than 4% of the total $HO^{\bullet}$ radicals. For this reason, it has not been taken into account in our study.

Irradiations were performed on 5 mL of aerated solution, and 5 mL of non-irradiated sample was systematically taken as control. Prior to each experimental set, glassware was carefully washed with TFD4 soap (Franklab S.A., France), rinsed with ultra pure water (18 MΩ, Maxima Ultra Pure Water, ELGA Labwater, Le Plessis-Robinson, France), and finally heated at 400 °C for 4 h to avoid any pollution by organic compounds.

2.3. Analysis of the chemical markers of oxidation

Conjugated dienes have been measured quantitatively by differential absorption spectrophotometry at $\lambda = 234$ nm, using a UV–Vis spectrophotometer (Beckman DU 70, Beckman Instruments Inc., Fullerton, CA, USA), using a molar extinction coefficient of 28000 L mol^{-1} cm^{-1} [36]. The optical path length was equal to 0.1, 0.2 or 1 cm, depending on the linoleate concentration.

Non-oxidised linoleate and lipid hydroperoxides have been determined by high performance liquid chromatography (HPLC) with both spectrophotometric and spectrofluorimetric detections. The method has been previously developed by Thérond et al. [37]. Operating conditions were as following: isocratic running with an eluent constituted by MeOH and ammonium acetate 10 mmol L^{-1} (96/4 v/v), flowrate of 1.2 mL min^{-1}, and 200 μL of solution injected. Two columns were used: a 25 cm reverse-phase C18 followed by a 15 cm reverse-phase C8 (both 4.6 mm ID), maintained at 40 °C. Hydroperoxides were quantified by chemiluminescence, after reaction with microperoxidase (10 mg/L) and isoluminol (55 mg/L) dissolved in a borate buffer (0.1 M, pH 9.2). Hydroperoxide concentrations were deduced from calibration curves established with standard 13(S)-HPODE (50 μg/mL). Non-oxidised LH was detected and quantified at 205 nm by absorption spectrophotometry. However, while the quantification of linoleate alone (15–600 μmol L^{-1}) in the absence of BSA was easy, it was impossible in the presence of BSA because of an important chromatographic peak broadening.

The structure of hydroperoxides generated depends on the initial concentration of LH $[LH]_0$ [6]: for $[LH]_0$ lower than the critical