Effects of Oxidation on Changes of Compressibility of Bovine Serum Albumin

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Abstract. The methods of ultrasound velocity and density measurements were used to study the adiabatic compressibility of bovine serum albumin (BSA) during its oxidation by the prooxidants Cu^{2+} and 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH). We did not find changes of compressibility of BSA in the presence of copper ions at rather high molar ratio Cu^{2+}/BSA = 0.66 mol/mol. This can be explained by binding of the Cu^{2+} to the binding site of BSA and thus protecting the prooxidant action of the copper. However, AAPH-mediated oxidation of BSA resulted in an increase of its apparent specific compressibility (φ_k/β_0). These changes could be caused by the fragmentation of the protein.

Key words: Bovine serum albumin — Protein oxidation — Adiabatic compressibility — Density

Introduction

Oxidative stress is one of the major factors impairing the cell function. Due to the physical or chemical factors the reactive compounds could appear in a biocolloids such as: hydroxyl $^{\bullet}OH$, hydroperoxide $HO_2^{\bullet}$, superoxide $O_2^{\bullet -}$ radicals and hydrated electrons, that induce the oxidation of cell membranes, biopolymers or their complexes with the lipids (Wilhelm 1990). For example the oxidation of lipoproteins is considered as an important in the development of atherosclerosis (Steinberg et al. 1989). Among a wide variety of lipoproteins, low density lipoproteins (LDL) are of special interest. LDL is the major sterol transporter in the circulation between blood plasma and the cell membranes. It is composed of an apolar core of cholesteryl esters and triglycerides, surrounded by a monolayer of phospholipids, unesterified cholesterol and one protein component, appolipoprotein B-100 (apoB)

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(see Kostner and Laggner 1989). It is assumed that the structural state of LDL is directly related to its oxidative susceptibility (Esterbauer and Jürgens 1993). In our recent work (Hianik et al. 2003) we have shown that copper-mediated oxidation of LDL resulted in a decrease of apparent specific compressibility (φ_k/β_0), of lipoproteins. The changes of ultrasound velocity and φ_k/β_0 value started much earlier than the beginning of the propagation phase as determined from the increase in conjugated diene concentration, measured by absorption at 234 nm. We assume, that changes of compressibility could be due to an increase of phospholipid ordering as a consequence of Cu^{2+}-mediated oxidation. Recently, we have shown (unpublished results) that also the prooxidant 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) induced decrease in adiabatic compressibility of LDL. The effect of AAPH was significant at the temperature 37 ℃, while at 25 ℃ we did not observe significant changes of adiabatic compressibility with and without AAPH. This is due to the fact that AAPH generates free radicals thermally at a constant rate at higher temperature, i.e. at 37 ℃ (Niki et al. 1991). Studies on biomembranes also showed considerable effects of oxidation on membrane ordering. Due to cross-linking between oxidized lipids both in the polar and hydrophobic regions, the rigidity of the membranes increased (Hegner 1980; Tirosh et al. 1997). Due to peroxidation, membrane proteins might also be cross-linked, and their rotational and lateral mobility might decrease (Richter 1987). Peroxidation causes also the damage of the proteins (see Wilhelm 1990 and references herein). Hydroxyl radicals induce cleavage of the protein molecule into small fragments (Wolf and Dean 1986; Wilhelm 1990).

Recent studies (Kveder et al. (2003) and references herein) have demonstrated that Cu^{2+}-mediated oxidation of LDL affect both protein and lipid components of LDL. However, despite of extensive studies of protein oxidation, it is not fully understood how the physical properties of the protein change during this process (Davis 2003). Particularly, it is not clear how fragmentation of protein affects its physical properties, e.g. compressibility. Because isolation and handling of apoB protein is difficult, a model protein – bovine serum albumin (BSA) was used to study how oxidation mediated by prooxidants Cu^{2+} and AAPH will change physical properties of the protein such as density and adiabatic compressibility.

Materials and Methods

Oxidation of BSA

Oxidation of BSA was induced by addition of: 1. AAPH at different final concentrations of 5, 10 and 20 mmol/l, or 2. 50 μmol/l $CuSO_4$ to the solution of BSA dissolved in 10 mmol/l phosphate buffer (PBS), pH 7.4 (concentration of BSA was 5 mg/ml). BSA and AAPH were purchased from Sigma (St. Louis, MO, USA), other chemicals were of analytical grade and used as received.

Sound velocity and density measurements

The measurement of ultrasound velocity allows us to evaluate the elastic properties of aqueous media, such as liposome, protein or lipoprotein suspensions. This evaluation is based on a simple relationship

$$\beta = 1/(\rho u^2) \tag{1}$$

where β is the coefficient of adiabatic compressibility, ρ is the density and u is the ultrasound velocity. In the study of mechanical properties of solutions, measuring a relative change in a physical characteristics *per* unit of solute concentration rather than its absolute value is often more important, precise and easier (Sarvazyan 1991). The relative concentration increments of compressibility, density and sound velocity can be defined as: $[\beta] \equiv (\beta - \beta_0)/\beta_0 C$; $[\rho] \equiv (\rho - \rho_0)/\rho_0$C; and $[u] \equiv (u - u_0)/u_0 C$, where C is the molar concentration of the solute and values related to the solvent are denoted by a subscript 0. The relationship between these relative increments at low solute concentration can be obtained by differentiating the equation (1):

$$[\beta] = -2[u] - [\rho] \tag{2}$$

If taking into account the definition of the quantities of apparent molar volume $\Phi_V \equiv (V - V_0)/CV = M/\rho_0 - [\rho]$ and the apparent molar compressibility $\Phi_k \equiv (\beta V - \beta_0 V_0)/CV$, where M is the molar mass of the solute, then from the above definitions and equation (2), the following relationship can be obtained

$$\Phi_k/\beta_0 = -2(u - u_0)/u_0 C - M/\rho_0 + 2\Phi_V \tag{3}$$

and for specific values:

$$\varphi_k/\beta_0 = -2[\mathrm{u}] - 1/\rho_0 + 2\varphi_V \tag{4}$$

where φ_k/β_0, φ_V are the specific apparent compressibility and specific volume, respectively. The value of $[u] = (u - u_0)/u_0 c$ (c is concentration in mg/ml) can be determined by ultrasonic velocity measurements and the value of φ_V (Kratky et al. 1973)

$$\varphi_V = [1 - (\rho - \rho_0)/c]/\rho_0 \tag{5}$$

by means of density measurements.

Ultrasonic velocity was measured using a differential fixed-path velocimeter consisting of two resonators as described elsewhere (Sarvazyan 1982 and Hianik et al. 1998).

For precise density measurements the vibrating tube principle was used to determine the apparent partial φ_V (see equation (5)) of lipoproteins (Kratky et al. 1973), employing the densitometer system DMA 60 with DMA 602M (Anton Paar KG, Graz, Austria). In order to obtain a higher resolution, the measurements were performed with two sample cells DMA 602M. One contained the BSA and the other

one – the reference buffer. The cells for both ultrasonic and density measurements were thermostated with a Lauda RK 8 CS ultrathermostat.

The measurements of u have been started after addition of AAPH (at different final concentration: 5, 10, 20 mmol/l) or $CuSO_4$ (at final concentration of 50 μmol/l) into the measuring and reference cells upon continuous stirring. The measurements were performed every 10 min. In contrast with measurement of u, the cells for density measurements does not allow stirring the protein solution during measurements. Due to the sedimentation of BSA, the long term measurements of its density could leads to errors. We therefore incubated BSA suspension with AAPH and took small volume of the BSA at certain interval of time (approx. 5 min) prior to the measurements.

Experimental errors

The uncertainty in the concentration of BSA was smaller than 0.25 %. The temperature of the measuring chambers was controlled in a range of ± 0.02 ℃. The relative error in the resonance frequencies of the ultrasonic resonator chambers was ± 5 Hz and less then $\pm 10^{-3}$ ml/g for $[u]$ and φ_V, respectively. The error in the determination of density was less than $\pm 10^{-3}$ g/ml. Each series of experiments was performed at least twice.

Results and Discussion

Proteins in water solution can be characterized by φ_V and φ_k/β_0. The volume compressibility of macromolecules in water solution is determined both by the intrinsic compressibility and the compressibility of the hydrated shell surrounding the protein. Because the adiabatic compressibility is an additive value, the total adiabatic compressibility of macromolecules in water solution is the sum of the compressibilities of the macromolecule ($(\varphi_k/\beta_0)_{\mathrm{M}}$) and the hydrated shell ($(\varphi_k/\beta_0)_{\mathrm{H}}$) (Sarvazyan and Kharakoz 1977)

$$\varphi_k/\beta_0 = (\varphi_k/\beta_0)_{\mathrm{M}} + (\varphi_k/\beta_0)_{\mathrm{H}} \tag{6}$$

The value $(\varphi_k/\beta_0)_{\mathrm{M}}$ is positive, however, the sign of $(\varphi_k/\beta_0)_{\mathrm{H}}$ depends on the temperature. For example, at $t = 20$ ℃ this value is negative. This is associated with lower compressibility of the hydrated shell in comparison with the volume of free unbound water molecules. At higher temperatures the differences between compressibility of the hydrated shell and free water molecules become less pronounced. Thus the overall compressibility of aqueous solutions of macromolecules should depend on the degree of hydration and on the temperature. At $t = 20$ ℃, the value of φ_k/β_0 is positive for globular proteins, but it is negative for fibrilar proteins (Sarvazyan and Kharakoz 1977). The negative value of adiabatic compressibility of fibrilar proteins is connected with their high level of hydration. The typical values of φ_V, $[u]$, and φ_k/β_0, of BSA water solution, LDL and liposomes from egg phosphatidylcholine are shown in Table 1. We can see that the compressibility of BSA

Table 1. Properties of BSA, LDL and unilamellar liposomes of eggPC at different temperatures. [a] Sarvazyan and Kharakoz (1977), [b] Hianik et al. (1997), [c] Hianik et al. (2000). The mean and S.D. were calculated from results of 4–6 independent experiments

Object	t, ℃	φ_V, ml/g	$[u]$, ml/g	φ_k/β_0, ml/g
BSA	20	0.733[a]	0.168[a]	0.13[a]
	25	0.75	0.14 ± 0.02	0.22 ± 0.02
	37	0.79 ± 0.02	0.13 ± 0.02	0.32 ± 0.02
LDL[b]	25	1.0 ± 0.03	0.016 ± 0.002	0.98 ± 0.09
	37	1.08 ± 0.03	−0.023 ± 0.002	1.17 ± 0.09
eggPC[c]	20	0.991	0.013	0.957

φ_V, specific volume; $[u]$, concentration increment of sound velocity; φ_k/β_0, specific compressibility; BSA, bovine serum albumin; LDL, low density lipoproteins; eggPC, egg phosphatidylcholine.

is considerable lower in comparison with LDL and liposomes and strongly increases with temperature. As we have already mentioned, we can expect the changes of compressibility of the hydrated shell as well as that of macromolecules if the temperature changes. For LDL this is shown also in Table 1. At $t = 37$℃, when LDL is in liquid crystalline state, the $[u]$ value is negative. This means that sound velocity in solvent (buffer) is higher than that in LDL suspensions. For BSA, as it is evident from Table 1, only monotonous changes of $[u]$ and φ_V take place. Therefore in the temperature range of 20–37℃ we can not expect the unfolding in BSA and, consequently, no substantial changes of BSA structure.

Oxidation of lipoproteins can be induced by Cu^{2+} or by AAPH (Ondriaš et al. 1994; Hofer et al. 1996). The mechanism of action of Cu^{2+} and AAPH is different. The Cu^{2+} binds to proteins (e.g. apoB) and localizes to the macromolecule surface, while AAPH induces initiation of free radicals in aqueous solution. Free radicals, which are formed due to degradation of AAPH finally cause the oxidation of the hydrophobic core of the lipoprotein (Niki et al. 1991) or protein molecules.

We studied how these prooxidants influence the compressibility of BSA in a model protein. The concentration increment $[u]$, practically does not depend on BSA concentration in the range 1–10 mg/ml (i.e., 15–150 μmol/l) (Fig. 1). The concentration of albumin in a blood serum is, however, much higher (approx.

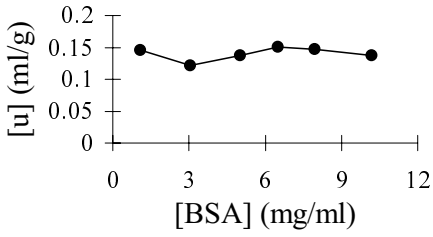

Figure 1. Dependence of $[u]$ on the BSA concentration, $t = 25$℃.

0.6 mmol/l (Kragh-Hansen 1990)). However, the sound velocimetry is most precise in rather diluted solutions. On the other hand, for study the properties of the complexes Cu^{2+}/BSA the molar ratio of BSA and Cu^{2+} is more important than the concentrations of these compounds. At used concentration of BSA (5 mg/ml, i.e. 76 μmol/l) and 50 μmol/l Cu^{2+} the molar ratio Cu^{2+}/BSA = 0.66 is rather high and comparable with conditions used in other works (see e.g. Gryzunov et al. 2003).

First, the Cu^{2+} influence on the changes of $[u]$ of the BSA suspension was examined. As was already mentioned in the Introduction, Cu^{2+} induces strong oxidation of LDL at relatively low concentrations (10 μmol/l). This oxidation resulted in an increase in $[u]$ and decrease in φ_V. Under these conditions a decrease in adiabatic compressibility of LDL was observed (Hianik et al. 2003). The $[u]$ and φ_V of BSA, however, did not change at the presence of even higher concentrations of $CuSO_4$ (50 μmol/l). The typical time course of $[u]$ values of BSA suspensions containing 50 μmol/l $CuSO_4$ (molar ratio Cu^{2+}/BSA = 0.66 mol/mol) at two different temperatures 25 ℃ (curve 1) and 37 ℃ (curve 2) are shown on Fig. 2. There are no significant changes of $[u]$ with time. The obtained results that physical properties of BSA do not change at the presence of Cu^{2+} are not surprising. The albumins are known as transporters of the copper to many target enzymes in the cells and in the biological fluids for which this metal plays important catalytic function (see Gryzunov et al. 2003 and reference herein). The binding of the Cu^{2+} to the active site of the albumin at the same time prevents the prooxidant action of the copper. Thus, undamaged and unmodified albumins play also role of effective antioxidants. The obtained results are in agreement with that obtained by Gryzunov et al. (2003) for native form of similar albumin – human serum albumin (HSA). Using EPR method they did not observed formation of free radicals for the complexes Cu^{2+}/HSA. However, they shown that the incubation of HSA with fatty acids converts albumins from antioxidant to a prooxidant.

In contrast with Cu^{2+} the effect of AAPH on physical properties of BSA was rather strong. Fig. 3 (A,B,C,D) shows an example of the time course of values $[u]$ (A), density, ρ (B), φ_V (C) and φ_k/β_0 (D) at presence of 20 mmol/l AAPH ($t = 37$ ℃). It is seen that the increment $[u]$ strongly decreases (almost 35 % within

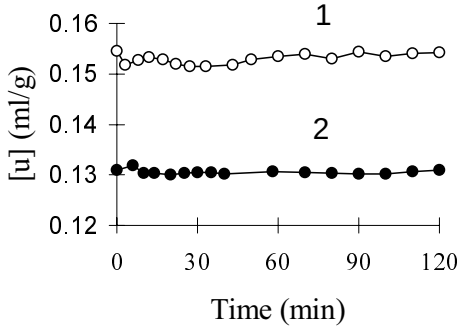

Figure 2. Time course of $[u]$ for the water solution of BSA at presence of 50 μmol/l $CuSO_4$, $t = 25$ ℃ (1), $t = 37$ ℃ (2).

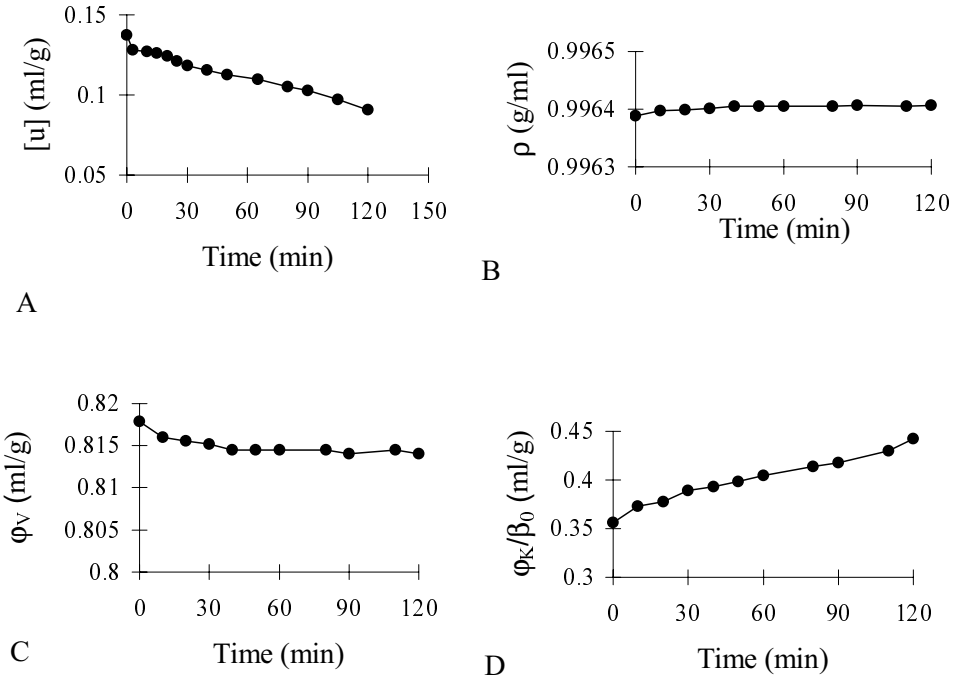

Figure 3. Time course of $[u]$ (A), ρ (B), φ_V (C) and φ_k/β_0 (D) for water solution of BSA following its oxidation at the present of 20 mmol/l AAPH at $t = 37$°C.

120 min) (Fig. 3A), however, only a slight decrease in the φ_V (approx. 0.5%) took place (Fig. 3C). As a result, the adiabatic compressibility increases by ∼20% (Fig. 3D). The results were well reproducible and an almost identical behavior has been obtained in three independent experiments. The influence of AAPH on physical properties of BSA strongly depends on concentration of this prooxidant. This effect is shown in Fig. 4, where the time course of relative changes of the concentration increment $\Delta[u]$, ($\Delta[u] = ([u] - [u]_0)/[u]_0$), where $[u]_0$ is the concentration increment at time $\tau = 0$ and $[u]$ at $\tau > 0$).

At least two effects could contribute to the changes of $[u]$ of BSA solution at the presence of AAPH: changes in hydration and/or structural changes of BSA. Let us discuss these possibilities.

Changes of hydration of AAPH

The decrease in the $[u]$ could be associated with a decrease in hydration of the compounds studied (see e.g. Buckin 1988). However, AAPH is hydrophilic and should not significantly penetrate into the hydrophobic core. We therefore do not expect that the observed changes of BSA compressibility are caused by penetration of AAPH into the protein interior, thus changing its hydration.

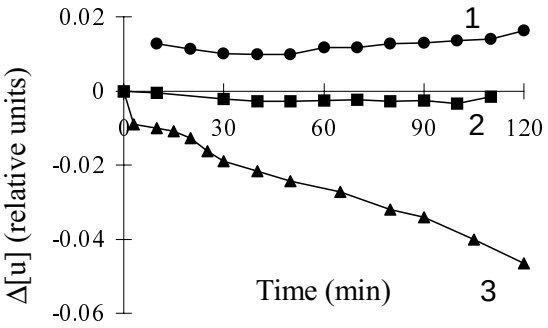

Figure 4. Time course of the relative changes of $\Delta[u]$ ($\Delta[u] = ([u] - [u]_0)/[u]_0$, where $[u]_0 = [u]$ ($\tau = 0$), $[u] = [u]$ ($\tau > 0$)) for water solution of BSA at presence of different concentrations of AAPH: 5 mmol/l (1), 10 mmol/l (2), 20 mmol/l (3) at $t = 37$℃.

Changes of BSA structure

The changes of $[u]$ values at the presence of AAPH indicate that this prooxidant might induce structural changes of BSA. As we mentioned above, AAPH is hydrophilic and is likely not to penetrate into the core of BSA. Thus the free radical chain reaction mainly starts at the BSA surface. The usually formed free radicals (see e.g. Wilhelm 1990) then induce further chemical and structural changes in the core of the protein. The increase in adiabatic compressibility of BSA at relatively small changes of BSA density and specific volume provides evidence for BSA fragmentation. The mechanism of this process is not well understood and requires further investigation.

Experiments on adiabatic compressibility of LDL during oxidation by AAPH performed in our laboratory (unpublished results) showed that, in contrast to BSA, low concentration of AAPH (5 mmol/l) induced a decrease in LDL adiabatic compressibility. This effect, which is similar to that obtained with Cu^{2+} (Hianik et al. 2003) was explained by possible cross linking between lipids and proteins facilitated by peroxide radicals. The changes of apparent φ_k/β_0 of BSA following oxidation by AAPH ($\sim$0.08 ml/g) were, however, larger than that observed for LDL following oxidation by Cu^{2+} (0.015 ml/g). In the above mentioned work, it was not possible to distinguish between volume compressibility of lipid and protein core of LDL and thus to decide what is the main target for oxidation – proteins or lipids. Our experiments with BSA indicate that prooxidant AAPH could have opposite effect on the protein and the lipid component of LDL. On the other hand, the apoB in LDL is surrounded by lipid components that could considerably influence the conformation of the protein and as a result also the oxidative susceptibility of the protein. Particularly, Gryzunov et al. (2003) demonstrated that binding of fatty acids to human serum albumin caused changes of HSA conformation. Therefore further studies of the effect of proxidant on the lipid and protein components should be focused also on the analysis of the action of prooxidants on the complexes of proteins and fatty acids.

Acknowledgements. This work has been supported by the project in framework of the programme "Aktion Austria–Slovakia", collaboration in science and education (Project

No. 25s46) and in part by Slovak Grant Agency (Project No. 1/8310/01) and by the Fonds zür Forderung der Wissenschaftlichen Forschung in Österreich (Project S7103). We thank to R. Krivánek for expert technical assistance.

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Final version accepted: August 15, 2003