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Oxidation of Natural and Thermal Denatured Bovine Serum Albumin Hydrazyl Free Radicals in the Presence of Cyclodextrins

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Abstract

The influence of certain thermal treatments on the reactivity of bovine serum albumin (denaturation followed by renaturation in three different cooling conditions) was studied monitoring the kinetics of oxidation of BSA with two water-soluble stable hydrazyl radicals. The results showed that the addition to the reaction mixture of α-cyclodextrin and β-cyclodextrin decreases the oxidation rate, probably due to the encapsulation of terminal amino acid rests by the cyclodextrins cavity. β-Cyclodextrin protects more efficiently the albumin probes than α-cyclodextrin. The denatured albumin probes are more reactive than natural albumin as a consequence of the reorienting of the hydrophobic rests of albumin molecule to their surface.

Introduction

Albumins represent a class of the transport proteins responsible for the transport, distribution and metabolism of many endogenous and exogenous ligands [1]. Particularly, the macromolecule of bovine serum albumin (BSA) has a prolate ellipsoid form with axes of 40 and 140 A in solution [2]. In the native state, the BSA molecules take a conformation with the polar residues of amino acids oriented towards the outside of the molecule, and the nonpolar (hydrophobic) residues directed towards the inside of the molecule [3]. The structure of BSA explains its capacity to interact by hydrophobic or hydrophilic forces with other molecules (especially with those of biological significance).

Increasing the temperature of BSA solutions, followed by different cooling procedures, has as effect the modifications of native albumin conformation. We expect that thermal denaturation of BSA could be responsible for changes in some physical properties or chemical reactivity. Our previous research showed that the chromatographic [4, 5], electrophoretic [4] or optical properties [6, 7] of albumin probes obtained after thermal treatment are considerably changed besides the natural BSA probe. Also, the interaction of BSA with some alkaline halide [5] or dyes [7] is modified after thermal treatment.

The goal of this paper is to discuss the changes in the chemical reactivity of albumin, modified after different thermal treatments. In two other papers [8, 9] we studied the kinetics of oxidation of amino acids by stable free radicals in the absence or in the presence of natural cyclodextrins. We used the same hydrazyl water-soluble type radicals (the sodium salts of 2-*p*-phenylsulfonic-acid-2-phenyl-1-picrylhydrazyl (**1**) and 2,2′-di-*p*-phenylsulfonic-acid-2-phenyl-1-picrylhydrazyl (**2**) see Figure 1a) to oxidize the albumin samples. The hydrophobic residues of amino acids from BSA structure are available to the cyclodextrins cavity. Some of these residues could be oxidated by hydrazyl radicals and the kinetic investigation on albumin's oxidation process, in the presence of cyclodextrin, was made in order to evidence, in an indirect way, the interaction between cyclodextrins and this protein.

The ability of cyclodextrins to form inclusion compounds with organic molecules in water solutions, as a consequence of their toroidally shaped cavity, has attracted attention, and the literature reported many papers in the last 50 years. Our study deals with two cyclodextrins (CDs), α and β; these are the most common and they are composed by linking of 6 and 7D-glucopyranose unity, respectively. The majority of literature data present equilibrium studies on inclusion complexes of CDs, using various physico-chemical methods, as spectrascopic techniques [10], circular dichroism [11], NMR [12, 13], competitive inhibition of catalytic reactions [14], microcalorimetry [15], solubility [16], chromatography [17–19], potentiometry [20]. However, there are also many studies on the effects of CDs on the organic reaction which are explained by two models: 'the enzyme model' and 'the extra reaction field model', designed by the hydrophobic cavity of cyclodextrins [21].

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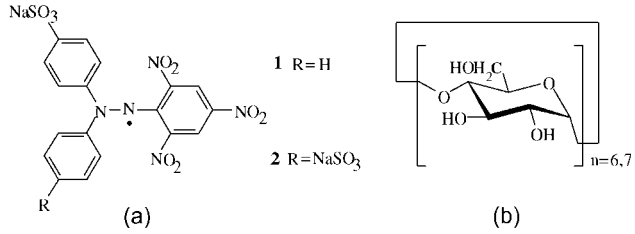

Figure 1. Structure of free radicals **1** and **2** (a) and of α- and β-cyclodextrins ((b), $n = 6$ or 7, respectively).

Experimental

Substances

BSA (fraction V) was purchased from Merck. The persistent free radicals **1** and **2** (Figure 1a) were obtained as described in the literature [22]. α- and β-CD (Figure 1b) were purchased from Aldrich.

Method and apparatus

The water solution of BSA with 2.8 mg/mL concentration was kept as reference (named sample **I**). Other water solutions with the same concentration of BSA were heated at 65°C for 15 min and cooled in different procedures as follows: gentle cooling in Dewar vessel (2°C/min) sample **II**, at room temperature (sample **III**) and using fast cooling procedure at 4°C (sample **IV**).

Kinetic measurements were made in aqueous solution at 293 K with a large excess of albumin and cyclodextrin (10^{-2} M) over free radicals, (the final concentration being approximately 10^{-4} M). Monitoring the disappearance of the radicals 1 and 2 at 515 nm followed the oxidation of albumin samples. At this wavelength, the absorbance of the BSA or of other reaction products is negligible. The measurements were carried out with a Specord M80 spectrophotometer. The course of the reaction was studied for at least two half lives and the rate constants in each case were evaluated from linear plots of absorbance against time.

Results and discussion

In our previous research we studied the disappearance of radicals **1** and **2** as a result of oxidation of amino acids in the absence and in the presence of α- and β-cyclodextrin in order to mime a biochemical process – transamination or deamination of amino acids and to establish if cyclodextrins have a protective effect against oxidation with free radicals.

In earlier papers [8, 9] we presumed that the free radicals **1** and **2** oxidize the amino group from amino acids. In the case of the present experiment, we consider that the hydrazyl radicals primarily react with the free amino group of the BSA amino acid residues existing at its surface. There are relatively numerous amino groups in the whole protein molecule (which contains 59 lysine residues, 23 argine residues and 17 histidine residues) [2]. This process is similar to that of the oxidative amino acid deamination presented in Figure 2 [8, 23].

The free radicals **1** and **2** undergo a slow decomposition in aqueous solution, the rate constant of decomposition of these radicals being 0.45 and 0.75×10^{-3} min^{-1}, for **1**, respectively **2**. These values were taken into account in order to determine the kinetic constants attributed to the disappearance of radicals **1** and **2** in the presence of albumin samples. The results are presented in Table 1 for radical **1** and in Table 2 for radical **2**. Thus, the disappearance of hydrazyl free radicals **1** and **2** in the presence of albumin samples is a result of two parallel processes: (1) reaction with water and (2) reaction with albumin

$$\mathbf{1}\ (\text{or } \mathbf{2}) + \text{HOH} \rightarrow \text{Products} \qquad (1)$$

$$\mathbf{1}\ (\text{or } \mathbf{2}) + \text{Albumin} \rightarrow \text{Products}. \qquad (2)$$

In the presence of α-CD or β-CD (at 10^{-2} M concentration) the rate constants of decomposition of these radicals in water solution are similar to those obtained in the absence of cyclodextrins. Otherwise, the recorded UV–Vis spectra of the corresponding hydrazine to the radicals **1** and **2** had no modification in the presence of cyclodextrins. The significance of these data is that the radicals **1** or **2** (and their hydrazines) do not interact with the cyclodextrins.

Previous results reveal that for amino acids that have in their structure NH_2, SH or aromatic groups the kinetic of disappearance of radicals **1** and **2** is accelerated

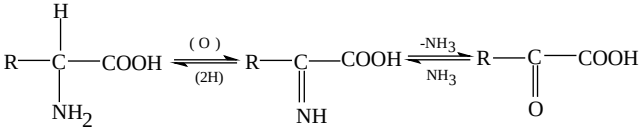

Figure 2. Oxidative desamination of amino acids.

Table 1. Rate constants (×10^4 min^{-1}) obtained for the disappearance of radical **1** in the absence of CD (k_1), in the presence of α-CD (k_1^{α}) and in the presence of β-CD (k_1^{β}) for albumin probes **I–IV**

Probe	I	II	III	IV
k_1	6.6	6.5	11.8	11.4
k_1^{α}	4.5	5.1	10.0	7.4
k_1^{β}	3.9	4.9	7.3	5.4

Table 2. Rate constants (×10^4 min^{-1}) obtained for the disappearance of radical **2** in the absence of CD (k_2), in the presence of α-CD (k_2^{α}) and in the presence of β-CD (k_2^{β}) for albumin probes **I–IV**

Probe	I	II	III	IV
k_2	9.5	9.5	12.4	13.6
k_2^{α}	6.8	7.4	10.3	8.9
k_2^{β}	6.4	4.7	6.5	7.6

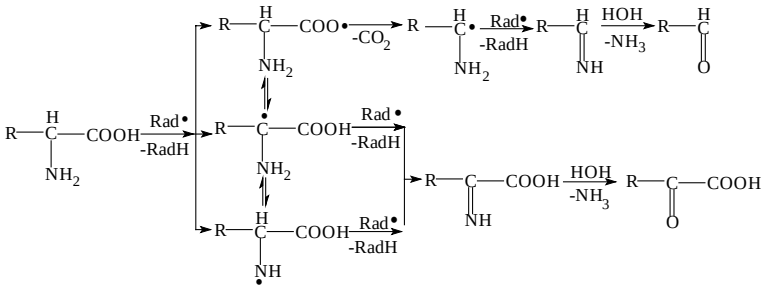

Figure 3. Pathway of oxidation of amino acids by free hydrazyl type radicals (noted as Rad).

both in the absence and in the presence of CDs [8, 9]. These results imply that hydrazyl radicals attack such groups presented in the structure of the albumin molecule. We can presume three possible oxidation mechanisms of amino acids (and BSA) (Figure 3).

In the case of albumin oxidation, the last pathway becomes important; in that situation the presence of other reactive groups (like SH, OH, aromatic) in residues of amino acids play their own role.

From data shown in Tables 1 and 2 we noticed first that the presence of CDs reduce the rate constants of hydrazyl radicals **1** and **2** for all albumin samples, the effect being higher for β-CD. Probe **I** represent the natural BSA (with a compact conformation), while probes **II–IV** are thermally denatured BSA samples, with different final conformational structures. Taking into account the heating and cooling procedures of the albumin (associated with protein unfolding) it is expected that the conformation of protein from sample **II** will be closer to sample **I** and differ more for samples **III** and **IV**.

Our previous study [4, 5] on the chromatographic and electrophoretic behavior of similar BSA samples sustains our description. Kinetic data from Tables 1 and 2 showed a higher reactivity of samples **III** and **IV** compared with samples **I** and **II**, both in absence and in presence of CDs.

It is evident that the molecule of albumin is too bulky to be completely included in the CD cavity and the peptide backbone may reduce the formation of inclusion complexes as a consequence of topological constrains. This is why we expect only a local interaction between CD and albumin. In the literature there are spectroscopic studies about the incorporation of L-tryptophan and L-tyrosine residues from a synthetic peptide into derivatised β-CD [24]. On the other hand, CDs can be used to solubilise and stabilize various biomedical peptides and proteins such as albumin [25], aspartame [26], β-amyloid-peptide [27] and γ-globulin [25].

The enhancement of the albumin reactivity in the cases **III** and **IV** is a consequence of the changes induced by the thermal treatments (defolding and refolding) on the three-dimensional structure of albumin. In the new conformation of albumin probes **III** and **IV**, the hydrophobic residues of amino acids are exposed to the solution and in this way could be attacked by the free radicals **1** and **2**.

The protection of BSA against oxidation by radicals **1** and **2** is greater in the presence of β-CD because its cavity is larger than α-CD and the local interaction with protein is favored.

The differences between kinetic data corresponding to probe **III** and **IV** in the case of radical **1** are negligible in the absence of CDs, while in the presence of those molecules the values of kinetic constants are significantly distinct. This can be interpreted in terms of a competitive interaction between the reactive amino acid residues of albumin to interact with CDs.

We can conclude that the interaction of CDs with hydrophobic residues from natural BSA and denatured BSA has a protective effect on the radicals attack. But the most important conclusion is that, using the kinetic data on the oxidation reaction of albumin samples with hydrazyl radicals (in the absence and in the presence of CDs), we have differentiated between different protein three-dimensional structures.

References

1. X.M. He and D.C. Carter: *Nature* **358**, 209 (1992).
2. T. Peters Jr., *All about Albumin*, Academic Press, New York, 1996.
3. A.L. Lehninger: *Biochemistry*, 2nd Ed., Worth Publishers, NY, 1975.
4. G. Ionita, C. Postolache, C. Tilimpea, D. Dinu, and V.E. Sahini: *J. Plan. Chromatogr.* **4**, 308 (2003).
5. G. Ionita and V.E. Sahini: *Analele Universitatii Bucuresti* **II**, 153 (2002).
6. V.E. Sahini: 11th Physical Chemistry Conference, Timisoara, Romania, September 2–5 (2003).
7. L. Barla, A. Tarsoaga, and V.E. Sahini: *Analele Universitatii Bucuresti* **I–II**, 161 (2003).
8. G. Ionita, V.E. Sahini, Gh. Semenescu, and P. Ionita: *Acta Chimica Slovenica* **47**, 111 (2000).
9. G. Ioniţă, P. Ionita, V.E. Sahini, and C. Luca: *J. Inclusion Phenom. Macrocyclic Chem.* **39**, 269–271 (2000).
10. R.P. Rohrbach, I.J. Rodriguez, E. Eyring, and J.F. Wojik: *J. Phys. Chem.* **81**, 944 (1977).
11. K. Harata: *Bioorg. Chem.* **10**, 255 (1981).
12. R.J. Bertgson, M.A.Channing, and K.A. McGovern: *J. Am. Chem. Soc.* **100**, 2878 (1978).
13. T. Nakajima, M. Sunagawa, T. Hirohashi, and K. Fujoka: *Chem. Pharm. Bull.* **32**, 383 (1984).

14. R.L. Vanetten, J.F. Sebastian, G.A. Glowes, and M.L. Bender: *J. Am. Chem. Soc*. **89**, 3242 (1967).
15. G.E. Hardee, M. Otagiri, and J.H. Perrin: *Acta. Pharm. Succ*. **15**, 188 (1978).
16. H. Schlenk and D.M. Sand: *J. Am. Chem. Soc*. **83**, 2312 (1961).
17. K. Uekama, F. Hirayama, and T. Irie: *Chem. Lett*. 661 (1978).
18. D.W. Armstrong, F. Nome, L.A. Spino, and T.D. Golden: *J. Am. Chem. Soc*. **108**, 1418 (1986).
19. K. Fujimura, T. Veda, M. Kitagawa, H. Takayanagi, and T. Ando: *Anal. Chem*. 2668 (1986).
20. T.P. Nguyen, M. Turme, P. Lettelier, N.M. Gosselet, and B. Sebille: *Pharm*. **216**, 53 (1997).
21. K. Takahashi: *Chem. Rev*. **98**, 2013 (1998).
22. G.V. Putirszkaja and T. Siladi: *Acta Chim. Hung*. **72**, 329 (1972).
23. D. Laloo and M.K. Mahanti: *J. Chem. Soc. Dalton Trans*. 311 (1990).
24. K. Matsubara, T. Irie, and K. Uekama: *Chem. Pharm. Bull*. **45**, 378 (1997).
25. M. Katakam and A.K. Banga: *J. Pharm. Pharmacol*. **47**, 103 (1995).
26. R.J. Prankerd, H.W. Stone, K.B. Dloan, and J.H. Perrin: *Int. J. Pharm*. **88**, 189 (1992).
27. P. Camilleri, N.J. Haskins, and D.R. Howlett: *FEBS Lett*. **341**, 256 (1994).