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Changes in the secondary structure of proteins labeled with ^{125}I: CD spectroscopy and enzymatic activity studies

Y. M. Efimova,[1]* B. Wierczinski,[1] S. Haemers,[1] A. A. van Well[2]**

[1] *Department of Radiochemistry*
[2] *Department of Neutron Scattering and Mössbauer Spectroscopy,*
Interfaculty Reactor Institute, Delft University of Technology, Mekelweg 15, 2629 JB Delft, The Netherlands

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Bovine serum albumin (BSA) and lysozyme (LSZ) were radiolabeled with ^{125}I. Three different methods for protein iodination with ^{125}I were optimized. Parameters like incubation time and ratio of oxidizing agent and amount of protein were established. During protein iodination with ^{125}I, structural damages caused by the introduction of iodine into the protein may occur. These damages depend on the oxidizing agent used and may lead to considerable changes in the protein structure and, hence, their biological activity. Changes in secondary structure of LSZ and BSA were examined by circular dichroism (CD). Enzymatic activity tests were performed with lysozyme to check its biological activity. The Iodo Bead was found the best oxidizing agent for protein iodination.

Introduction

Many different substances can be labeled by radioiodination. Such labeled molecules are of major importance in a variety of investigations, e.g., studies of intermediary metabolism, quantitative measurements of physiologically active molecules in tissues and biological fluids, the adsorption kinetics and exchange of proteins on interfaces.

Iodine-125 is most commonly used for iodination of compounds for in vitro procedures. It has a half-life of 60 days and a γ-ray energy of 35 keV. Thus, ^{125}I is the radionuclide of choice for radioiodination. Various methods have been developed for the radioidination of peptides and proteins.[1–5] They differ in the nature of the oxidizing agent (e.g., Chloramine-T, Iodogen, Iodo Beads) for converting ^{125}I into the reactive species $^{125}I_2$ or $^{125}I^+$. Those reactive species are incorporated into amino acid residues of the proteins. Mostly, substitution into tyrosine residues of the protein takes place, but substitution into other residues, such as histidine,[6] cysteine, and tryptophan may also occur.

There are four levels of protein structure complexity. Primary structure is the amino-acid sequence specific for each protein. The shape of the polypeptide chain determines the secondary structure of the protein. There are four common secondary structures in proteins namely alpha helices, parallel or antiparallel beta sheet, turns, and random coil. Tertiary structure is the folding and twisting of secondary structures and the quaternary structure is defined as two or more polypeptide chains, which are assembled together. We assumed that changes in the secondary structure could be used as a probe for the structural stability of the protein.

During the process of the protein iodination by ^{125}I different types of changes can occur in the protein structure.[1]

(1) Damage caused by the iodination process. This is an important factor: if the labeling procedure itself damages the protein structure, considerable changes in biological activity in a variety of biological systems can take place. This kind of damage may depend on the oxidizing agent used. Therefore, we have considered three different oxidizing agents: Iodogen, Chloramine-T and Iodo Beads. It is known that Chloramine-T is a strong oxidizing agent and some proteins are denaturated under the relatively strong oxidizing conditions.[7] Methods that use Iodogen or Iodo Beads are supposed to be more gentle.

(2) Radiation damage. If very high activities of ^{125}I are used for the iodination, the radiation emitted during the decay of ^{125}I may cause bond breakage. Whenever possible, lower levels of radioactivity are used.

(3) The introduction of an iodine atom by itself may have an influence on the properties of the protein.

We have applied radiolabeling to study adsorption and exchange processes of proteins on solid surfaces. For these experiments it is very important that the structure of the labeled proteins does not change during the labeling process. We have optimized the labeling procedure for two proteins: Lysozyme (LSZ) is a "hard" globular protein with a stable secondary structure and shape, and bovine serum albumin (BSA) which is a "soft" globular protein and may easily change its internal structure. Changes of the secondary structures after radiolabeling were determined by using circular dichroism (CD) spectroscopy that measures the difference in absorbance of right- and left-circularly

* E-mail: efimova@iri.tudelft.nl
** Present address: Institut für Radiochemie, Walther-Meissner-Strasse 3, 85748 Garching, Germany.

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Akadémiai Kiadó, Budapest

polarized light. It has been shown that the CD spectra can be analyzed in terms of different secondary structural types. A number of review articles are available describing the technique and its application.[8–12] Furthermore, the enzymatic activities of iodinated and native LSZ were compared using *Micrococcus lysodeikticus*, which is a probe of the tertiary structure as well.

Experimental

Materials

Proteins BSA (A4378) and LSZ (L6876) were purchased from Sigma and used without further purification. Tris buffer with a concentration of 50 mM and pH 7.5 was prepared by mixing 50 mM of Tris-(hydroxymethyl)-aminomethane and 50 mM of HCl in an appropriate ratio. The buffer solution was made using Super Q-Millipore water and was filtrated through a membrane (Rotilabo-Spritzenfilter) with a pore size of 0.22 μm and stored at 4 °C until use. Oxidizing agents Chloramine-T (C9887) and Iodogen (T0656) were obtained from Sigma, Iodo Beads from PIERCE (No. 28665). Chloroform was from J. T. Baker (No. 7386). The ^{125}I in a NaOH solution (370 MBq/100 μl) was from Amersham Bioscience.

PD 10 desalting columns with Sephadex G-25 as medium were purchased from Amersham Pharmatia Biotech. *Micrococcus lysodeikticus* has been obtained from Sigma (M3770). All activities were measured using a Wallac 1480 "WIZARD 3" sodium iodide counter. The protein concentrations were measured on a Shimadzu UV-1601 spectrophotometer.

Labeling procedures

Optimization: For fair comparison of the different labeling methods the optimum combination of the incubation time and the ratio between the amount of oxidizing agent and protein were determined.

Iodogen and Chloramine-T methods: Both labelings were performed by mixing 100 μl of the protein solution ([protein] = 5 mg/ml in Tris buffer), 50 μl of $Na^{125}I$ solution, and 20 μl of Tris buffer or inactive NaI solution in a small Eppendorf cup. In the case of Chloramine-T labeling, 10 μl of Chloramine-T solution ([Chloramine-T] = 2 mg/ml) was also added to the mixture and the total reaction volume was approximately 180 μl. Exact volumes were determined by weighing. For the Iodogen labeling a solution of Iodogen in chloroform ([Iodogen] = 2 mg/ml) was evaporated in the Eppendorf cup before adding the reaction solutions of total volume of about 170 μl. The solution is allowed to stand for 30 minutes after mixing by gentle shaking.

To reduce the probability of protein denaturation the procedure is run on ice. Finally 300 μl of sodium metabisulfite solution ($[Na_2O_5S_2] = 1$ mg/ml in H_2O) is added to stop the radioiodination reaction.

Iodo Beads method: Put one Iodo Bead into the ^{125}I buffered solution (70 μl) and let stand for 5 minutes at room temperature. Add 100 μl of the protein solution ([protein] = 5 mg/ml in Tris buffer) and incubate for 10 minutes. The iodination process was stopped by separation of the Iodo Bead from the reaction volume, which was around 170 μl.

Separation from free iodide

After labeling the radiolabeled proteins were separated from free iodine by use of a PD 10 desalting column. Before use the column is equilibrated with 25 ml Tris buffer. The solution containing the free iodide and the labeled protein is applied onto the column and the mixture is eluted with Tris buffer. Fractions of about 0.5 ml are collected. Figure 1 shows a typical elution profile for the separation of free iodide and radiolabeled protein.

Enzymatic activity test of LSZ

The rate of lysis of *Micrococcus lysodeikticus* using turbidity measurements was determined.[13] Native and radiolabeled LSZ were dissolved at a concentration of 150–500 units/ml with reagent grade water. Pipette 2.9 ml of *Micrococcus lysodeikticus* cell suspension ([*Mic lys*.] = 0.3 mg/ml in Tris buffer pH 7.5) into a cuvette and after adding 0.1 ml of dissolved LSZ record the changes in A_{450} (absorbance at wavelength 450 nm) per minute from the initial linear part of the curve. One unit of LSZ activity is equal to a decrease in turbidity of 0.001 per minute at 450 nm, pH 7.5 and 25 °C and the specific enzymatic activity of LSZ was calculated by:

$$\frac{\text{Units}}{\text{mg}} = \frac{1000 \cdot \Delta A_{450}/\text{min}}{\text{mg (enzyme in reaction mixture)}} \qquad (1)$$

Circular dichroism (CD) spectroscopy

The CD spectra were recorded on a JASCO (J-715) spectropolarimeter. The temperature was kept constant at 20±0.1 °C using a JASCO PTC-348WI thermostat. The spectra were scanned and then analyzed by Dicroprot 2000 software.[14]

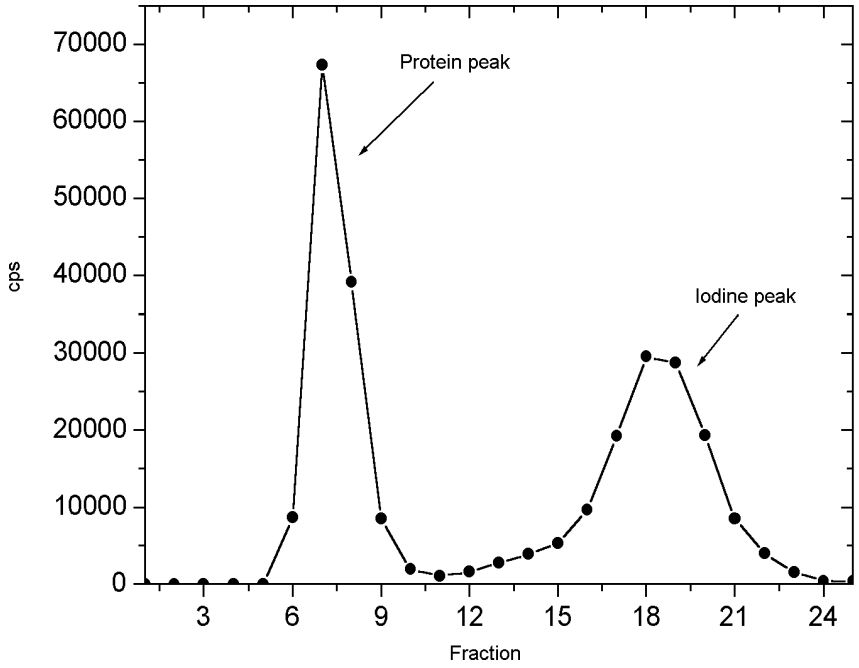

Fig. 1. Separation of free iodine and radioiodinated protein with a PD-10 column. Fractions of 0.5 ml were collected and activities of each fraction were measured

Results and discussion

Labeling procedure

For a better understanding of the process of incorporation of iodine into the protein molecules we have plotted "master" curves for each method and each type of protein. We define the "master" curve as the relative number of labeled protein molecules N_{pr*}/N_{pr} (labeling efficiency) versus the total number of radioactive and inactive iodine atoms (N_{I*}/N_I) in the reaction mixture. From a chemical point of view radioactive ^{125}I and inactive iodine are identical. Therefore, to study the high iodine concentrations only a small part of radioactive ^{125}I was used and the rest consisted of inactive iodine. The amount of labeled protein molecules after iodination (N_{pr*}) and the initial amount of proteins (N_{pr}) can be calculated by:

$$N_{pr*} = \frac{A_{pr}}{\lambda} \cdot \frac{N_I}{N_{I*}} \qquad (2)$$

$$N_{pr} = \frac{N_a m_{pr}}{M_{pr}} \qquad (3)$$

where A_{pr} [Bq] is the activity of the proteins after the iodination process, λ [s^{-1}] is the decay constant of ^{125}I, N_I/N_{I*} is the ratio of inactive and radioactive iodine. N_a [mol^{-1}] is the Avogadro's constant. The protein mass in the reaction mixture is m_{pr} [g] and the M_{pr} [Da] is its molecular weight. (M_{LSZ} = 14307 Da, M_{BSA} = 66430 Da).

The "master" curves for BSA and LSZ using three different labeling methods are presented in Fig. 2. The curves were fit with a linear function with an approximate slope 1 which justified our assumption that the labeling is a first order reaction. Table 1 gives the exact fitting results for the curves and the average of the fits for the three different procedures are presented as solid lines in Fig. 2 for both proteins. This means that Iodogen, Chloramine-T and Iodo Beads methods have a similar linear dependence of an increasing labeling efficiency with increasing amount of iodine atoms in the reaction mixture.

Table 1. Slopes from fitting results of master curves for each method and their average for LSZ and BSA (Fig. 2, solid lines)

Labeling method/ protein	LSZ	BSA
Chloramine-T	1.02 ± 0.01	1.02 ± 0.02
Iodo Beads	1.04 ± 0.03	1.03 ± 0.01
Iodogen	1.03 ± 0.01	1.06 ± 0.02
Average:	1.03 ± 0.01	1.06 ± 0.02

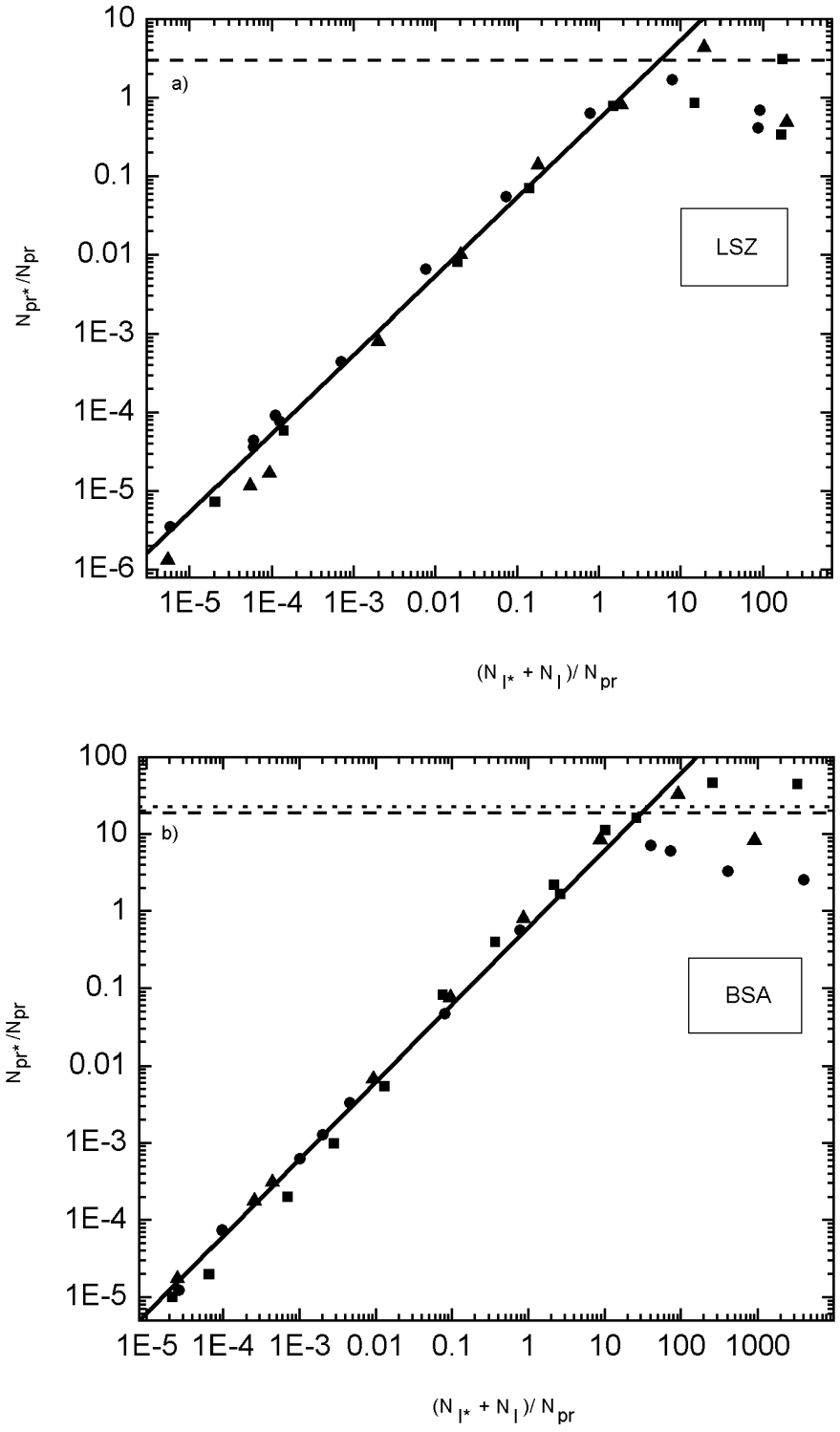

Fig. 2. Ratio of labeled proteins and the initial amount of protein molecules as a function of the total number of iodine atoms (inactive iodine plus active) in the reaction mixture (volume of reaction mixtures is given in "Labeling procedures") for LSZ (a) and BSA (b); ■ Iodogen method, ● Chloramine-T method, ▲ Iodo Beads method. The relative errors of the measurements were a few percent and are not visible in these graphs. The solid lines are the averages of the fits for the three different procedures. The dashed lines indicate the numbers of tyrosine (the most sensitive for iodine) residues in the proteins

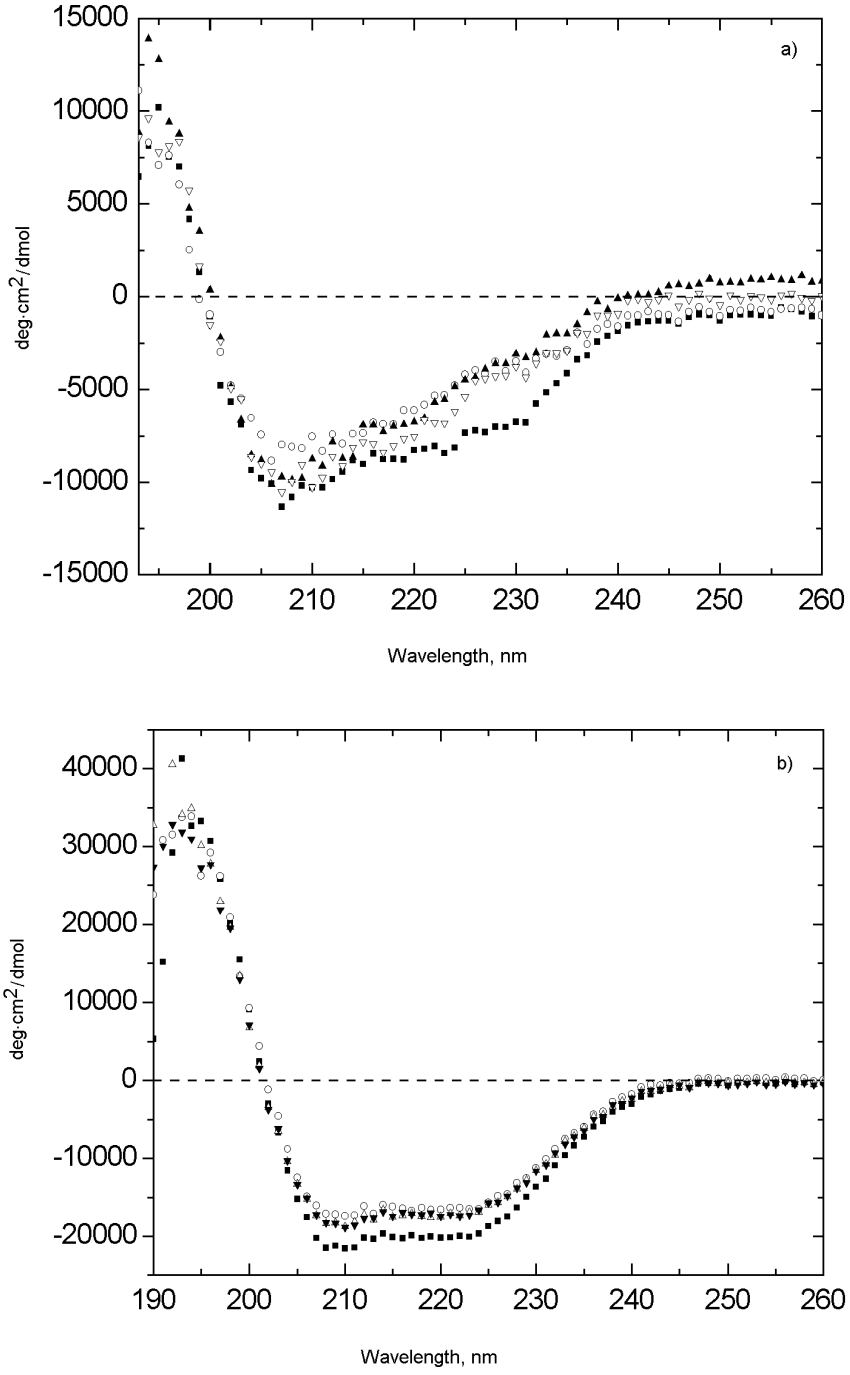

Fig. 3. CD spectra of the native proteins (■) LSZ (a) and BSA (b) compared to the corresponding spectra after using different labeling methods: ○ Chloramine-T, Δ Iodogen, ▼ Iodo Beads

Table 2. Secondary structures of BSA and LSZ in native state, labeled with Iodogen, Iodo Beads andChloramine-T

Structure	LSZ				BSA			
	Native	Iodogen	Iodo Beads	Chlora-mine-T	Native	Iodogen	Iodo Beads	Chlora-mine-T
α-Helix, %	28*	26	24	22	67	58	59	55
β-Sheet, %	20	22	23	25	7	8	8	9
β-Turns, %	18	18	19	19	12	13	13	13
Unordered, %	34	34	34	34	14	21	20	23

* In our experience, the relative error of the experiments within the same batch is a few percent.

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