Conformational changes in seventeen cystine disulfide bridges of bovine serum albumin proved by Raman spectroscopy

Koji Nakamura[a], Seiichi Era[a,*], Yukihiro Ozaki[b], Masaru Sogami[c], Tomoya Hayashi[a], Masataka Murakami[d]

[a]*Department of Physiology, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500, Japan*
[b]*Department of Chemistry, School of Science, Kwansei Gakuin University, 1-1-155 Uegahara, Nishinomiya 662, Japan*
[c]*Gifu University, 1-1 Yanagido, Gifu 501-11, Japan*
[d]*Department of Molecular Physiology, National Institute for Physiological Sciences, 38 Myodaiji, Okazaki 444, Japan*

Received 22 September 1997; revised version received 13 October 1997

Abstract Conformational changes in cystine disulfide bridges of bovine serum albumin during acid-induced isomerization (N→F and F→E transitions) have been studied with Raman spectroscopy. In an X-ray crystallographic study of human serum albumin, Carter and Ho reported that all disulfide bridges of the albumin molecule are in the *gauche-gauche-gauche* conformation [1]. On the other hand, the solution structure of bovine serum albumin examined by Raman spectroscopy differs from its crystal structure in the conformation of some of the disulfide bridges. Two Raman bands were detected at 520 and 505 cm^{-1} in the disulfide stretching mode region, suggesting that the 17 disulfide bridges in the N-form of bovine serum albumin solution take both the *gauche-gauche-gauche* and *gauche-gauche-trans* conformations. The ratio of the peak intensities at 520 and 505 cm^{-1} (I_{505}/I_{520}) is increased from 1.6 to 2.1 and from 2.1 to 6.3 on going from the N- to the F-form and from the F- to the E-form, respectively, indicating that the *gauche-gauche-trans* conformation of the disulfide bridges is converted to a *gauche-gauche-gauche* one which is the most energetically stable form during the acid-induced isomerization. However, small amounts of *gauche-gauche-trans* conformation still remain even in the E-form.

Key words: Acid-induced isomerization; Bovine serum albumin; Disulfide bridge conformation; Raman spectroscopy

1. Introduction

Serum albumin is the most abundant protein in mammalian plasma and has been the subject of many investigations because of its easy isolation in large quantities, its high stability and its solubility. Albumin is the principal protein contributing to the colloid osmotic pressure of the blood and also the transport protein for numerous endogenous and exogenous compounds [2]. This molecule, having a molecular mass of 66 kDa, consists of a single polypeptide chain containing about 580 amino acids [2]. Bovine serum albumin (BSA) undergoes remarkable reversible changes in conformation, usually under non-physiological conditions. In the pH range from 4.40 to 2.80, the reversible conformational changes occur in two steps [3]. The first step, between pH 4.40 and pH 3.75 (0.10 M KCl), is called the N→F transition, while the second step, between pH 3.60 and pH 2.80 (0.10 M KCl), is called the F→E transition [3]. Physicochemical techniques such as intrinsic viscosities [4], effective radii obtained by moving boundary electrophoresis [5,6], helical contents [7–10], tryptophyl fluorescence spectra [11], rotational correlation times of labeled spin-probe [12] etc., showed the two-step changes corresponding to N→F and F→E transitions (acid-induced isomerization). The physiological significance of these transitions, especially that of the N→F transition, may be suggested by the conservation of this transition among various species (human, bovine, rat [13] and turkey [1]).

Three-dimensional protein structure is influenced and stabilized to a great extent by cystine disulfide bridges. Serum albumin has one cysteinyl residue at position 34 (Cys-34) and 17 disulfide bridges per molecule [2]. The unique feature of albumin structure is the 17 disulfide bridges pattern. A feature to this pattern is the presence of two neighboring cysteinyl residues (disulfide pairing) throughout the sequence and these disulfide pairings are located between helical segments [1,2].

Raman spectroscopy has been employed extensively to probe the conformation of disulfide bridges in proteins and peptides because the frequency of an S-S stretching mode of the disulfide bridge is sensitive to its conformation [14]. Raman bands at ~510, ~525 and ~540 cm^{-1} due to the S-S stretching mode can be ascribed to *gauche-gauche-gauche* (*ggg*), *gauche-gauche-trans* (*ggt* or *tgg*) and *trans-gauche- trans* (*tgt*) configurations of the Cβ-S-S′-Cβ′ disulfide bridges, respectively [15,16]. Raman spectra of BSA have already been investigated in some detail by several research groups [17–21], and it was reported that only a single Raman band was observed near 510 cm^{-1} in the S-S stretching band region. Based upon this observation it was concluded that the 17 disulfide bridge conformation of BSA can only be a *ggg* conformer [20,21]. In addition, recent X-ray crystallographic studies of human serum albumin (HSA) [1] are undoubtedly in good agreement with the conclusion from Raman spectroscopy [20,21].

In the present study, the conformation of BSA in solutions has been investigated by Raman spectroscopy in order to explore whether the conformation of 17 disulfide bridges is indeed only a *ggg* conformer and whether conformational changes occur during acid-induced isomerization.

*Corresponding author. Fax: (81) (58) 267-2962.
E-mail: era@cc.gifu-u.ac.jp

Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; *ggg*, *gauche-gauche-gauche*; *ggt*, *gauche-gauche-trans*; HSA, human serum albumin; *tgt*, *trans-gauche-trans*

PII S0014-5793(97)01326-4

2. Materials and methods

Crystallized BSA (lot Y74806) was purchased from Armour Pharmaceutical Co. and used without further purification. For pH study, BSA solution had an ionic strength of 0.10 M NaCl. BSA solution was filtered through a Milex HV filter (0.45 μm) to minimize Tyndall scattering on recording the Raman spectra. All other chemicals employed were of reagent grade.

The Raman measurements were carried out using a Jasco R-800 laser Raman spectrometer at ambient temperature. The 514.5 nm line from an argon ion laser (stabilite 2017, Spectra Physics Co.) was used for excitation, and the laser power at the sample position was 150–400 mW. An average of four or eight scans was collected for each spectrum. Data acquisition was performed with a Hewlett Packard 9122 personal computer. The obtained spectra in the 550–470 cm^{-1} region were subjected to numerical curve fitting (Grams/386; Galactic Ind. Co.); the band shapes were approximated by a Gaussian function. The baseline was approximated by a straight line between two points at 550 and 480 cm^{-1}, chosen at both sides of the band envelope.

Concentration of BSA was determined with a Hitachi 320 spectrophotometer assuming E (1%, 1 cm) at 279 nm to be 6.67. Concentration was 2.50% for the series of pH experiments. The pH measurements were made with a Hitachi-Horiba F-7SS, equipped with an expanded scale, using a Radiometer GK-2401C combined electrode.

3. Results and discussion

Fig. 1 shows the Raman spectrum of a BSA solution (2.50%, 0.10 M NaCl) at pH 5.0 (the N-form). The observed frequencies of Raman bands are almost identical to those reported previously [17–21], and assignments for the bands have already been made in detail [19,20]. Serum albumin has one cysteine residue and 17 disulfide bridges, and they play important roles in the intramolecular sulfhydryl-disulfide exchange reaction (*N-A* isomerization, molecular aging of BSA) [3,10,22–24]. To obtain qualitative information about these disulfide bridges conformation is a matter of great importance. Of note in Fig. 1 is that cystine disulfide stretching vibrations of BSA generate a strong Raman band at 508 cm^{-1} with a weak shoulder at 517 cm^{-1}. A relative intensity of the two bands at 517 and 508 cm^{-1} may be estimated by curve fitting of the experimental band profile in the 550–470 cm^{-1} region.

Fig. 2 shows results for the curve fitting in the region of 550–470 cm^{-1} for the Raman spectra of the N- (pH 5.29), F- (pH 3.78) and E-forms (pH 2.60) of BSA. After the curve fitting of the band profile to the minimum number of Gaussian components, we were able to identify two disulfide stretching bands at ~520 and ~505 cm^{-1}. In the previously reported papers, only a single Raman frequency was reported in the 550–500 cm^{-1} region, and it was concluded that the 17 disulfide bridges of BSA can only assume a *ggg* conformer [20,21]. As described above, the Raman spectrum of BSA solution (0.10 M NaCl) at pH 5.0 (Fig. 1) and their Raman frequencies are similar to those reported previously [17–21]. However, on the high-frequency side of the 505 cm^{-1} band, we successfully observed the weak feature near 520 cm^{-1}, assignable to a disulfide stretching mode of a *ggt* (or *tgg*) conformer by the curve fitting for the experimental band profile. Thus, it may be concluded that the 17 disulfide bridges in the N-form of the BSA solution structure assume mainly the *ggg* conformer but also take the *ggt* (or *tgg*) conformation. The ratio of peak intensities at 520 and 505 cm^{-1} (I_{505}/I_{520}) in the N-form (pH 5.29) of BSA is 1.6, indicating that about 10 out of 17 disulfide bridges of the molecule take *ggg*, the re-

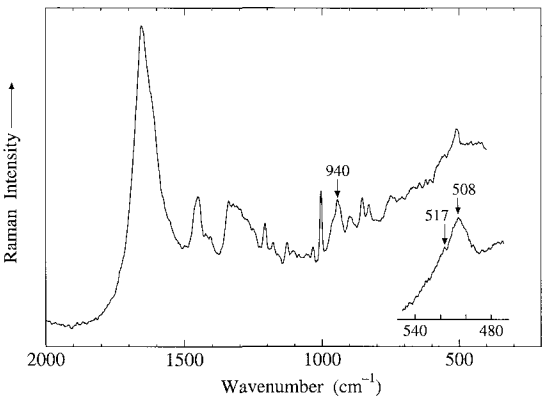

Fig. 1. A Raman spectrum (2000–470 cm^{-1}) of BSA (2.50%) in 0.10 M NaCl at pH 5.0 (the N-form).

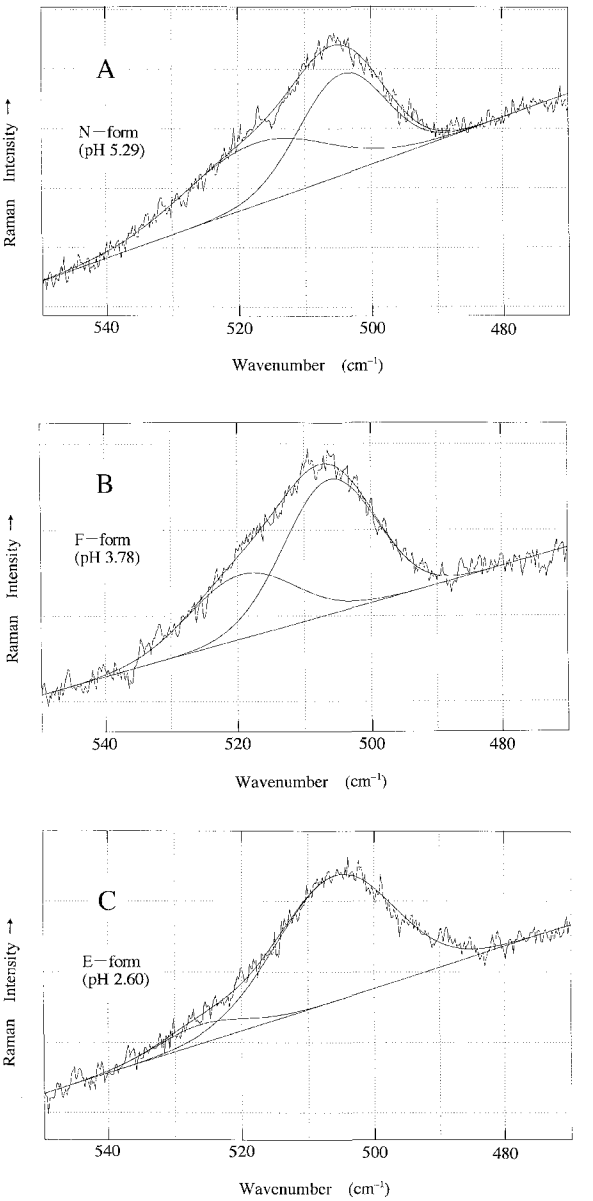

Fig. 2. Curve fitting for the cystine disulfide band profile in the region of 550–470 cm^{-1} of the Raman spectra of BSA (2.50%) in 0.10 M NaCl. A: N-form (pH 5.29). B: F-form (pH 3.78). C: E-form (pH 2.60).

maining ones take the *ggt* (or *tgg*) conformer, and no *tgt* conformer is presented in the N-form of BSA. Of course, it is rather difficult to estimate the exact numbers of *ggg* and *ggt* (or *tgg*) conformers in the protein because the Raman scattering intensity of the S-S stretching band of each conformer may be different and the results of the curve fitting have some ambiguity. With the decrease in pH of the BSA solution, the value for I_{505}/I_{520} increased from 1.6 to 2.1 and from 2.1 to 6.3, as shown in Fig. 2A,B,C. It is therefore very likely that the *ggt* conformation of the disulfide bridges in the N-form is converted to the *ggg* one during acid-induced isomerization.

According to the X-ray crystallographic data [1], the albumin molecule is composed of three homologous domains (I, II and III) and each domain is the product of two subdomains, which are predominantly helical. About 67% of the albumin structure is helical, ~23% is in an extended chain conformation, and ~10% is in turn [1]. Therefore, the albumin molecule is predominantly α-helix-rich protein as examined by various spectroscopic [7–10] and X-ray crystallographic [1] methods.

As already mentioned, the 17 disulfide bridge pattern is characterized by the presence of two neighboring cysteinyl residues (disulfide pairing) throughout the sequence and these disulfide pairings are located between the helical segments (subdomains). This property not only makes the albumin molecule very compact but is also responsible for its stability. From the home-made three-dimensional helical model (Maruzen Co., Japan) of BSA based on X-ray crystallographic data [1] and the complete amino acid sequence [2], quite surprisingly, one can find that six disulfide bridges (Cys-53·Cys-62 and Cys-167·Cys-176 in domain I; Cys-244·Cys-252 and Cys-359·Cys-368 in domain II; Cys-436·Cys-447 and Cys-557·Cys-566 in domain III) are located in the very short turns between the helical subdomains. Furthermore, disulfide pairings such as Cys-168 next to Cys-167 in domain I, Cys-245 next to Cys-244, Cys-360 next to Cys-359 in domain II and Cys-437 next to Cys-436, Cys-558 next to Cys-557 in domain III are located within these short turns in each domain. These peculiar disulfide bridges restrict the potential packing arrangement between the subdomains. Based on this consideration, together with the present Raman result, it may be concluded that about six disulfide bridges, which are noted above, adopt the energetically unfavorable *ggt* (or *tgg*) conformation in the N-form of BSA. The N→F transition can be interpreted as the isomerization of domains I and II, without loss of helix content, and the unfolding of domain III in the cooperative process [25–28]. Therefore, the conformation of two disulfide bridges in domain III may convert from *ggt* (or *tgg*) to *ggg* conformers during the N→F transition. Finally, the value for I_{505}/I_{520} in the E-form of BSA becomes 6.3, indicating that most disulfide bridges in the E-form of the BSA solution structure are in the *ggg* conformation, the most energetically stable disulfide conformation. However, small amounts of *ggt* (or *tgg*) conformation still remain, even in the E-form (pH 2.60).

According to the result obtained with circular dichroism (CD)-resolved secondary structure of BSA previously reported by us [8], the helical contents of the N-, F- and E-forms of BSA solution are 72, 62 and 52%, respectively. It follows that BSA molecules still have a highly helical content even in the E-form. A Raman band of BSA, which serves as an indicator for the α-helix structure, is identified at 940 cm^{-1} (the skeletal C-C stretching vibration). The strong intensity of this marker is indicative of a high α-helix content [14]. The intensity decrease in this band during acid-induced isomerization is consistent with the CD results [8] (data not shown).

From the sets of the present Raman results and CD-resolved secondary structural changes of BSA [8], one can speculate that acid-induced isomerization, i.e. the N→F and F→E transitions, corresponds to the loss of the intradomain helices associated with the sequential domain-domain and/or subdomain-subdomain dissociations, and some of the 17 disulfide bridges undergo a conformational change from *ggt* to *ggg* conformers during acid-induced isomerization.

Recently, recombinant serum albumin has been achieved by gene expression in yeast [29–31], in order to serve as a serum replacement product. Seventeen disulfide bridges of serum albumin undoubtedly stabilize the structure of this molecule very effectively and its conformation is unusually stable under a variety of harsh conditions.

What is needed now is knowledge of the three-dimensional structure of the recombinant serum albumin such as the disulfide bridge conformation, by various physicochemical and X-ray crystallographic techniques.

Acknowledgements: The authors are grateful to Professor M. Mimuro (Faculty of Science, Yamaguchi University, Yamaguchi, Japan) for his valuable advice on the use of the Raman spectrometer. The authors wish to thank Ms. Y. Makino (National Institute for Basic Biology, Okazaki, Japan) for her technical assistance in this work and Ms. J. Hayashi in this laboratory for her kind help in preparing the manuscript.

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