

# Laser-Raman Spectra, Sulfhydryl Groups, and Conformation of the Cystine Linkages of $\beta$ -Lactoglobulin

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## INTRODUCTION

Laser-Raman spectra of  $\beta$ -lactoglobulin have been reported by several authors,<sup>1,2</sup> but the region where S-H stretching vibrations are expected has not been examined. S-H stretching bands of cysteine residues have been observed around  $2580\text{ cm}^{-1}$  for eye lens proteins, which are relatively rich in cysteine residues, and have been used to measure the sulfhydryl content of the intact lens.<sup>3,4</sup> Native bovine  $\beta$ -lactoglobulin A occurs as a dimer with a molecular weight of 36,724, composed of two identical spherical monomers, each of which has only one free sulfhydryl group and two cystine bridges.<sup>5</sup> We investigated the S-H stretching region of this protein to ascertain if Raman spectroscopy can be used to determine the presence and follow the reactions of sulfhydryl groups in proteins with a very low cysteine content.

The high-resolution x-ray crystallographic structure of  $\beta$ -lactoglobulin has not been determined, but it is known from circular dichroism studies<sup>6</sup> and from infrared studies<sup>7</sup> that it has a relatively high content of antiparallel chain pleated-sheet structure ( $\beta$ -structure) for a globular protein, ca. 45–50%.<sup>6,7</sup> X-ray data at 0.6-nm resolution<sup>8</sup> indicate that the free sulfhydryl group is near the surface of the molecule, but precise information on the backbone structure and the conformation of the cystine linkages is lacking. New information concerning the conformation of the two disulfide bridges<sup>5</sup> would therefore substantially add to an overall understanding of the structure of this protein. Such information can be obtained by a careful study of the S-S and C-S stretching frequencies which occur in the  $500\text{--}700\text{-cm}^{-1}$  spectral region.<sup>9–16</sup> The Raman data available in the literature are not sufficiently detailed for such a study. We therefore reinvestigated the Raman spectra of  $\beta$ -lactoglobulin in this spectral region as well. The results suggest that both cystine bridges have a *gauche-gauche-gauche* conformation.

## MATERIALS AND METHODS

$\beta$ -lactoglobulin A was a three-times recrystallized and lyophilized product prepared in this laboratory from the milk of homozygous A/A cows by the method of Aschaffenburg and Drewry.<sup>17</sup> The preparations were treated with 10 mM EDTA prior to recrystallization. Protein solutions for measurements in  $\text{H}_2\text{O}$  were prepared one day before use and stored overnight at  $4^\circ\text{C}$ . To prepare deuterated samples, a suitable amount of crystalline protein was allowed to equilibrate repeatedly for 24-h periods as a slurry with a small quantity of  $\text{D}_2\text{O}$  in a stoppered vial at  $4^\circ\text{C}$ ,

followed by high-speed centrifugation and addition of fresh D<sub>2</sub>O, for a total of three exchanges. The solutions were prepared by direct addition of solid Na<sub>2</sub>HPO<sub>4</sub> and KCl; the pD was adjusted by addition of 0.1N NaOD in D<sub>2</sub>O. Concentrations of the protein were determined spectrophotometrically using an absorptivity value of 0.96 mL mg<sup>-1</sup> cm<sup>-1</sup> at 278 nm.<sup>18</sup>

15.5% solutions (w/w) at pH 6.8 in 0.04M phosphate and 0.2M KCl were placed in 5-mm-o.d. nmr tubes to obtain Raman spectra on a Spex Ramalog System equipped with a model 1401 monochromator. The 514.5-nm line of a Spectra Physics model 165-03 argon ion laser was used for excitation. The laser power at the sample was ca. 250 mW and the spectral slit width was 5 cm<sup>-1</sup>.

## RESULTS AND DISCUSSION

Figure 1 shows the Raman spectrum of  $\beta$ -lactoglobulin A at pH 6.8 in the 2500–3100-cm<sup>-1</sup> spectral region. The S–H stretching band, observed at 2570 cm<sup>-1</sup>, is also shown at 10 times higher amplification. Its relative intensity can be estimated by comparison with the C–H stretching bands centered around 2925 cm<sup>-1</sup>. The S–H band is caused by the single cysteine residue at the 119 or the 121 position.<sup>5,8</sup> Inserts A and B of Fig. 1 show that the band is totally polarized. Insert C gives the S–D stretching band of deuterated  $\beta$ -lactoglobulin A, which is observed at 1865 cm<sup>-1</sup> in D<sub>2</sub>O solution at pD 6.8. The isotopic shift  $\nu(\text{SH})/\nu(\text{SD}) = 1.378$  confirms the assignment. (The theoretical ratio for an idealized pure SH/SD mode would be 1.393, about 1% higher than the observed value.) The disappearance of the S–H band upon deuteration cannot be observed because in D<sub>2</sub>O solution the 2600 region is obscured by O–D stretching bands, but the near-theoretical isotopic shift appears to be sufficient to corroborate the assignment of the 2570-cm<sup>-1</sup> and

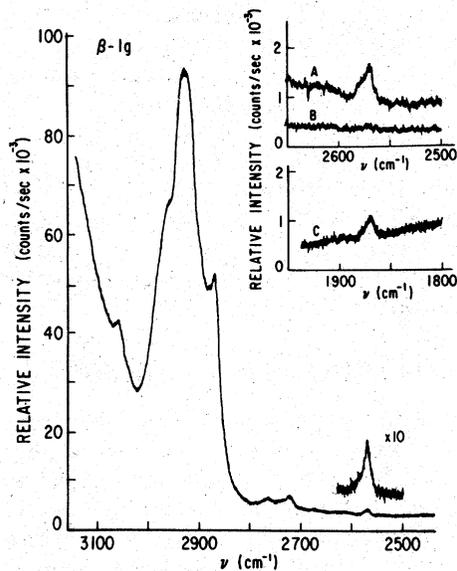


Fig. 1. Laser Raman spectrum of  $\beta$ -lactoglobulin A in the 2500–3100-cm<sup>-1</sup> region. Insert A: 2570-cm<sup>-1</sup> S–H stretching band as observed with parallel polarization. Insert B: 2500–2600-cm<sup>-1</sup> region observed with perpendicular polarization. Insert C: S–D stretching band of deuterated  $\beta$ -lactoglobulin at 1865 cm<sup>-1</sup>.

the 1865-cm<sup>-1</sup> bands to S-H and S-D stretching vibrations, respectively. The S-H band is also very close to the corresponding band in eye lens proteins.<sup>3,4</sup> The data presented in Fig. 1 thus indicate that Raman spectroscopy can indeed be used to estimate and monitor the sulfhydryl content of proteins containing very few cysteine residues—in the case of  $\beta$ -lactoglobulin, one out of 162.<sup>5</sup>

Figure 2 shows the Raman spectrum of  $\beta$ -lactoglobulin A from 470 to 700 cm<sup>-1</sup>. The medium-intensity band at 508 cm<sup>-1</sup> and the very weak band at 537 cm<sup>-1</sup> could be assigned to S-S stretching modes of the two cystine linkages<sup>5</sup> by comparison with previous work on related molecules.<sup>1,2,9-16</sup> There is general agreement that the S-S stretching frequency does not depend on the C-S-S-C dihedral angle  $\chi_1$  if the angle is larger than 60°,<sup>9,10,16</sup> despite some earlier controversy on the subject.<sup>13-15</sup> Normal coordinate calculations,<sup>12</sup> based on a Urey-Bradley force field, suggest that the S-S stretching frequency is nearly independent of the dihedral angle over all possible values of  $\chi_1$ . In any case, crystallographic evidence shows that in cystine linkages of proteins the angle  $\chi_1$  is always larger than 60°,<sup>14,19</sup> and vibrational spectroscopy of model compounds establishes the stable conformation about the S-S bond of disulfide groups as *gauche*, with a dihedral angle of nearly 90°. <sup>9,10</sup> According to a survey of x-ray evidence, the great majority of cystine linkages (C-CH<sub>2</sub>-S-S-CH<sub>2</sub>-C) in proteins are in a *gauche-gauche-gauche* (“left-handed spiral” or “right-handed hook”), or a *trans-gauche-trans* extended conformation.<sup>19</sup>

The S-S stretching frequency does strongly depend on the dihedral angles  $\chi_2$  and  $\chi_2'$  about the C-S bonds of the C-CH<sub>2</sub>-S-S-CH<sub>2</sub>-C linkage.<sup>9-12</sup> This dependence has been used by Nakanishi et al. to evaluate the conformation of the cystine linkages in bovine  $\alpha$ -lactalbumin.<sup>11</sup> We follow a similar reasoning.

Sugeta et al. have suggested that the S-S stretching frequency lies around 510 cm<sup>-1</sup> for a *gauche-gauche-gauche* conformation, around 525 cm<sup>-1</sup> for a *trans-gauche-gauche* conformation, and around 543 cm<sup>-1</sup> for *trans-gauche-trans* conformations.<sup>9,10</sup> In  $\alpha$ -lactalbumin there are four cystine linkages.<sup>5</sup> By analogy with lysozyme (which has a known crystallographic structure<sup>20</sup> and a primary structure very similar to  $\alpha$ -lactalbumin<sup>21</sup>), Nakanishi et al.<sup>11</sup> conclude that in  $\alpha$ -lactalbumin three of the cystine bridges have two *gauche* C-S bonds each, but in the fourth, the conformation must be different. Indeed a Raman band is found at 507 cm<sup>-1</sup> and another one at 540 cm<sup>-1</sup>. The intensity ratio is very roughly 3:1. The authors

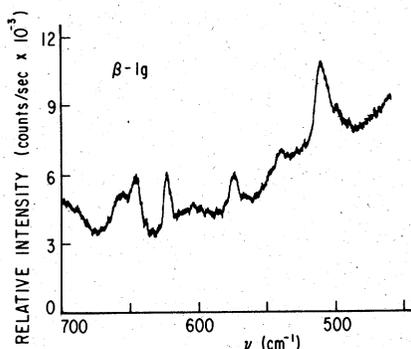


Fig. 2. Laser Raman spectrum of  $\beta$ -lactoglobulin A in the 470–700-cm<sup>-1</sup> region.

conclude that the 507-cm<sup>-1</sup> band is caused by three bridges with two *gauche* C-S bonds each and the 540 cm<sup>-1</sup> band by one linkage with two *trans* C-S bonds. These observations strongly suggest that (a) cystine linkages in proteins with different C-S dihedral angles are distinguishable by Raman spectra and (b) the intensities of the S-S stretching bands for the *gauche-gauche-gauche* and *trans-gauche-trans* conformations are approximately equal.

In  $\beta$ -lactoglobulin we have a relatively strong band at 507 cm<sup>-1</sup> and a very weak one at 537 cm<sup>-1</sup>, as shown in Fig. 2. If one of the two cystine linkages were *gauche-gauche-gauche* and the other *trans-gauche-trans*, we would expect two bands of roughly comparable intensity. This evidently is not the case. We therefore conclude that both cystine linkages are in a *gauche-gauche-gauche* conformation and that the very weak 537-cm<sup>-1</sup> band is caused by tryptophan residues, as previously suggested for lysozyme and  $\alpha$ -chymotrypsin.<sup>11,22</sup>

The conclusion that both cystine linkages in  $\beta$ -lactoglobulin are in a *gauche-gauche-gauche* conformation is supported by an investigation of the C-S stretching bands. In simple models *gauche-gauche-gauche* conformations give rise to C-S frequencies around 640 cm<sup>-1</sup>, *trans-gauche-gauche* conformations around 670 cm<sup>-1</sup>, and *trans-gauche-trans* conformations presumably at still higher frequencies.<sup>10</sup> In  $\beta$ -lactoglobulin there are bands at 645 and 655 cm<sup>-1</sup>. We assign the 645-cm<sup>-1</sup> band to *gauche-gauche-gauche* C-S stretching vibrations and the 655-cm<sup>-1</sup> band to tyrosine residues.<sup>1,2</sup> Alternatively, the 645- and 655-cm<sup>-1</sup> bands could be assigned to coupled in-phase and out-of-phase C-S stretching modes of the *gauche-gauche-gauche* conformation, but there is no basis for such distinction in the spectra of small model compounds.<sup>10</sup> To sum up, the bands between 500 cm<sup>-1</sup> and 700 cm<sup>-1</sup> in the Raman spectra of  $\beta$ -lactoglobulin can be assigned as follows. 507 cm<sup>-1</sup>: S-S stretching, *g-g-g*, both linkages<sup>11</sup>; 537 cm<sup>-1</sup>: tryptophan residues<sup>11,22</sup>; 574 cm<sup>-1</sup>: tryptophan residues<sup>1,2,22</sup>; 622 cm<sup>-1</sup>: phenylalanine residues<sup>1,2</sup>; 645 cm<sup>-1</sup>: C-S stretching, *g-g-g*, both linkages<sup>10</sup>; and 655 cm<sup>-1</sup>: tyrosine residues.<sup>1,2</sup> The C-S bonds of methionine residues give rise to appreciably higher stretching frequencies<sup>1,22</sup> and do not interfere with our analysis.

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