FT-IR study of the Ca^{2+}-binding to bovine α-lactalbumin

Relationships between the type of coordination and characteristics of the bands due to the Asp COO^- groups in the Ca^{2+}-binding site

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Abstract Fourier-transform infrared spectroscopy (FT-IR) was applied to examine relationships between the type of coordination and the COO^- antisymmetric and symmetric stretches of the COO^- groups in the Ca^{2+}-binding site of bovine α-lactalbumin. The peaks at 1593, 1578, 1425, and 1403 cm^{-1} were assigned to the COO^- groups of Asp-82, 87, and 88 coordinating to Ca^{2+} in the pseudo bridging mode, according to the results of X-ray crystallography. The bands due to the COO^- groups were quite similar to each other between α-lactalbumin and EDTA which is the model compound for the pseudo bridging state.

Key words: α-Lactalbumin; Ca^{2+}-binding protein; FT-IR spectroscopy; Coordination type

1. Introduction

The COO^- groups can coordinate to metal ions in a number of ways, i.e. unidentate, bidentate, bridging, and pseudo bridging modes. When the metal ion interacts with only one oxygen atom of the COO^- group, the system is regarded as the unidentate form. In the bidentate form, the metal ion interacts equally with the two oxygen atoms of the COO^- group. In the bridging coordination mode, one metal ion is bound to one of the two oxygens in the COO^- group and another metal ion to the other oxygen atom. If one of these metal ions is replaced by a hydrogen atom of a water molecule, the system is in the pseudo bridging mode. Extensive infrared studies have been made on the relationships between the COO^- stretching frequencies and the types of coordinations [1,2]. Deacon and Phillips [1] have found a general tendency in the relationship between $\Delta\nu_{a-s}$ (frequency separation between the COO^- antisymmetric and symmetric stretching vibrations) and the types of coordination of the COO^- group to metal ions by examining the structures and vibrational frequencies observed for a number of acetate salts in the solid state. It was clarified by ab initio molecular orbital calculations that such correlation is related to changes in the CO bond lengths and the OCO angle [3].

The COO^- antisymmetric stretching vibrations of the glutamyl side-chain give rise to a band at 1567 cm^{-1} in D_2O solutions [4]. In the second derivative and Fourier-self deconvolved spectra for the Ca^{2+}-bound pike parvalbumin, a strong band found at 1553 cm^{-1} reflects the bidentate coordinations of the COO^- groups of Glu residues in the Ca^{2+}-binding sites [5]. This assignment is supported by the X-ray analysis of the Ca^{2+}-bound form of pike parvalbumin. The band due to the COO^- antisymmetric stretching vibration shifts from 1567 cm^{-1} to 1553 cm^{-1} as a result of the coordination of the COO^- groups of Glu residues to calcium ions in the bidentate mode.

α-Lactalbumin possesses a high-affinity Ca^{2+}-binding loop that consists of a 3_{10}-helix and an α-helix with a short turn region separating the two helices [7,8]. The Ca^{2+}-binding sites in the crystal structures of baboon, human, guinea pig, goat and bovine α-lactalbumin are essentially identical in terms of both conformation and ligand coordination. The Ca^{2+} is coordinated by seven oxygen atoms that form a slightly distorted pentagonal bipyramid. These oxygens are supplied by the main chain (Lys-79 and Asp-84) carbonyl groups, side-chain (Asp-82, 87, and 88) carboxyl groups and two water molecules [7–10]. In solution, the COO^- groups of Asp-82, 87, and 88 in the Ca^{2+}-binding site are accessible to water molecules, so it is reasonable to consider that these side-chain COO^- groups coordinate to Ca^{2+} in the pseudo bridging mode, where a divalent metal cation is bound to one of the two oxygens in the COO^- group and a water molecule is hydrogen-bonded to the other oxygen.

Although there are certain similarities between the typical EF-hand motif and the Ca^{2+}-binding loop of α-lactalbumin which has been referred to in literature as the α-lactalbumin elbow, distinct differences have been found from the X-ray analysis [7]. ^{113}Cd NMR study as well as ^{43}Ca NMR have suggested that the geometry of the EF-hand motif is different from that of the α-lactalbumin elbow [11,12].

Because FT-IR spectra are composed of many overlapping bands that have inherently large half-bandwidths, resolution enhancement methods such as Fourier-self deconvolution, curve fitting, second derivative analysis, and difference spectroscopy are usually necessary to detect spectral changes.

In the present article, we study the FT-IR spectra of bovine α-lactalbumin for the purpose of establishing the correlation between the COO^- stretching frequencies of Asp side-chains and the type of coordination, namely the pseudo bridging mode. We also investigate the FT-IR absorption spectra of EDTA to confirm the information obtained from the spectra of bovine α-lactalbumin.

2. Materials and methods

Bovine α-lactalbumin was prepared by the methods described previously [13]. Guaranteed reagent grade disodium dihydrogen ethylene-

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diamine tetraacetate dihydrate was obtained from Kanto Chemical Co. and used without further purification. Suprapur sodium chloride and calcium chloride were from E. Merck. The Ca^{2+}-free form of bovine α-lactalbumin was obtained by chromatography on a Bio-Gel P-4 column equilibrated with 0.01 M HCl, followed by lyophilization. Complete deuteration of the NH groups of the main chain is required to examine the region of the COO^- antisymmetric stretching vibration (1610–1550 cm^{-1}), where the amide II band (the NH bending mixed with the CN stretching) gives rise to a strong absorption. The freeze-dried apo-bovine α-lactalbumin was dissolved in D_2O (99.8%) and incubated at 38°C for at least 60 min, long enough to ensure the complete exchange of any amide protons for deuterons. The solution was lyophilized after cooling at room temperature. Apoprotein solution was obtained by dissolving the powder of deuterated Ca^{2+}-free bovine α-lactalbumin in a D_2O solution containing 0.2 M NaCl and 20 mM Tris buffer (pH 7.3). No NH proton signals were observed in the ^1H NMR spectra of the deuterated proteins dissolved in D_2O solution. Therefore, the bands around 1580 cm^{-1} are not contributed by the absorption of unexchanged amide II. All pH values were not corrected for isotope effects. Sample solution for the Ca^{2+}-bound form of bovine α-lactalbumin was obtained by adding $CaCl_2$ stock solution to the Ca^{2+}-free protein solution described above. The concentration of this stock solution was established by atomic absorption spectroscopy (Hitachi 170-100). All reagents used in this study had been D_2O exchanged before use. The protein concentrations for the FT-IR measurements were 5.5 mM.

FT-IR measurements were carried out at room temperature on a JEOL JIR-7000 Fourier-transform infrared spectrophotometer equipped with a MCT detector (IR-DET 100) at 2 cm^{-1} resolution. To improve the signal to noise ratio, a total of 1000 sample scans and 1000 reference scans were taken for each spectrum, by using a shuttle device. Dry air was constantly pumped into the spectrophotometer to eliminate water vapor which absorbs into the spectral region of interest. About 0.013 ml of the protein sample was placed between two CaF_2 plates separated by a 0.015 mm thick Teflon spacer. FT-IR absorption spectrum of the buffer solution was collected for each spectrum in the same way and carefully subtracted from the sample spectrum. Second derivative, Fourier-self deconvolved and difference absorption spectra were calculated using the software supplied by JEOL. A (triangle)^2 apodizing function was used with deconvolving parameters 2σ and L given in the figure captions. The concentrations of the solutes and pathlengths were kept as constant as possible for each difference spectrum, but there were small variations that could affect the calculated difference spectra. To correct for these variations, the buffer subtracted spectra were normalized according to the total peak area in the 1610–1700 cm^{-1} or 1430–1480 cm^{-1} regions.

3. Results

For the purpose of our investigation described above, we concentrated on the features of the bands in the region of the COO^- antisymmetric and symmetric stretches, since they are potentially useful for deriving information on the interaction between Ca^{2+} and the COO^- groups. Fig. 1 shows the second derivative and Fourier-self deconvolved spectra for Ca^{2+}-free and Ca^{2+}-bound form of bovine α-lactalbumin in D_2O (left) and H_2O (right) solutions. FT-IR spectra in H_2O solutions can give more reliable information in the region of the COO^- symmetric stretches around 1400–1420 cm^{-1}, since the amide II′ absorptions are overlapping in the spectra in D_2O solutions. The COO^- antisymmetric and symmetric stretching bands of the deconvolved spectra for bovine α-lactalbumin are enlarged and given in Fig. 2. Fourier-self deconvolved spectrum of Ca^{2+}-free bovine α-lactalbumin mainly shows two component bands at 1588 and 1578 cm^{-1} in the COO^- antisymmetric stretching region whose frequencies are identical with those of Ca^{2+}-bound form. However, the intensity of the band at 1578 cm^{-1} of the Ca^{2+}-bound form seems to be stronger than that of the Ca^{2+}-free form. The other apparent difference is the presence of the band at 1593 cm^{-1} in the

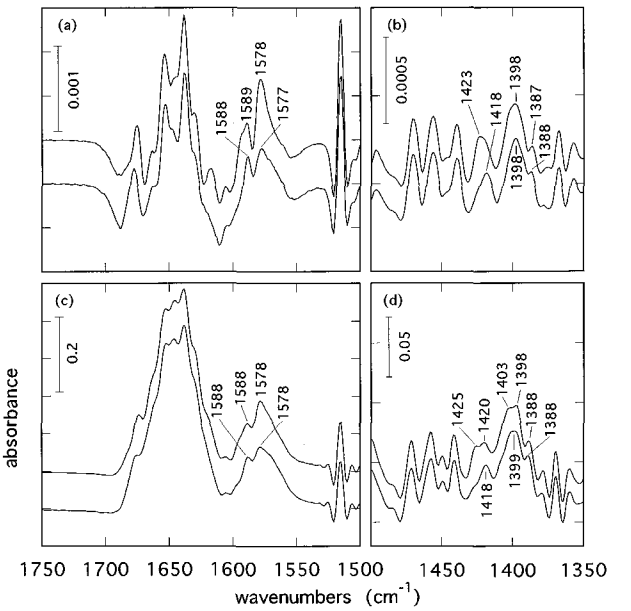

Fig. 1. Second derivative (a and b) and Fourier-self deconvolved (c and d) spectra of bovine α-lactalbumin in D_2O (a and c) and H_2O (b and d) solutions. (Top in each panel) Ca^{2+}-bound form in 20 mM Tris at pH 7.3 in the presence of 200 mM NaCl and 5.2 mM $CaCl_2$. (Bottom in each panel) Ca^{2+}-free form in 20 mM Tris at pH 7.3 in the presence of 200 mM NaCl. Second derivatives are multiplied by −1. Deconvolving parameters: 2σ, 16 cm^{-1}; L, 0.162 cm.

spectrum of the Ca^{2+}-bound form, not present in the spectrum of the Ca^{2+}-free form (Fig. 2a).

Marked differences are clearly seen in the region of the COO^- symmetric stretching vibration as shown in Fig. 2b. The most notable features in the deconvolved spectra of bovine α-lactalbumin are as follows: Ca^{2+}-binding results in the loss of the peak area at about 1388 cm^{-1} and gains of intensities around 1425 and 1403 cm^{-1} bands compared with Ca^{2+}-free bovine α-lactalbumin. These observations indicate that parts of the absorption around 1388 cm^{-1} in the spectrum of Ca^{2+}-free form shifts to higher wavenumbers with splitting into two components at 1403 and 1425 cm^{-1} by binding of Ca^{2+} to the COO^- groups in the high affinity Ca^{2+}-binding site.

These results are also observed in the second derivative spectra in Fig. 1, although the bands at 1593 and 1403 cm^{-1} of the Ca^{2+}-bound form are not clearly resolved.

FT-IR difference absorption spectra of bovine α-lactalbumin give essentially the same information obtained from the second derivative and deconvolved spectra (Fig. 3). Difference spectra were obtained by subtracting FT-IR absorption spectrum of the Ca^{2+}-free form from that of the Ca^{2+}-bound form. Thus, positive peaks in the difference spectrum correspond to gains of intensities in the absorption spectrum upon Ca^{2+} binding, and vice versa. In the difference spectrum, the binding of Ca^{2+} gives rise to positive bands at 1592 and 1578 cm^{-1} in the region of the COO^- antisymmetric stretching vibration (Fig. 3a). In the region of the COO^- symmetric stretch, the Ca^{2+}-binding results in a loss of peak area at about 1388 cm^{-1} and gains of intensities around the 1425 and 1406 cm^{-1} bands (Fig. 3b).

Fig. 4 shows the FT-IR absorption spectra for the Ca^{2+}-free and Ca^{2+}-bound form of EDTA in D_2O solutions (25 mM). The infrared spectra for the complexes of EDTA with

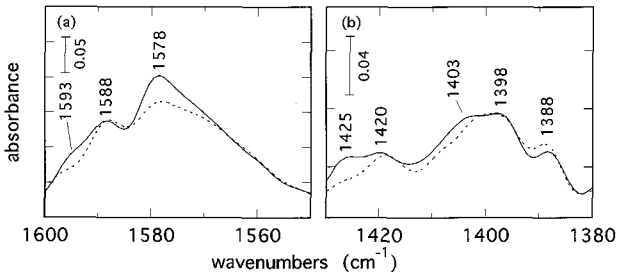

Fig. 2. Fourier-self deconvolved spectra enlarged in the regions of the COO^- antisymmetric and symmetric stretching vibrations for bovine α-lactalbumin in D_2O (a) and H_2O (b) solutions. Solid and dotted lines represent the Ca^{2+}-bound and Ca^{2+}-free form, respectively. Deconvolving parameters: 2σ, 16 cm^{-1}; *L*, 0.162 cm.

metal ions have been studied previously. At neutral pH, two bands due to the COO^- antisymmetric stretches appear at around 1620 and 1590 cm^{-1}. The former becomes weaker and the latter stronger as the pH is raised. This phenomenon is interesting, but the theoretical basis for it has not been fully clarified yet [14,15]. For the purpose of the present paper described above, we measured the FT-IR spectra of EDTA at pH 14, where the single band at 1585–1588 cm^{-1} is observed. The band due to the COO^- antisymmetric stretching vibrations for the Ca^{2+}-free and Ca^{2+}-bound EDTA appears at 1585 and 1588 cm^{-1}, respectively. The bands at 1407 and 1414 cm^{-1} are assignable to the COO^- symmetric stretches.

4. Discussion

In the solid form, the COO^- groups in EDTA coordinate to a metal ion in the unidentate form [16–19], where only one of the two oxygen atoms in the COO^- group interacts with a metal ion. The COO^- groups contact with water molecules in aqueous solution, so we must consider that these COO^- groups coordinate to metal ion in the pseudo bridging mode. The difference between the frequencies of the Ca^{2+}-bound and Ca^{2+}-free state is 3 cm^{-1} for the COO^- antisymmetric stretches, and 7 cm^{-1} for the COO^- symmetric stretches (Fig. 4). It is indicated that the band due to the COO^- symmetric stretches shows a larger shift than that of the COO^- antisymmetric stretches upon Ca^{2+} binding. On the other hand, the intensity of the COO^- antisymmetric stretching band becomes stronger by the binding of Ca^{2+}, not apparent in the case of the COO^- symmetric stretching band.

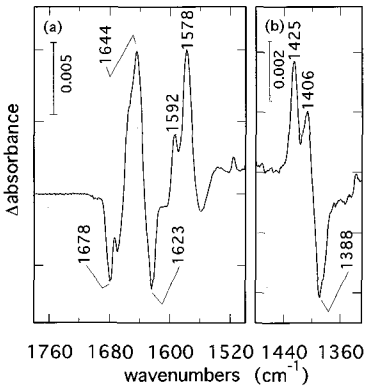

Fig. 3. FT-IR difference spectra induced by Ca^{2+}-binding of bovine α-lactalbumin in D_2O (a) and H_2O (b) solutions.

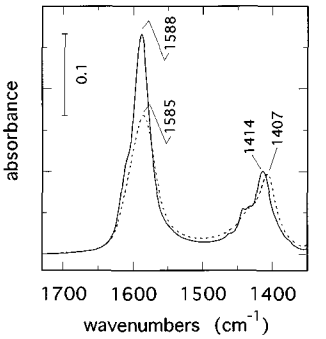

Fig. 4. FT-IR absorption spectra of Ca^{2+}-EDTA complex (solid line) and Ca^{2+}-free EDTA (dotted line) in D_2O in the presence of 200 mM NaCl at pH 14.

Therefore, we consider these characteristics as a result of the coordination of the COO^- groups to Ca^{2+} in the pseudo bridging mode.

We have observed FT-IR absorption spectrum of aspartic acid in D_2O in the presence of 200 mM NaCl at pH 7.3 (data not shown). The antisymmetric and symmetric stretches of the β-COO^- group give rise to bands at 1585 and 1393 cm^{-1}, respectively. Therefore, we consider that the peak positions of Asp COO^- groups which do not interact with Ca^{2+} are 1585 and 1393 cm^{-1}. The releasing of Ca^{2+} from the Asp sidechains results in the increase of peak area at 1388 cm^{-1} (Fig. 2b).

The COO^- groups of Asp-82, 87, and 88 coordinating to Ca^{2+} in the pseudo bridging mode give rise to the bands at 1593, 1578, 1425, and 1403 cm^{-1}, according to the recent X-ray crystallography [10]. The band due to the COO^- symmetric stretches shows a larger shift than in the case of the COO^- antisymmetric stretches upon Ca^{2+} binding to bovine α-lactalbumin. The frequencies of the COO^- antisymmetric stretches for the Asp COO^- groups coordinating to Ca^{2+} are 7–8 cm^{-1} higher or lower than that of the free aspartate. The frequency of the COO^- symmetric stretches for Asp sidechains shifts to a higher frequency by 10–32 cm^{-1} from that of free aspartate upon Ca^{2+} binding. On the other hand, the band due to the COO^- antisymmetric stretches gains its intensity as shown in the second derivative and deconvolved spectra (Figs. 1 and 2). Therefore, the characteristics of the FT-IR spectra for bovine α-lactalbumin are similar to those for EDTA in the region of the COO^- antisymmetric and symmetric stretching vibration. These observations support the fact that the COO^- groups of Asp-82, 87, and 88 in the Ca^{2+}-binding site of bovine α-lactalbumin coordinate to Ca^{2+} in the pseudo bridging mode in solution.

It is difficult to explain why two bands corresponding to the pseudo bridging mode are observed at 1578 and 1593 cm^{-1} for antisymmetric stretches, and at 1403 and 1425 cm^{-1} for symmetric stretches in the spectra for bovine α-lactalbumin (Fig. 2). However, the bands at 1578 and 1425 cm^{-1} probably correspond to two of Asp-82, 87, and 88. The bands at 1593 and 1403 cm^{-1} seem to reflect the structural environment of the other Asp residue of these three. The reason for this estimation is that the intensity is proportional to the number of the COO^- groups coordinating to Ca^{2+} and the increase of intensity at 1578 (1425) cm^{-1} is approximately twice as strong as that of the band at 1593 (1403) cm^{-1}.

Taking the above estimation into consideration, the value

of $\Delta\nu_{a-s}$ (frequency separation between the COO^- antisymmetric and symmetric stretching vibration) for the side-chain COO^- groups of Asp residues in the pseudo bridging mode is estimated to be 153 cm^{-1} and 190 cm^{-1}, which are relatively close to the value for the side-chain COO^- group of free aspartate (192 cm^{-1}). On the other hand, the value of $\Delta\nu_{a-s}$ is 178 cm^{-1} for the Ca^{2+}-free EDTA and 174 cm^{-1} for the Ca^{2+}-bound EDTA of which the COO^- groups are in the pseudo bridging mode in solution. These results are consistent with the rule obtained by Deacon and Phillips [1], that the value of $\Delta\nu_{a-s}$ is substantially greater than that of the free COO^- group is indicative of the unidentate carboxylate coordination, that of $\Delta\nu_{a-s}$ for the COO^- group in the bidentate form is significantly less than the free COO^- group, and that of $\Delta\nu_{a-s}$ for the pseudo bridging species is close to the value for the free COO^- group. The calculated $\Delta\nu_{a-s}$ values of the acetate ion interacting with Ca^{2+} in the unidentate, bidentate, and pseudo bridging form are 260 cm^{-1}, 102 cm^{-1}, and 197 cm^{-1}, respectively and that of the free acetate ion is 193 cm^{-1} [3].

The most striking differences between EF-hand motif and α-lactalbumin elbow lie in the fact that the former has Glu COO^- group coordinates to Ca^{2+} in the bidentate mode; this is not observed in the latter [7–10,20]. Examinations of the FT-IR spectra for the EF-hand Ca^{2+}-binding protein such as pike parvalbumin and bovine brain calmodulin reveal that the band of Glu side-chains shifts from 1567 cm^{-1} to 1552 cm^{-1} as a result of the coordination of the COO^- groups to calcium ions in the bidentate mode [5,6]. The characteristic of the bands due to the COO^- groups in the bidentate mode is clearly different from that in the pseudo bridging mode in the region of the COO^- antisymmetric stretching vibration. Shift to a lower frequency is a characteristic of the bidentate state. An increase in its intensity, which is characteristic of the pseudo bridging mode, is not observed in the case of the bidentate mode. Furthermore, it is proposed that if the COO^- groups of Asp residues are in the bidentate mode, the band may be observed at 1553 cm^{-1} [6]. Our present study shows no changes around 1553 cm^{-1}. The discussions described above explain that the bidentate coordination of the Asp COO^- group to Ca^{2+} does not exist in the Ca^{2+}-binding site of bovine α-lactalbumin.

It is necessary, for a better understanding of the interactions between the side-chain COO^- group and metal ion, to examine information obtainable from the various FT-IR spectra of other Ca^{2+}-binding proteins and model compounds with their high resolution X-ray structures. Application of these correlations will be useful for the structural analysis of some biologically important Ca^{2+}-binding proteins.

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