

Comparative Fourier Transform Infrared and Circular Dichroism Spectroscopic Analysis of α_1 -Proteinase Inhibitor and Ovalbumin in Aqueous Solution

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α_1 -Proteinase inhibitor (α_1 Pi) and ovalbumin are both members of the serpin superfamily. They share about a 30% sequence identity and exhibit great similarity in their three-dimensional structures. However, no apparent functional relationship has been found between the two proteins. Unlike α_1 Pi, ovalbumin shows no inhibitory effect to serine proteases. To see whether or not a conformational factor(s) may contribute to the functional difference, we carried out comparative analysis of the two proteins' secondary structure, thermal stability, and H-D exchange using FT-IR and CD spectroscopy. FT-IR analysis reveals significant differences in the amide I spectral patterns of the two proteins. Upon thermal denaturation, both proteins exhibit a strong low-wavenumber β -sheet band at 1624 cm^{-1} and a weak high-wavenumber β -sheet band at 1694 cm^{-1} , indicative of intermolecular aggregate formation. However, the midpoint of the thermal-induced transition of α_1 Pi ($\sim 55^\circ\text{C}$) is 18°C lower than that of ovalbumin ($\sim 73^\circ\text{C}$). The thermal stability analysis provides new insight into the structural changes associated with denaturation. The result of H-D exchange explains some puzzling spectral differences between the two proteins in D_2O reported previously. © 2000 Academic Press

Key Words: α_1 -proteinase inhibitor; serpins; ovalbumin; FT-IR; thermal denaturation; hydrogen exchange.

α_1 -Proteinase inhibitor (α_1 Pi)² and ovalbumin are members of a superfamily of proteins known as the serine proteinase inhibitors (serpins) (1). Some 60 different serpins with diverse physiological functions have been identified from a range of organisms, including viruses, plants, insects, and animals (2). Members of this family share a similar three-dimensional structure but each has a unique inhibitory specificity with serine proteinases, with a few exceptions (e.g., ovalbumin, angiotensinogen, and thyroxine-binding globulin) (3). Human plasma α_1 Pi, a glycoprotein of 394 amino acid residues, is the best characterized member of inhibitory serpins. Avian egg white ovalbumin, a glycoprotein of 386 amino acid residues, is the best characterized member of noninhibitory serpins (1, 4–6).

High-resolution X-ray crystal structures of several serpins are currently available, including α_1 Pi and ovalbumin in both uncleaved and cleaved forms (1, 5, 7, 8). The major structural feature shared by inhibitory serpins is the so-called β -sheet A, a five-stranded antiparallel β -pleated sheet present in the cleaved form (1, 5, 7). Structural comparison of the uncleaved and cleaved serpins reveals a large conformational change upon the cleavage of the reactive-center loop (Gly³⁴⁴ to Glu³⁶³ in α_1 Pi). In intact α_1 Pi, the reactive-center loop adopts either a distorted helical or an extended conformation (8, 9), whereas in the cleaved form, the N-terminal side (the residues designated P14 to P1) of the reactive-center loop inserts into the β -sheet A, forming a new β -strand (s4A). It appears that the conformation of the uncleaved reactive-center loop of serpins is con-

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² Abbreviations used: α_1 Pi, α_1 -proteinase inhibitor; FT-IR, Fourier transform infrared; CD, circular dichroism; H-D exchange, hydrogen-deuterium exchange.

siderably mobile and can take either an extended or a distorted/regular α -helix (8, 10). Cleavage of the reactive-center loop results in a large conformational change, separating the P1 and P1' residues to opposite poles of the molecule with a distance change of 70 Å (1). It is also found that the central β -sheet A of the intact α_1 Pi is largely similar to that of noninhibitory serpin ovalbumin (8, 9), in which the strand s4A is missing from the β -sheet A (1). The structural comparison of the noninhibitory ovalbumin and its proteolytically cleaved counterpart, plakalbumin, reveals no similar conformational change to that of inhibitory serpins and the P1 and P1' residues remain in close proximity upon cleavage (5).

However, despite extensive studies, some questions remain about functionally related conformational changes in inhibitory vs noninhibitory serpins. What role, if any, does the primary sequence of the reactive-center loop play in postcleavage conformational changes? Does the conformation of the reactive-center loop adopted by various serpins affect the insertion of the s4A strand upon cleavage? What effect, if any, does the β -sheet A folding preference have on the insertion of s4A strand? It seems that the preference for cleavage-induced insertion of the s4A strand in inhibitory serpins cannot be fully accounted for by either the primary sequence or conformation of the reactive-center loop (11–14). It is known that the active form of plasminogen activator inhibitor-1 can convert spontaneously to an inactive latent form with the insertion of the s4A β -strand without cleavage (11, 12). Latent α_1 Pi and antithrombin have also been obtained by pasteurization with various concentrations of citrate (13, 14). Furthermore, the conversion of noninhibitory ovalbumin to an inhibitory form (I-ovalbumin) with a 30-min heat treatment at 97°C has also been reported (15).

The conformational factor that prevents the insertion of the s4A β -strand in the noninhibitory ovalbumin upon proteolytic cleavage of the reactive-center loop equivalent is not fully understood. Detailed comparison of the inhibitory and noninhibitory serpins in terms of secondary structure and conformational dynamics may provide some insight into this puzzle. It is well known that ovalbumin has a higher thermal stability than that of α_1 Pi (16–18). What is missing, however, is the structural explanation for the observed difference in the thermal stabilities of the two proteins. Infrared spectroscopy has proved to be a valuable tool for studying conformational dynamics of proteins because of the secondary structural information that it can provide (19–22). It has advantages in its ability to resolve different types of secondary structures with high sensitivity without time-averaging effects, while permitting a rapid, relatively inexpensive analysis of the bulk properties of proteins under various physical states (20, 23, 24). FT-IR spectroscopy has been used in

a comparative study of the native and cleaved serpins in D₂O and provided invaluable structural information on cleavage-induced conformational changes of several serpins (25). However, it has also revealed some puzzling spectral features of the native serpins, such as lack of a clearly defined α -helix band in the native forms and a much stronger α -helix band in the cleaved serpins in D₂O. Searching for explanations to this and other questions, we carried out a further comparative study of α_1 Pi and ovalbumin in H₂O using FT-IR and CD spectroscopies.

MATERIALS AND METHODS

Sample preparations. Recombinant human α_1 Pi was expressed and purified as previously described (26, 27). The concentration of the α_1 Pi solution was 17.5 mg/ml in 0.1 M Tris-HCl buffer, pH 8.0. Ovalbumin (VII, chicken egg) was purchased from Sigma and prepared in 0.1 M Tris-HCl buffer at a concentration of 19 mg/ml and used without further purification. I-Ovalbumin was prepared according to the procedure described previously by Mellet and co-workers (15), with minor modification. The protein solution (2 mg/ml) was heat-treated at 95°C for 30 min. After cooling, the sample was filtered with a 0.20- μ m filter and centrifuged for 2 min using a bench-top ultracentrifuge. The resultant supernatant was then loaded on a size-exclusion HPLC column to determine the aggregation status of the protein before being concentrated for FT-IR measurement with a Centricon 10 microconcentrator (Amicon) at 4000g.

Size-exclusion chromatography. Size-exclusion chromatography was performed on a Tosohaas G2000SWXL size-exclusion column (7.8 mm \times 30 cm) and a Hewlett-Packard HP1090 HPLC system. The running buffer was 100 mM sodium phosphate (pH 6.9) at a flow rate of 0.6 ml/min.

Hydrogen-deuterium exchange. H-D exchange was carried out by a 10-fold dilution of protein stock solutions (17.5 mg/ml for α_1 Pi and 19 mg/ml for ovalbumin) with D₂O buffer. The mixtures were concentrated by centrifugation using a Centricon 10 microconcentrator (Amicon) at 4000g. The dilution/concentration procedure was repeated twice in the first 90 min and twice in the following 90 min. FT-IR spectra were measured at 90-min and 3-h time points after initiation of H-D exchange. The remaining samples were heated at 50°C for 30 min and followed by FT-IR measurement.

Infrared spectroscopy. FT-IR spectra were measured with a Magna-IR 550 spectrometer (Nicolet) equipped with a dTGS detector. Protein samples were prepared in a heatable cell (P/N 20500, Graseby) with CaF₂ windows and a 6- μ m spacer for H₂O and 25- μ m spacer for D₂O. For each spectrum, a 256-scan interferogram was collected in the single beam mode with a 4-cm⁻¹ resolution. Reference spectra were recorded under identical scan conditions with only the corresponding buffer in the cell. Protein spectra were obtained according to previously established criteria and a double-subtraction procedure (22, 28). The resultant spectra were smoothed with a seven-point Savitsky-Golay smooth function to remove the white noise. Second-derivative spectra were obtained by the derivative function of Omnic software (Nicolet). The inverted second-derivative spectra were obtained by factoring by -1 and baseline correcting as previously described (24).

Thermal denaturation experiments were carried out using a custom-built Peltier IR cell temperature controller. Sample temperature was measured with a thermal probe inserted into the half-hole of the top CaF₂ window. The desired temperature was controlled within $\pm 0.5^\circ\text{C}$. The average heating rate between spectral acquisition temperatures was about 1°C/min. Spectral acquisition at a given temperature required approximately 5 min (i.e., dwell time at the given temperature).

Circular dichroism spectroscopy. The far-UV CD melting scans were recorded with an Aviv Model 62DS spectrometer (Lakewood, NJ) using a strain-free cuvette with a path length of 0.01 mm. Bandwidth was set at 1.5 nm. The temperature change rate limit was set at 2°C/min for an effective heating rate of approximately 1.5°C/min. Spectral data were collected at 1°C intervals, using an averaging time of 5 s.

RESULTS

Primary infrared spectra. Figure 1 shows the overlay of primary spectra of α_1 Pi and ovalbumin in 0.1 M Tris-HCl, pH 8.0, at 25°C. The amide I band (1700–1600 cm^{-1}) arises primarily from the C=O stretching vibration of the peptide linkages that constitute the backbone structure and is known to be sensitive to protein secondary structures and conformational changes (19, 20). The band centered near 1551 cm^{-1} is known as the amide II band, which arises mainly from an out-of-phase combination of N-H in-plane bending and C-N stretching vibrations of the peptide linkages (19). The α_1 Pi exhibits the amide I band maximum at 1637 cm^{-1} with a shoulder near 1653 cm^{-1} , whereas the ovalbumin exhibits two nearly equal peaks at 1654 and 1640 cm^{-1} . The frequencies of the amide I band maxima indicate the presence of a large amount of β -sheet and α -helix structures in the two proteins (22). Although the informational content of the primary spectra is rather low, the conformational difference between the two proteins is readily detectable.

Second-derivative infrared spectra. To gain more detailed information regarding the secondary structural composition, we carried out the second-derivative and curve-fitting procedures to resolve the overlapping components under the amide I contour. Figure 2 shows the curve-fitted, inverted second-derivative spectra of α_1 Pi and ovalbumin measured at 25°C. The spectrum of α_1 Pi exhibits two major bands at 1655 and 1635 cm^{-1} and three minor bands at 1691, 1684, and 1673 cm^{-1} , while the spectrum of ovalbumin exhibits three major bands at 1657, 1638, and 1626 cm^{-1} and two minor

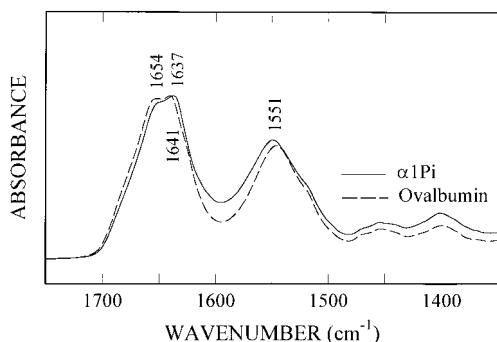


FIG. 1. Primary spectra of α_1 Pi and ovalbumin at 25°C. The spectral contributions from liquid and gaseous water were removed from the protein spectra as described under Materials and Methods.

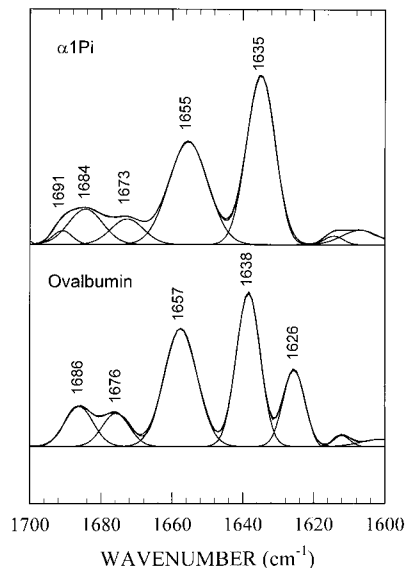


FIG. 2. Curve-fitted inverted second-derivative amide I spectra of α_1 Pi (top) and ovalbumin (bottom) at 25°C.

bands at 1686 and 1676 cm^{-1} . Assignments of the bands can be made on the basis of previous infrared studies of over 50 proteins in H_2O solutions (22, 28, Protein Infrared Database³). The bands at 1638/1635 and 1626 cm^{-1} can be assigned to low-wavenumber β -sheets, the bands at 1655/1657 cm^{-1} to α -helix, the bands at 1686/1684 and 1676/1673 cm^{-1} to β -turns, and the band at 1691 cm^{-1} to a high-wavenumber normal mode associated with β -sheet structures. The presence of the 1626- cm^{-1} β -sheet band in the spectrum of ovalbumin has also been confirmed by the Fourier self-deconvolution method (data not shown). Quantitative analysis (Fig. 2) revealed that α_1 Pi contains 43.2% β -sheets and 36.1% α -helix and ovalbumin contains 47.5% β -sheets and 34.6% α -helix. These results are consistent with the values determined by X-ray crystallography (\sim 45% β -sheet and \sim 35% α -helix) (1, 5, 7); FT-IR in D_2O by factor analysis (\sim 38% β -sheet and \sim 36% α -helix) (25), and CD spectroscopy (44% β -sheet and 35% α -helix) (29). This result sets up a baseline for the spectral interpretation of the following experiments.

Thermal denaturation. Figures 3 and 4 show the second-derivative spectra of α_1 Pi and ovalbumin as a function of temperature. As temperature increased, the intensities of the 1635/1638- and 1655/1657- cm^{-1} bands of both proteins were altered dramatically, indicating a thermally induced conformational change in the β -sheet and α -helix structures. However, the intensity change in the 1635/1638- cm^{-1} band seems to be

³ Protein Infrared Database: <http://www.unco.edu/chemist/aichun/irdata.htm>.

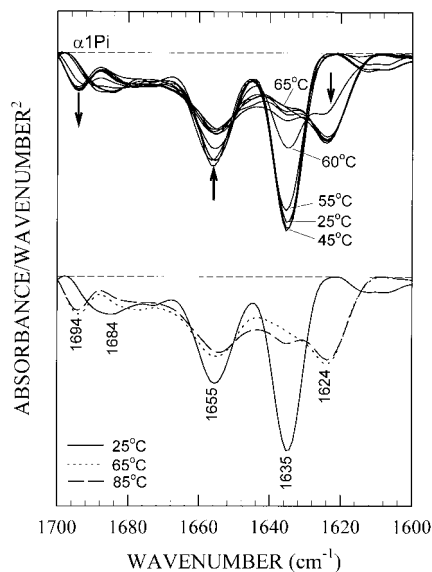


FIG. 3. Second-derivative amide I spectra of α_1 Pi as a function of temperature. (Top) Spectra measured at 25, 35, 45, 55, 60, 65, 70, 75, 80, and 85°C. (Bottom) Spectra measured at 25, 65, and 85°C. The arrows indicate the directions of spectral change as a function of temperature.

more sensitive to the changes in temperature, and thus can be used as a better probe to monitor the aggregation process of the proteins. Figure 5 shows a plot of relative intensity change at the 1635/1638- cm^{-1} band as a function of temperature. The midpoints of struc-

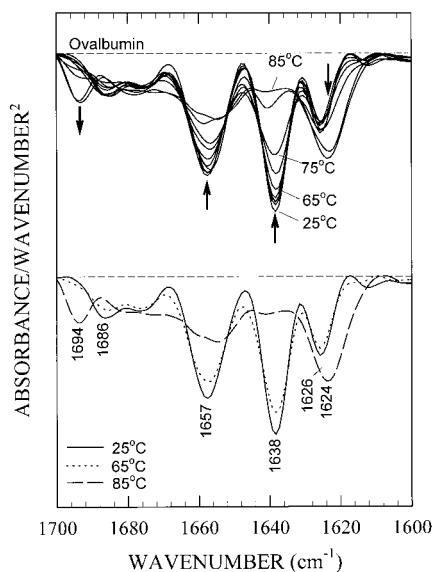


FIG. 4. Second-derivative amide I spectra of ovalbumin as a function of temperature. (Top) Spectra measured at 25, 35, 45, 55, 60, 65, 70, 75, 80, and 85°C. (Bottom) Spectra measured at 25, 65, and 85°C. The arrows indicate the directions of spectral change as a function of temperature.

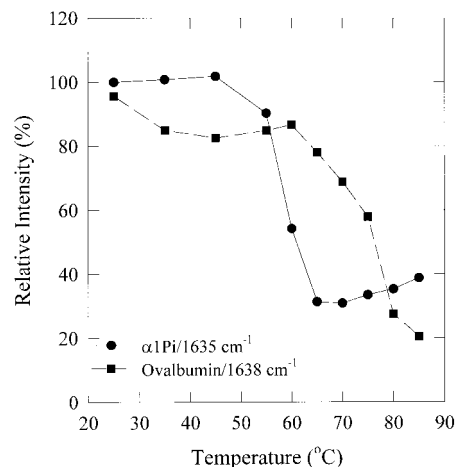


FIG. 5. Temperature-dependent intensity changes at the bands assigned to the native β -sheet structure: (●) 1635- cm^{-1} band of α_1 Pi; (■) 1638- cm^{-1} band of ovalbumin.

tural transition are found around 55°C for α_1 Pi and 73°C for ovalbumin. The result confirms observations reported separately for ovalbumin (16, 18) and α_1 Pi (17) using CD spectroscopy and centrifugation. Furthermore, the intensity decrease at the 1635/1638- cm^{-1} band was accompanied by an intensity increase at the 1624- and 1694- cm^{-1} bands, with two isosbestic points near 1690 and 1630 cm^{-1} . This observation indicates that thermal treatment of the two proteins results in a structural transition from a native β -sheet to an intermolecular β -sheet aggregate (24).

To see whether a comparable thermal aggregation process of α_1 Pi and ovalbumin can be detected by another spectroscopic technique under these conditions, the temperature dependence of the secondary structure of the two proteins was monitored using CD spectroscopy. The ellipticity at 222 nm was measured over the temperature range 25–90°C (Fig. 6). The wavelength was selected because the negative band near 222 nm is indicative of the α -helical content of the protein and the β -sheet is relatively insensitive by CD spectroscopy. Upon raising the temperature, α_1 Pi displayed a cooperative structural transition centered near 60°C, which is consistent with the FT-IR data. By comparison, however, ovalbumin exhibited no apparent structural transition under similar conditions, which deviates significantly from that observed by FT-IR.

Hydrogen-deuterium exchange. H-D exchange has been used extensively to determine the structural dynamics of proteins (30–33). The rate of H-D exchange is known to be sensitive to differences in secondary structural composition and compactness of three-dimensional structures of proteins (31, 33). Differences found between proteins with similar secondary structural conformation and overall three-dimensional

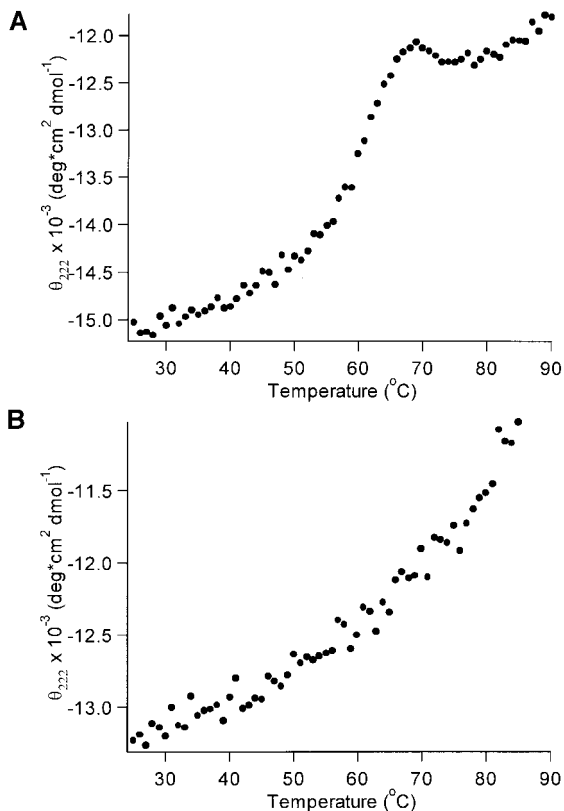


FIG. 6. Thermal aggregation transitions for α_1 Pi (A) and ovalbumin (B) monitored by circular dichroism. Ellipticity data at 220 nm are plotted against temperature. A cooperative denaturation transition for α_1 Pi is observed near 60°C.

structures are even more meaningful (34). Figure 7 shows the second-derivative amide I spectra of α_1 Pi and ovalbumin recorded after 90 and 180 min of H–D exchange at 25°C and a 30-min heat treatment at 50°C, respectively. The spectra of the two proteins obtained in H₂O solution are included for easier comparison. Since α_1 Pi was purified in H₂O-based solution, we carried out H–D exchange using several cycles of D₂O dilution and concentration instead of the commonly used lyophilization–deuteration procedure to avoid possible lyophilization-induced structural damage (23). However, constrained by the procedure, we performed no detailed comparison on the H–D exchange rate between the two proteins. Although some minor changes continued after the first 90-min H–D exchange, the overall spectral appearance of both proteins remained similar. Comparing with the spectra in H₂O, significant changes were observed in the bands assigned to the α -helix structure (\sim 1655 cm⁻¹), while little or no change was observed in the bands assigned to the β -sheet structure (1642 and 1625 cm⁻¹). The spectral changes in the α -helical band of α_1 Pi are most prominent. A single strong α -helical band at 1657 cm⁻¹ in H₂O changes to three weaker bands in D₂O at 1664,

1652, and 1646 cm⁻¹. However, no similar change was observed in the 1655-cm⁻¹ α -helix band of ovalbumin, although its intensity decreases significantly. This result suggests that, while the β -sheet structures in both proteins are fairly resistant to H–D exchange, the α -helical structure of α_1 Pi is more prone to H–D exchange than that of ovalbumin. The deuteration-induced spectral changes in α_1 Pi are consistent with those observed in α_1 -antichymotrypsin, another member of the inhibitory serpins (25). As noted in our previous study, the deuteration-induced amide I spectral changes in proteins are due to an isotopic effect through the hydrogen/deuterium bonding alterations, not the structural changes (42).

Infrared spectrum of heat-treated ovalbumin. To see whether or not the so-called I-ovalbumin exhibits an amide I spectral pattern similar to that of inhibitory serpin α_1 Pi, we prepared I-ovalbumin following the procedure of Mellet and colleagues (15). After filtrating with a 0.20- μ m filter and centrifugation, the IR spectrum of I-ovalbumin was collected and analyzed. Second-derivative analysis reveals that the I-ovalbumin sample contains large amounts of antiparallel β -sheet aggregate as evidenced by a strong low-wavenumber β -sheet band near 1622 cm⁻¹ and a weaker high-wavenumber β -sheet band near 1694 cm⁻¹ (Fig. 8). The spectral pattern is typical for thermally induced intermolecular β -sheet aggregate (24). This finding is supported by the size-exclusion chromatographic analysis of the I-ovalbumin preparation (Fig. 9). The elution profile from size-exclusion chromatography shows that

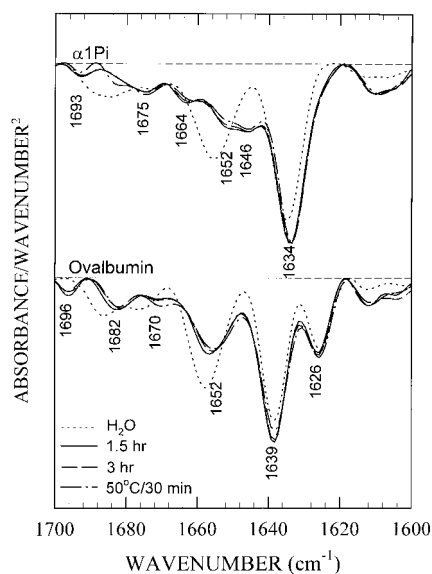


FIG. 7. Second-derivative amide I spectra of α_1 Pi and ovalbumin measured during the H–D exchange: spectra recorded at 25°C after 90-min (—) and 3-h (---) H–D exchange, and spectra recorded at 25°C after 3-h H–D exchange and 30-min 50°C treatment (— · —). The spectra recorded in H₂O are included for comparison.

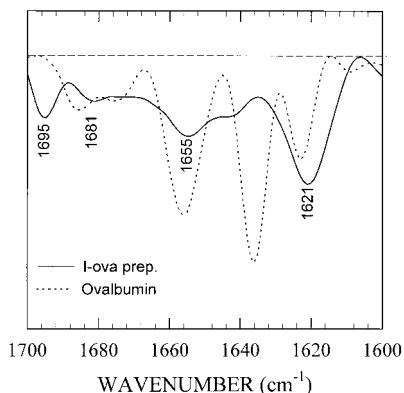


FIG. 8. Second-derivative amide I spectrum of I-ovalbumin preparation (solid line). The spectrum of ovalbumin in the native state (dashed line) is included for comparison.

about ~89% of the ovalbumin molecules elute as high molecular weight aggregates and only ~11% of ovalbumin molecules elute as monomers. This result may explain the unusually high thermal stability of the I-ovalbumin preparation reported (15). The inhibitory effect of I-ovalbumin observed by Mellet and colleagues (15) is likely associated with the remaining fraction of monomeric molecules. Unfortunately, our attempts to separate and concentrate the fraction of monomeric I-ovalbumin for infrared analysis failed due to the instability of the heat-treated protein, which continues to aggregate upon concentration.

DISCUSSION

α_1 Pi and ovalbumin are both members of the serpin superfamily and share about 30% sequence identity (35). The two proteins exhibit great similarity in their three-dimensional structures and in their proportions of major secondary structural elements (~35% α -helix and ~45% β -sheet structures) (1, 5). A similar conformation is also shared by other serpins such as α_1 -antichymotrypsin (36) and antithrombin (13). It has been shown that the central β -sheet A of the intact α_1 Pi is largely similar to that of noninhibitory serpin ovalbumin (8, 9), except that strand s4A is missing from the β -sheet A in ovalbumin (1). Therefore, there must be a more subtle structural explanation for the functional differences observed between the two proteins. Our results indicate that although α_1 Pi and ovalbumin contain similar amounts of α -helix and β -sheet structures, they exhibit significantly different amide I spectral patterns, especially in the β -sheet region (Figs. 2 and 7). Considering the single strong, symmetrical band component at 1635 cm^{-1} , the β -sheet structures of α_1 Pi appear to be uniformly packed, whereas the corresponding β -sheet structures in ovalbumin resonate at two different frequencies (1638

and 1626 cm^{-1}). The higher frequency feature is twice as intense as the other, indicating that it corresponds to approximately two-thirds of the residues in the β -sheet conformation. It has been suggested that lower wavenumber IR vibration for the β -sheet structure is due to stronger hydrogen bonding (19). Together, these data suggest that about one-third of the β -sheet residues may involve tighter hydrogen bonding than found in the rest of the ovalbumin molecule or in α_1 Pi. Although definitive assignments cannot be made without specific labeling of the protein, the IR data are consistent with stronger interactions within the β -sheet structure of the noninhibitory ovalbumin than within the inhibitory α_1 Pi. The β -sheet band at 1626 cm^{-1} appears to be a unique feature of the noninhibitory ovalbumin, since it is also absent from the IR spectra of the inhibitory serpins α_1 -antichymotrypsin and C1 inhibitor in D_2O solution (25).

It has been known for some time that there is a significant difference in the thermal stabilities of α_1 Pi and ovalbumin. It is not clear, however, what the structural basis for such a difference is. By monitoring the temperature-dependent conformational change using FT-IR spectroscopy, we observed a close relationship between the conformational change in the β -sheet region and the cooperative aggregation of the two proteins (Fig. 5). The midpoints of aggregation curves at $\sim 55^\circ\text{C}$ for α_1 Pi and $\sim 73^\circ\text{C}$ for ovalbumin determined by FT-IR are consistent with the values determined by CD for ovalbumin (18) and α_1 Pi (17) and by centrifugation for ovalbumin (16). This result suggests that the difference in thermal stability likely originates from differences in the folding of the β -sheet structures of the two proteins.

In the search for answers to the noninhibitory nature of ovalbumin, much of the attention has focused on the reactive-center loop. One mechanistic explanation is

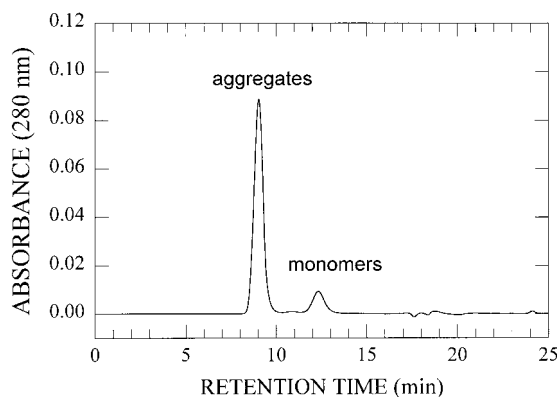


FIG. 9. Size-exclusion chromatographic elution profile of I-ovalbumin preparation monitored at 280 nm. After filtration and centrifugation, $50\ \mu\text{l}$ of the sample was applied to a Tosohaas G2000SWXL size-exclusion column ($7.8\text{ mm} \times 30\text{ cm}$). The running buffer was 100 mM sodium phosphate (pH 6.9) with a flow rate of 0.6 ml/min.

that ovalbumin cannot be cleaved to form a reactive-center loop product. However, several studies have shown that this is not the case. Although no inhibitory properties have been identified for ovalbumin, its reactive-center loop is readily accessible to proteolytic cleavage by subtilisin Carlsberg (37), thermolysin, and elastase (38). Of these, neutrophil elastase cleaves ovalbumin at the P1–P1' bond, without any additional cleavage (38). Ovalbumin fails to act as an inhibitor, despite recognition of the P1–P1' bond by elastase. On thermodynamic grounds, Wright and colleagues (7) hypothesized that the inability of the reactive-center loop to insert into the β -sheet A in ovalbumin may be due largely to a charged residue (Arg³³⁹) at position P14, which locates near the hinge region of the proposed loop insertion into the β -sheet. To test this hypothesis, Hood and colleagues (39) created an arginine-containing P14 variant (T345R) of α_1 Pi. The resulting variant exhibited a significantly slower rate of loop insertion, but nevertheless still inhibitory. X-ray crystallographic study of a P14 arginine variant of α_1 -antichymotrypsin has also shown that the P14 arginine does not prevent the insertion of a reactive-center loop into the β -sheet A (40). On the other hand, a recombinant α_1 Pi containing arginine at both P14 and P1 positions was found noninhibitory toward thrombin (41).

Recently, Huntington and colleagues (18) tested the hypothesis that ovalbumin could be turned into a protein inhibitor by increasing the rate of loop insertion through hinge region mutation. They replaced the P14 arginine residue of ovalbumin with serine, either alone or in combination with changes of P12–P10 to alanine, and observed a large increase in the rate of partial loop insertion into β -sheet A following cleavage at the P1–P1' bond by porcine pancreatic elastase. However, none of the three variants examined showed any detectable protein inhibitory properties. The inhibitory effect observed in heat-treated ovalbumin by Mellet and colleagues (15) is likely the result of reactive-center loop insertion upon cleavage after thermally induced modification at the β -sheet structure.

Consequently, it appears that ovalbumin is defective in its ability of β -sheet A to expand and fully accommodate the whole reactive-center loop, which is likely to be the principal reason for ovalbumin not being a proteinase inhibitor. Our results provide evidence supporting such a conclusion, as ovalbumin exhibits a spectral pattern that differs significantly from that of α_1 Pi in the β -sheet region. The differences can be interpreted as increased hydrogen bond strength within the sheet, retarding any insertion of the s4A strand critical for functionality

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