Structural Differences of Ovalbumin and S-Ovalbumin Revealed by Denaturing Conditions

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We found, by circular dichroism and Raman spectroscopy measurements, that the secondary structure of the native ovalbumin and of its heat-stable form, called S-ovalbumin, is a probe of the structural differences between the two proteins. Small angle X-ray scattering and circular dichroism measurements performed on the two proteins under denaturing conditions, with different concentrations of guanidine hydrochloride, show the changes of the tertiary and secondary structure and a different pathway in the unfolding process. These experimental data confirm that the conversion of native ovalbumin into S-ovalbumin is irreversible and reveal that the response of the two proteins to the same chemical environment is different.

Introduction

Recently we have reported the first comparative study of the ovalbumin and S-ovalbumin by means of small angle X-ray scattering (Congiu Castellano *et al.*, 1996). Our results indicate a different size of the two proteins in the native and in some chemical denatured forms. While the ovalbumin in the native form has been extensively studied also by means of light and X-ray scattering and rheological measurements (Matsumoto and Chiba, 1990), its heat-stable form named S-ovalbumin is still poorly characterised (Smith, 1964; Smith and Back, 1965; Smith and Back, 1968a; Smith and Back, 1968b; Nakamura *et al.*, 1981; Nakamura and Ishimaru, 1981). The data on the secondary structure of the two proteins inferred from Raman spectroscopy, are contradictory (Painter and Koenig, 1976; Kint and Tomimatsu, 1979).

Ovalbumin is a globular protein with a molecular weight of ca. 43000 dalton which exists in a compact conformation (Taborsky, 1974). It is a member of the serpins (serine protease inhibitors) but shows no inhibitory activity. It accounts for about 5.9% of egg white and comprises ca. 65% of the total protein in egg white. During embryonic growth ovalbumin could be transferred into the yolk from the white of the egg, becoming an amino acid store. In this step it probably loses its phosphate content, which is perhaps utilised in the transfer process of the protein across the membrane. It is composed of a single polypeptide chain with 385 residues, with a single carbohydrate chain covalently linked to the nitrogen amide of an asparagine residue. The protein is crosslinked by one disulphide bond (cystine) and has four free sulfhydryl groups (cysteine) (Fothergill and Fothergill, 1970). With respect to the degree of phosphorylation of two of its serine side chains the protein may be bound to a carbohydrate chain; the N terminal and C terminal are acetyl glycine and proline, respectively. The complete amino acid sequence of ovalbumin was determined by Nisbet *et al.* (1981) and the crystallographic structure of the ovalbumin molecule was investigated in detail by Stein *et al.* (1991). It is worth analysing the conformational changes of the proteins in various environmental conditions because the transformation of the ovalbumin to S-ovalbumin is related, in *vivo* and in *vitro,* to these conditions. In fact it is found that in *vivo* the amount of S-ovalbumin as well as the value of pH increases with storage length of

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eggs and in *vitro* an irreversible switch of the ovalbumin to its stable form S-ovalbumin occurs under alkaline conditions. The thermal variations and the addition of acids and detergents are relevant to our understanding of the mechanism affecting the three-dimensional structure and the secondary structure of a protein, which cannot be considered as consisting of rigid molecules, but shows a high degree of flexibility which is essential for the functionality (Tanford, 1968; Chan and Dill, 1989; Dill and Shortle, 1991).

Several investigations have been carried out on the structure of the ovalbumin (Egelandsdal, 1986; Gorbunoff, 1969), although only limited information was available on its secondary and tertiary structure until recently. There is also a significant controversy over a possible renaturation of the ovalbumin after chemical denaturation. Several studies have been carried out on the effects of different denaturing agents on the ovalbumin (Glazer *et al.*, 1963; Gorinstein *et al.*, 1995; Takeda *et al.*, 1992). Analyses have been made of the globular conformation, the flexibility of the protein at low pH (Koseki *et al.*, 1988) and the importance of the role of the disulphide bond on the structure of the ovalbumin through the modification of sulfhydryl groups and disulphide reduction (Batra *et al.*, 1989). By means of circular dichroism studies on the irreversible conformational changes of ovalbumin in solutions containing urea, guanidine and SDS (sodium dodecyl sulfate), it was estimated that native ovalbumin could contain about 33% α-helix, 5% β-structure, 62% random coil (Batra *et al.*, 1989; Takeda and Moriyama, 1990). Moreover denaturation kinetics has recently been observed and the different conformations that ovalbumin assumes in presence of urea, guanidine hydrochloride and various surfactants (amphoteric, anionic, cationic) have been pointed out (Zemser *et al.*, 1994).

The objective of this work is to study the structural properties of the S-ovalbumin molecule by comparing it to native ovalbumin and to compare the denatured states of these proteins as a function of the temperature and of guanidine hydrochloride concentration by means of small angle X-ray scattering (SAXS) and circular dichroism.

The SAXS method allows to evaluate shape, size and compactness of the biopolymer in solution, following its structure, as well as the spatial distribution in folded, unfolded or intermediate states and hence also the small conformational changes due to the flexibility (Durchschlag *et al.*, 1991; Garrigos *et al.*, 1992; Abrego *et al.*, 1993). Moreover, we used spectral properties of the polypeptide chain to characterise and to follow changes in the folded conformation in aqueous solution: circular dichroism spectra was used to evaluate the changes of the secondary structure and hence the amount of helical structure by different denaturing conditions. In the native proteins the amount of ordered α and β structure has been confirmed by Raman spectroscopy (Williams, 1986).

Materials and Methods

Best grade albumin chicken egg, free of S-ovalbumin, was purchased from Sigma (St. Louis, MO, USA) and used without further purification.

Protein concentration (based on a molecular weight of 43000 daltons and a primary sequence of 385 amino acids) was determined spectrophotometrically at 280 nm using the molar extinction coefficient ε_{280}=32040 $M^{-1}cm^{-1}$ (Nakamura *et al.*, 1980). S-ovalbumin was prepared according to Smith and Back (1965) and elsewhere extensively described (Congiu Castellano *et al.*, 1996): a 5% solution of ovalbumin was adjusted to pH 9.9 at room temperature and then heated for 38 hours at 55 °C. After cooling, a small amount of denatured protein was precipitated at pH 4.7 (acetic acid 0.1 M), the solution was clarified by centrifugation and lyophilized after an extensive dialysis. Chromatography of S-ovalbumin on DEAE-cellulose and isoelectric focusing techniques show the absence of resolvable subfractions according to data elsewhere reported (Goux *et al.*, 1986; Nakamura *et al.*, 1980).

In order to determine whether the two proteins show different properties at different temperatures, SAXS and CD measurements were performed at 20 °C, 30 °C, 40 °C, 50 °C.

Scattering measurements were carried out in aqueous solution of native protein, buffered with Tris-HCl (tris hydroxymethylaminomethane hydrochloride) 0.05 M at pH 7.5. Four solutions for each sample of ovalbumin and S-ovalbumin, with concentrations ranging from 2 mg/ml to 8 mg/ml were employed in the SAXS experiments.

To study the denaturing process of ovalbumin and of S-ovalbumin with a strong denaturant, we

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prepared samples of proteins with different concentrations of guanidine hydrochloride (Gnd-HCl). We used buffered Tris-HCl 0.05 M pH 7.5 solutions containing the required concentration of guanidine hydrochloride (0.2, 0.6, 1, 2.4, 6 M). Under denaturing conditions, at these values of pH and ionic strength, the possible disulphide exchange is hindered (Batra *et al.*, 1989).

Scattering measurements for this set of samples, were performed at room temperature, after keeping them at 25 °C for at least 12 hours in order to reach equilibrium conditions. Samples were contained in calibrated quartz capillary tubes, 1 mm in diameter.

Small angle scattering data were collected at the experimental station D24 of the DCI storage ring of LURE Laboratory (Orsay, Paris) with the same experimental conditions already described (Congiu Castellano *et al.*, 1996).

The synchrotron radiation source was operated at an electron beam energy of 1.86 GeV with an average electron beam current of 300 mA. Scattering data were collected at fixed wavelength (λ= 1.4878 Å, $\Delta\lambda/\lambda = 10^{-3}$) on a position-sensitive proportional detector, 1080 mm from the sample. The experimental SAXS intensity for each concentration was determined as a function of the modulus of the scattering vector named "momentum transfer" $s = \frac{2}{\lambda}\sin\theta$, where 2θ is the scattering angle and λ the X-ray wavelength. The scattering intensity was measured in the s range between $2.6\text{x}10^{-4}$(Å^{-1}) and $5.7\text{x}10^{-2}$(Å^{-1}). The background scattering arising from the buffer, as well as from the experimental setup (capillary tube, air and slits), was recorded before analysing each protein sample for comparable acquisition times, and subtracted from the sample scattering curves.

Circular dichroism spectra were recorded on a Jasco 500 A spectropolarimeter equipped with an IBM computer as data processor. In this case, aqueous solution of proteins at a concentration of $5\text{x}10^{-6}$ M (MW 45000) were recorded with a sensitivity of 2 m°/cm using quartz cuvettes of 1 mm optical length. The molar ellipticity (deg cm^2 decimol^{-1}) is expressed as $[\Theta]_a$ on a mean residue ellipticity in the far UV region (200–250 nm; mean residue mass: *111 daltons)*. Temperatures were controlled by mean of a thermostatic bath (±0.05 °C) between 20–55 °C. Denaturation experiments were performed at 25 °C varying the guanidine hydrochloride concentration between 0–4 M. The denaturation curves were obtained by measuring the $[\Theta]_a$ at 222 nm.

The Raman measurements were recorded as reported elsewhere (Fontana *et al.*, 1995). The incoming beam 380 mW at L=514.5 nm from a Spectra Physics 171 Ar-ion Laser was focused on the sample and monitored during the data collection in order to normalize the scattered intensity to the instantaneous laser power. The light scattered by the sample at an average angle of 90° was collected by a lens with an angular acceptance of the order of 0.02 sr and its vertically polarized portion was focused on the entrance slit of the Jobin-Yvon-Ramanor U1000 double monochromator equipped with 1800 grooves/mm holographic gratings. All the spectra were collected with the entrance and exit slits of the monochromator set at 50 mm and the two intermediate slits set at 70 mm, yielding a resolution of 0.6 cm^{-1}.

The spectra were measured in the 1600–1720 cm^{-1} frequency range, with a step of 1 cm^{-1} and an integration time of 10 s.

Results

Native proteins. The characteristic scattering curves of native ovalbumin and native S-ovalbumin (8 mg/ml) are plotted in Fig. 1a and 1c. The intensity at the origin and the radius of gyration were obtained from a linear regression of the logarithm of the scattering intensity (ln I) versus the square of the momentum transfer (s^2) using Guinier's law (Guinier and Fournet, 1955)

$$I(s) = I_0 \exp\left(-\frac{4\pi^2 R_g^2 s^2}{3}\right) \quad (1)$$

where I_0 denotes the intensity at zero scattering angle and R_g corresponds to the radius of a thin hollow spherical shell of the same mass and moment of inertia as the protein and is the second moment of the electron density distribution within the molecule. The values of the radii of gyration obtained by the extrapolation at zero concentration are respectively 23.9±0.2 Å for ovalbumin and 24.8±0.2 Å for S-ovalbumin (Congiu Castellano *et al.*, 1996).

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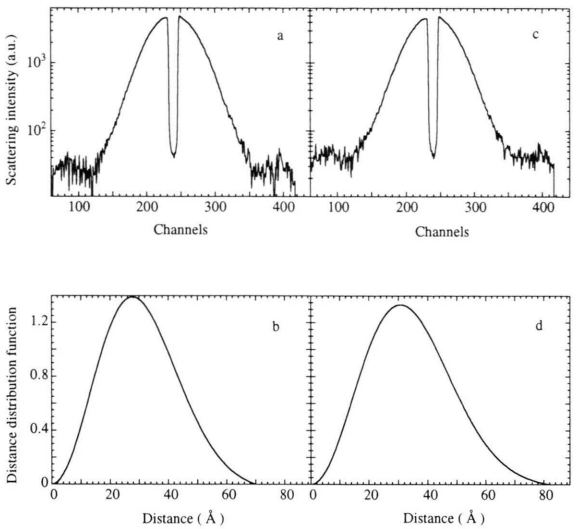

Fig. 1. Ovalbumin protein at 8 mg/ml concentration: a) experimental scattering curve and b) distance distribution function, $P(r)$ versus r;
S-ovalbumin protein at 8 mg/ml concentration: c) experimental scattering curve and d) distance distribution function, $P(r)$ versus r.

The distance distribution function $P(r)$ of the proteins was inferred from the experimental SAXS intensity I (s) as follows:

$$P(r) = \frac{1}{\pi}\int_0^{\infty} I(s)\, s\, r \sin(2\pi\, s\, r)\mathrm{d}s \qquad (2)$$

where r indicates the distance of two scattering points in the proteins and s is the momentum transfer. This function depends on the molecular shape and defines the maximum molecular dimension D_m.

Experimental $P(r)$ for ovalbumin and S-ovalbumin are plotted in Fig. 1b and 1d respectively, and the values of D_m obtained are about 70 Å and about 80 Å, respectively. To explain the difference in the radius of gyration due to scattering intensity in a relatively large range of scattering angle of SAXS, we also excluded the possible presence of some fluctuations in the electron density inside the dispersed phase and a charge density gradient in the spatial electron distribution of the two proteins. Nevertheless, these differences of the R_g values are probably related to some changes in the structural features of the proteins most likely due to alterations in the secondary (or tertiary) structure. The CD spectra shown for the two native proteins in Fig. 2 in the far UV in the peptide bond region are sensitive to secondary structure conformation. It was estimated (Takeda and Moriyama, 1990) that native ovalbumin could contain 33% of α-helix and the presence of two peaks at 222 and 208 nm in CD spectra of Fig. 2 reflects this conformational state. S-ovalbumin shows a slightly more intense peak around 217 nm, indicating a higher content of β structure with respect to the ovalbumin.

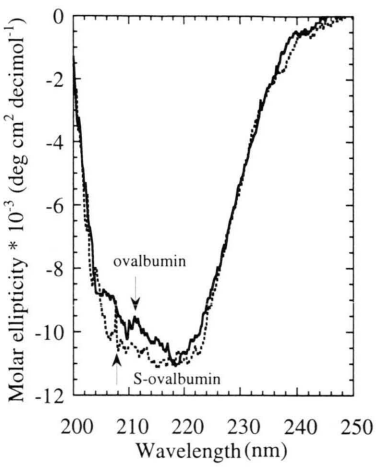

Fig. 2. Circular dichroism spectra of ovalbumin and S-ovalbumin proteins in the far UV: molar ellipticity [Θ] (deg cm^2 decimol^{-1}) versus wavelength.

The Raman spectra of the two native proteins are reported in Fig. 3a and their difference spectrum in Fig. 3b. These spectra allow the secondary structure on the basis of the amide I region to be estimated. The results are consistent with the CD predictions: the α-helical content is practically the same in the ovalbumin and in the S-ovalbumin, while in the S-ovalbumin there is an increase of the β extended strand and a decrease in the β-sheet.

Heat effect on proteins. Guinier plot of the ovalbumin and S-ovalbumin at T=50 °C are reported in Fig. 4. The Kratky plot of the ovalbumin and S-ovalbumin at T=50 °C indicate that both the proteins at this value of temperature are still globular.

We have analyzed the CD spectrum at 222 nm of the two proteins at different temperatures. The results reported in Fig. 5a indicate a weak variation of the ellipticity in the S-ovalbumin with respect to the ovalbumin with increasing temperature; at a temperature of 50 °C, the helicity seems to be the same.

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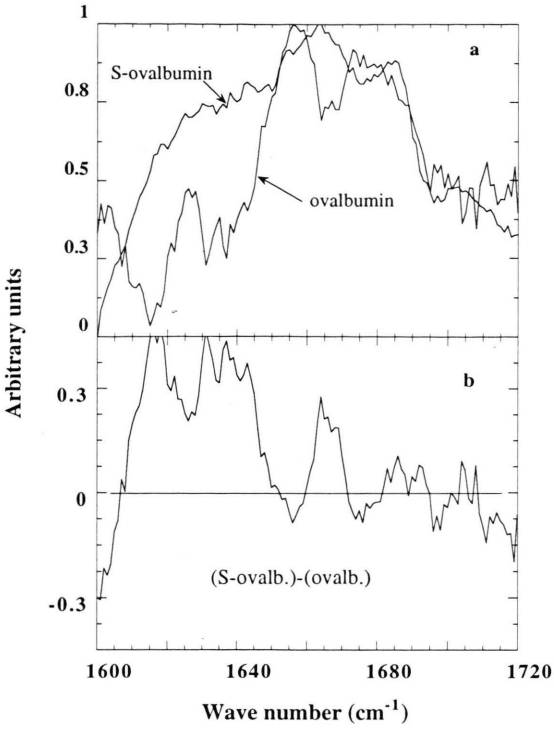

Fig. 3. Experimental Raman spectra of the two native proteins (a) and difference Raman spectra (b).

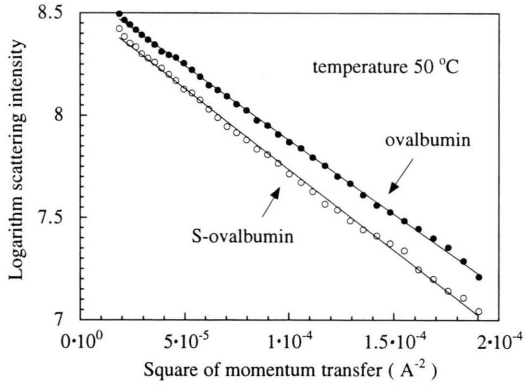

Fig. 4. Guinier plot and linear fits of the logarithm of the scattering intensity (ln I) as a function of the square of the momentum transfer (s^2) of the two native proteins compared at T=50 °C.

Guanidine hydrochloride denatured proteins. To reveal the differences between the two proteins we performed SAXS and CD measurements of ovalbumin and S-ovalbumin at various concentrations of guanidine hydrochloride. Urea and guanidine induce transitions by interfering with the hydrophobic regions in the interior as well as in the hydrogen bonding pattern involved in the polar regions of the peptide chain in a protein molecule. As already reported (Zemser *et al.*, 1994) guanidine hydrochloride causes the disorganization of the secondary structure of the ovalbumin: the α-helix content of the native ovalbumin evaluated about 33% results reduced to 6% in 6.4 M guanidine hydrochloride.

The denaturation curves of the proteins obtained by recording CD spectra at increasing denaturing concentrations are shown in Fig. 5b. While the denaturing effect on S-ovalbumin is very weak up to 1.8 M of guanidine hydrochloride a fast conformational change on ovalbumin is evident beginning from 0.2 M of guanidine hydrochloride.

Using these data the denaturation free energy $\Delta G°$ was calculated, on the basis of the equation $\Delta G_d° = \Delta G° + m$ [Gnd-HCl] where $\Delta G_d°$ is the apparent free energy change, $\Delta G°$ is determined by extrapolating to [Gnd-HCl]=0, and m measures the dependence of ΔG on denaturant concentration and

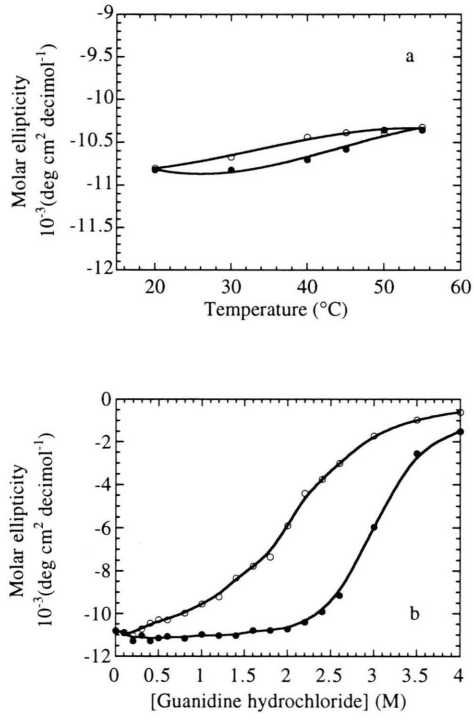

Fig. 5. a) Molar ellipticity $[\Theta]_a$ as a function of the temperature obtained from circular dichroism spectra at 222 nm of native ovalbumin and S-ovalbumin;
b) molar ellipticity $[\Theta]_a$ at 222 nm of ovalbumin (open circle) and of S-ovalbumin (dark circle) versus increasing concentrations of guanidine hydrochloride.

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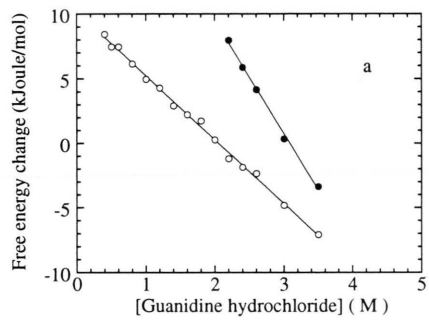
its absolute value is correlated with the number of amino acids exposed to the solvent during the unfolding process (Pace, 1975).

Fig. 6 a shows the straight line interpolating the calculated values of ΔG versus guanidine hydrochloride concentration. For ovalbumin $\Delta G°$ is 10.17 ± 0.04 kJoule/mol and m is 4.95 ± 0.02 kJoule l / mol^2, for S-ovalbumin $\Delta G°$ is 26.9 ± 0.2 kJoule/mol and m is 8.7 ± 0.1 kJoule l/mol^2. The low values of the m parameter suggest that the presence of crosslinks in the unfolded state will result in a more compact unfolded state, thus reducing the accessibility of the unfolded polypeptide chain to solvent (Myers *et al.*, 1995; Nakamura and Ishimaru, 1981). Our thermodynamic data represent hitherto unreported experimental evidence of the higher stability of the S-ovalbumin with respect to the native protein ovalbumin.

The increased stability also reflects modification of the overall shape of the protein, which can be evaluated in terms of radius of gyration, as shown in Fig. 6b where the R_g values, deduced from SAXS experiments on solutions of the two proteins at different Gnd-HCl concentrations, are compared. In Fig. 7 the Kratky plots show the differences between the globularity of ovalbumin and S-ovalbumin in solution of guanidine hydrochloride 2.4 M: the globularity of S-ovalbumin is almost unchanged, while the ovalbumin globular compactness is largely modified as shown by the unsymmetrical shape of the curve; therefore at guanidine hydrochloride 2.4 M the conformation of ovalbumin is largely denatured, while the S-ovalbumin conformation is still similar to the native form.



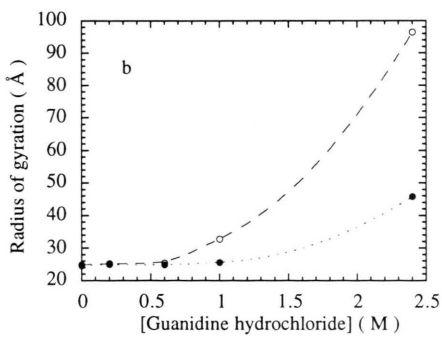


Fig. 6. a) Straight line interpolating the calculated values of the free energy change (ΔG) versus guanidine hydrochloride concentration in ovalbumin (open circle) and in S-ovalbumin (dark circle); b) radius of gyration (R_g) as a function of the guanidine hydrochloride concentration in ovalbumin (open circle) and in S-ovalbumin (dark circle).

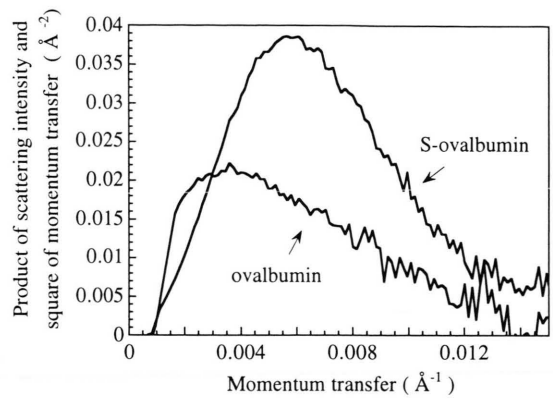


Fig. 7. Kratky plots (the product of the scattering intensity and the square of the momentum transfer, $I \cdot s^2$, as a function of the momentum transfer s) of ovalbumin and of S-ovalbumin in solution of guanidine hydrochloride 2.4 M.

Discussion

Our results allow to clarify the structural and thermodynamic differences between the ovalbumin and the S-ovalbumin both in the native form and in the denatured state.

Kratky plots of the two native proteins show that ovalbumin and S-ovalbumin are globular and despite the small difference in the radius of gyration of the two proteins, 24.8 Å instead of 23.9 Å, and in the maximum molecular dimension D_m, 80 Å instead of 70 Å, we have observed meaningful differences (irreversibly induced in vivo by favourable environmental conditions) of the thermal stability and the folding of these proteins. CD spectra of Fig. 2 show a slight difference in the secondary structure of the proteins which is confirmed by the

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Raman spectra of Fig. 3. Our Raman spectra indicate a larger content of β extended strand in S-ovalbumin protein (due to a partial α helix, to β structure transition or to a partial conversion of β sheet in extended β strands) which could be responsible for a stronger hydrophobic stabilization of the protein.

The tertiary structures remain practically constant when the two proteins are heated up to 50 °C. The radius of gyration undergoes a negligible modification and the Kratky plots show no aggregation or unfolding, as is confirmed also by forward scattering. Optical activity by Fig. 5a displays no meaningful variation of the secondary structure, even if in ovalbumin the denaturation curve is smoother and less cooperative. Nevertheless beyond this temperature (50 °C) we have observed a sharp change of trend in the two proteins: they aggregate at different temperatures giving rise to the formation of a precipitate. This is in according with the values of the denaturation temperature which is 84 °C for ovalbumin and 92 °C for S-ovalbumin (Donovan and Mapes, 1976).

The effect of chemical denaturants such as Gnd-HCl compared with heat induced effects, could contribute to our understanding of the reactive differences with the environments between the two proteins. The data in Fig. 5b and Fig. 6 amply describe the denaturation process and represent the experimental evidence that the thermodynamic stability of these proteins is mainly due to preferential interactions with the solvent as suggested by Nakamura *et al.* (1981). Moreover Batra *et al.* (1989), studying the denaturation of ovalbumin by SDS, urea and Gnd-HCl suggested that the single disulphide bond did not physically constrain the ovalbumin molecule. The denaturation pathway was followed by recording CD spectra at 222 nm and the modifications of the tertiary structure appeared as an increase in the radius of gyration. In the range of Gnd-HCl concentrations between 0 to 2.4 M, the conformational features of the S-ovalbumin remain almost constant. In the same conditions, ovalbumin conformation is deeply modified and more than the 70% of the protein is denatured. As the denaturant concentration increases, also S-ovalbumin loses its native conformation and exhibits a more cooperative behavior which can be ascribed to more stable non-covalent interactions. In the denaturation experiments, we obtained a reversibility of up to 80%. This was found by removing Gnd-HCl by dialysis and recording CD spectra for samples of the two proteins. Taking into account all the possible experimental errors, we found that, starting with a 2 M solution of Gnd-HCl, the α-helix content of the S-ovalbumin practically did not change while for ovalbumin in the same experimental conditions the α-helix content increased. This may be interpreted as experimental evidence that ovalbumin should not be considered a metastable conformation of the S-ovalbumin (Honeycutt and Thirumalai, 1992).

These results seem to confirm that S-ovalbumin is a different native protein produced by an irreversible transformation of the ovalbumin. In *vivo* this transformation seems to be promoved by changes in environmental acidity or by the biochemical mechanisms related to egg fecundation. In this way, irreversibility could guarantee the inhibition of the biological function of the ovalbumin.

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