Conformational Changes Involved in the Switch from Ovalbumin to S-Ovalbumin

Agostina Congiu Castellano[a], Mario Barteri[b], Antonio Bianconi[a], Fabio Bruni[a], Stefano Della Longa[c] and Corrado Paolinelli[a]

[a] Dipartimento di Fisica, Università "La Sapienza", 00185 Roma, Italy
[b] Dipartimento di Chimica, Università "La Sapienza", 00185 Roma, Italy
[c] Dipartimento di Medicina Sperimentale, Università dell'Aquila, 67100 L'Aquila, Italy

Z. Naturforsch. **51c,** 379–385 (1996); received December 19, 1995/February 6, 1996

Protein Folding, Conformational Change, Denatured State, Synchrotron Radiation, X-Ray Scattering

For the first time a comparative study on conformational differences between native ovalbumin and its heat-stable form, called S-ovalbumin, using small angle x-ray scattering, is reported. To detect a different pathway in the folding mechanism of the two proteins, scattering measurements have been performed on ovalbumin and S-ovalbumin denatured with different concentrations of guanidine hydrochloride, and by heating the proteins at acid pH. The intensity scattering curves provide evidence that the intermediate states in the unfolding process are globular for both proteins while their compactness changes. The reported experimental results suggest that the ovalbumin to S-ovalbumin transformation can be considered a protein-switch triggered by changes in the chemical conditions of the protein environment. Because the conformational changes are likely to be of functional importance, we infer that the occurrence *in vivo* of S-ovalbumin is thus determined by the transformation of ovalbumin, with a functional role for embryonic development, into a new protein with a different function.

Introduction

The dynamics of the process of polypeptide chain folding to its compact native state is clearly crucial to understand the structure of a protein. Recently it has been proposed (Sall *et al.*, 1994) that the folding process starts with a rapid collapse from the random coil state to a random semicompact globule, which then slowly proceeds to a transition state; finally the chain folds to the native state. In their work on the metastability hypothesis of protein folding, Honeycutt and Thirumalai (1992) suggested that the particular state into which the protein folds depends strongly on the initial thermodynamic conditions, pH, and ionic strength and report, as experimental evidence, the conformational change of ovalbumin protein in a more stable form, called S-ovalbumin. Within the framework of the metastability hypothesis, ovalbumin should thus be considered a distinct metastable state, which is transformed to S-ovalbumin (the lowest energy state) with very little structural change. Therefore, ovalbumin has been proposed by Honeycutt and Thirumalai as an ideal candidate for testing the hypothesis of the protein folding mechanism and the methods used by nature to change protein conformation.

Ovalbumin is a globular protein with a molecular weight of ca. 43000 daltons. 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 N terminal and C terminal are acetyl glycine and proline, respectively. Ovalbumin is synthesized under hormonal control by the bird oviduct and is deposited in the white of the growing egg. Despite being the major protein in avian egg white, its physiological function has not yet been clarified. Ovalbumin could play a role in the transport and storage mechanism of metal ions, and a single strong binding site for a variety of di- and trivalent metal ions has been found (Taborsky, 1974). It is very interesting that a more stable form, designated S (stable) ovalbumin is found *in vivo* in shell eggs stored at 20 °C, and its amount increases proportionally with storage length (Smith and Back, 1965; Smith and Back, 1968 a,b). Smith (1964) found that, upon warming the ovalbumin protein *in vitro* under alkaline conditions, an irreversible conformational change to S-ovalbumin, occurred. This form can be distinguished

Reprint requests to Dr. A. Congiu Castellano.
Telefax: +39-6-4957697.

0939–5075/96/0500–0379 $ 06.00

from the usual form of the protein by its increased resistance to heat denaturation: the conversion of the native protein to its S form shifts the denaturation temperature from 84.0 ± 0.5 to 92.0 ± 0.5 °C. Ovalbumin behaves as an anomalous non-inhibitor member (such as hormone binding globulins, angiotensinogen) of the serine protease inhibitor (serpin) superfamily of more than 20 homologous proteins, found in animals, plants and viruses, including plasma inhibitors, which control coagulation enzymes. The crystal structure at 1.95 Å resolution of uncleaved ovalbumin was obtained by Stein *et al.* (1991). It has been shown that a protruding isolated α-helix is the unexpected structure of the peptide loop forming the analogue to the reactive centre of the inhibitory serpins. While ovalbumin has been studied by means of light and X-ray scattering, and rheological and crystallographic measurements have been performed on it (Matsumoto and Chiba, 1990) there are no similar studies on S-ovalbumin.

The aim of this study is to characterize the S-ovalbumin molecule by comparing it to native ovalbumin and to some denatured states of these proteins by means of Small Angle X-ray Scattering (SAXS) to ascertain if S-ovalbumin has to be considered either the final state of the ovalbumin folding process, or a denatured form of ovalbumin or, a native protein. SAXS appears to be a suitable technique for this study due to its sensitivity to changes in size, shape and density of the scattering molecule, and is thus a useful and unique method to characterize the spatial distribution of molecules in solution (Kataoka *et al.*, 1993; Eliezer *et al.*, 1993).

Materials and Methods

Lyophilized chicken-egg albumin (free of S-ovalbumin) purchased from Sigma (St. Louis, MO, USA) was used without further purification as starting material for the preparation of ovalbumin and S-ovalbumin samples in solution. Scattering measurements were carried out using water solution of native protein containing as buffer Tris HCl (Tris hydroxymethyl aminomethane hydrochloride) 0.05 M at pH 7.5.

S-ovalbumin was prepared according to the method described by Smith and Back (1965): a 5% solution of ovalbumin in water was adjusted to pH 9.9 with $Na_2B_4O_7.10\ H_2O$ (Sodium tetraborate) 1 M buffer and by the addition of NaOH (Sodium hydroxide) 0.5 M at room temperature, stirred for 15 min and then heated for 16 hours at 55 °C. After cooling, acetic acid 0.1 M was added to give a pH of 4.7. A small amount of denatured protein (less than 1% of the total) was precipitated and the solution was clarified by centrifugation and filtered off with 0.45 μ millipore filter. The filtrate was dialysed overnight against bidistilled water and lyophilized. Protein concentration (based on a molecular weight of 42700 daltons and a primary sequence of 385 amino acids) was determined spectrophotometrically at 280 nm using the molar extinction coefficient $\varepsilon_{280} = 32040$. Four solutions for each sample of ovalbumin and S-ovalbumin, with concentrations ranging from 2 mg/ml to 8 mg/ml, were prepared by diluting a buffered stock solution of protein.

In order to determine whether SAXS measurements show any differences in the unfolding process of the two proteins, some samples of ovalbumin and S-ovalbumin with different concentration (0.2 M and 6 M) of guanidine hydrochloride (Gdn-HCl) were prepared using buffered (Tris HCl 0.05 M pH 7.5) solutions containing the required concentration of guanidine; scattering measurements for this set of samples, were performed after at least 12 hours to reach equilibrium conditions. Others samples of the two proteins were prepared by adjusting the solutions to pH 3 with 0.1 M hydrochloride acid (HCl) and then by heating the solutions at 55 °C for 90 min, as suggested by Smith (1964). At pH 3, ovalbumin is 95% denatured after 90 minutes at 55 °C whereas S-ovalbumin is 12% denatured under the same conditions (Smith and Back, 1965).

Samples were contained in calibrated quartz capillary tubes, about 1 mm in diameter. Small Angle Scattering (SAXS) data were collected, during three different runs, at the experimental station D24 of the D.C.I. storage ring of LURE Laboratory in Orsay (France) (Depauteux *et al.*, 1987). The Synchrotron Radiation Source was operated at an electron beam energy of 1.86 GeV with an average electron beam current of 300 mA. The X-ray beam had a cross-section of 0.5x2.0 mm at the sample level and 0.5x1.0 mm at the detector level. Let $\vec{S}_0$ be the characteristic vector of the incident beam (whose modulus is $1/\lambda$ where λ is the

wavelength of the X-rays) and let $\vec{S}_1$ be the characteristic vector of the scattered beam, the scattering vector is defined $\vec{S} = \vec{S}_1 - \vec{S}_0$. The modulus of $\vec{S}$ is $S = \frac{2}{\lambda} \sin \theta$, where 2 θ is the scattering angle and λ the X-ray wavelength used in the experiment. The experimental intensity scattering for each concentration was determined as a function of the S parameter in the range between 2.6×10^{-4} Å^{-1} and 5.7×10^{-2} Å^{-1}. Scattering data were collected at fixed wavelength (λ = 1.4878 Å, K-edge of nickel, $\Delta\lambda/\lambda = 10^{-3}$) on a position-sensitive proportional detector with delay-readout, 1080 mm away from the sample. The constant width for each channel, deduced by the experimental conditions, corresponds to an increment of the scattering parameter S equal to $dS = 2.601 \times 10^{-4}$ Å^{-1}/channel. The data acquisition system has already been described (Bordas *et al.*, 1980). The counting time was 4x400 s. The background scattering arising from the buffer, as well as from the experimental setup (capillary tube, air and slits) was recorded prior to analysis of each protein sample for comparable acquisition times, and subtracted from the sample scattering curves. Experiments were performed at room temperature.

Results

The SAXS intensity distribution of a protein in solution can be approximated by the Guinier relation (Guinier and Fournet, 1955)

$$I(q) = I_o \exp(-R_g^2 q^2 / 3) \qquad (1)$$

where I_o denotes the intensity at zero scattering angle, $q = 2\pi S$ (named momentum transfer), and R_g corresponds to the radius of a spherical shell of the same mass and moment of inertia as the protein. I_o is not directly accessible because of the beamstop, which protects the detector against radiation damage. Both quantities I_o and R_g are obtained by fitting experimental data to Eq. 1 using a non-linear fitting algorithm. Experimental data were also fitted with straight lines on a conventional Guinier plot (ln I against q^2) and the agreement on the values for I_o and R_g given by the two different fitting procedures was excellent.

Fig. 1 (panel a) and Fig. 2 (panel a) show the conventional straight line Guinier plots of scattering curves of ovalbumin and S-ovalbumin, at concentrations ranging from 2 mg/ml to 8 mg/ml. The

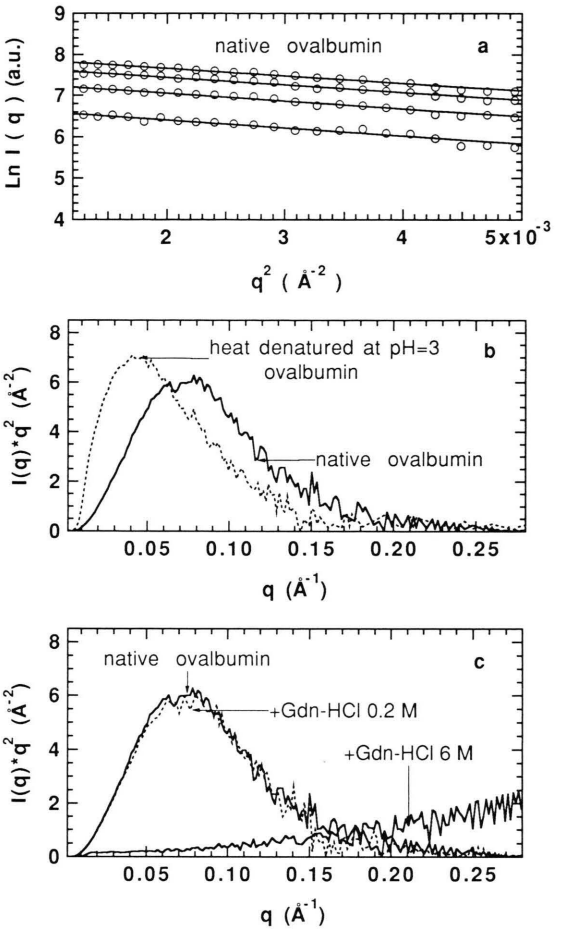

Fig. 1. Guinier plots and linear fits of the logarithm of the scattering intensity (ln I) as a function of the square of the momentum transfer (q^2) at 2,4, 6 and 8 mg/ml concentrations of native ovalbumin (panel a); Kratky plots (the product of the scattering intensity and the square of the momentum transfer, $I\,q^2$, as a function of the momentum transfer q), of ovalbumin (8 mg/ml): native and heat (pH 3) denatured (panel b), denatured with 0.2 M and with 6 M of guanidine hydrochloride (panel c).

radii of gyration determined by the fitting procedure and linearly extrapolated at zero concentration are shown in Table I and compared with those elsewhere reported (Matsumoto and Inoue, 1991; Matsumoto *et al.*, 1992). It is evident that: i) the native ovalbumin has a smaller R_g with respect to native S-ovalbumin; ii) 0.2 M Gdn-HCl produces on ovalbumin and S-ovalbumin an increase of R_g = 0.7 Å; iii) heat denaturation at pH 3 yields an R_g

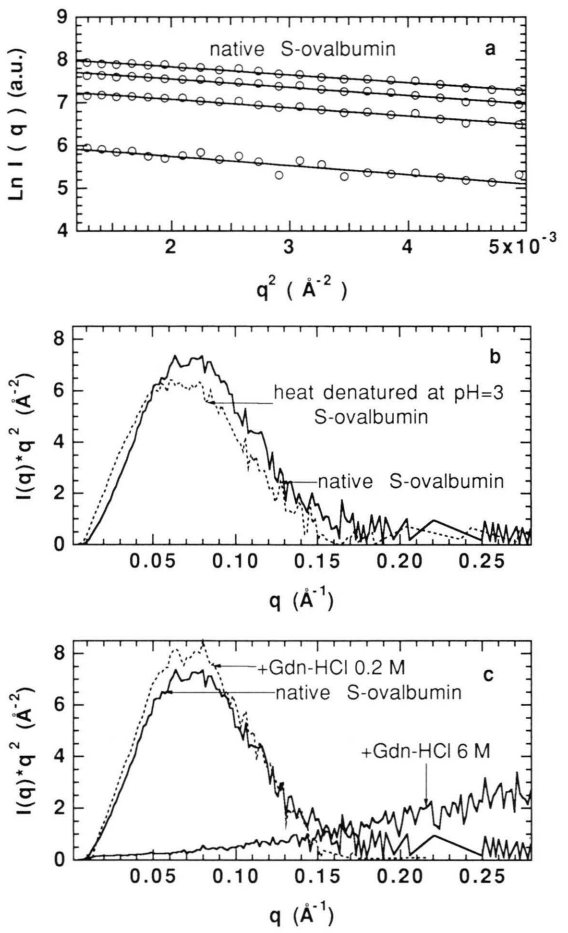

Fig. 2. Guinier plots and linear fits of the logarithm of the scattering intensity ($\ln I$) as a function of the square of the momentum transfer (q^2) at 2,4, 6 and 8 mg/ml concentrations of native S-ovalbumin (panel a) ; Kratky plots (the product of the scattering intensity and the square of the momentum transfer, $I\,q^2$, as a function of the momentum transfer q), of S-ovalbumin (8mg/ml): native and heat (pH 3) denatured (panel b), denatured with 0.2 M and with 6 M guanidine hydrochloride (panel c).

that is larger than those of the native and the Gdn-HCl denatured proteins.

The volume V of dispersed particles can be calculated from the intercept $\ln I_o$ on the $\ln I(q)$ axis in Figs 1a and 2a, according to

$$V = \frac{2\pi^2 I_o}{Q}$$

where Q is an invariant defined as $Q = \int_0^\infty Iq^2 dq$.

In Fig. 1 (panel b) and Fig. 2 (panel b) are shown the Kratky plots, $I(q)q^2$ versus q for native ovalbumin and native S-ovalbumin (8 mg/ml), respectively compared with the same samples but heat denatured at pH 3. In Fig. 1 (panel c) and in Fig. 2 (panel c) are shown the Kratky plots for ovalbumin and S-ovalbumin denatured with 0.2 M and 6 M of Gdn-HCl. The invariant Q can be thus calculated by integrating the curves in Fig. 1b and Fig. 2b. The Kratky plot, $I(q)q^2$ versus q, is a useful tool of the scattering profile to characterize the structure of an unfolded or folded chain. In fact, the scattering intensity of a globular protein has a region obeying Porod's law, $I(q)\ \alpha\ q^{-4}$, while the scattering intensity of a typical chain molecule has a region at moderate angles where it is proportional to q^{-2}, followed by a region proportional to q^{-1} at higher angles. Therefore, a peak in the Kratky plot indicates a compact globular structure, and the absence of a peak is an indication of a loss of compactness or globularity. The position of the peak depends on the value of R_g, and a shift to lower q indicates a larger R_g value. The peak positions indicate that the values display the same trend as in Table I. While native ovalbumin, native S-ovalbumin, and the samples either heat denatured or denatured with 0.2 M of Gdn-HCl show a globular state, the samples denatured with 6 M of Gdn-HCl have a chain-like conformation deduced from the absence of the peak in the Kratky plot; the same result has been obtained for the samples at the other concentrations.

If the protein molecule is assumed to be a sphere, the radius of gyration R_g is related to the radius of the sphere by means of $r = (3/5)^{-1/2} R_{gs}$. A shape factor f which is a measure of the deviation from a sphere can thus be defined as $f = R_g/R_{gs}$. Our experimental results (f = 1.1 for ovalbumin and f = 1.0 for S-ovalbumin) indicate that both ovalbumin and S-ovalbumin are almost spherical.

Discussion

The first question to address is whether ovalbumin is more compact than S-ovalbumin. In fact, previous experimental results are contradictory. Optical activity and hydrodynamic studies (Smith and Back, 1968) indicate that S-ovalbumin has a more compact conformation with a higher percentage of α-helical residues than ovalbumin. Con-

Table I. Our experimental data of the radius of gyration (R_g) of Ovalbumin and S-ovalbumin proteins in the native and denatured forms. The R_g (Å) value of the native ovalbumin is compared with those determined by other researches. The R_g of the S-ovalbumin and of denatured forms of the two proteins have not been elsewhere determined.

Protein	this work	X-ray [a,b]	calculated [c]
Ovalbumin	23.9 ± 0.2	25–28	20.9
S-ovalbumin	24.8 ± 0.2	–	–
Heat denatured (pH 3) ovalbumin	39.4 ± 0.2	–	–
Heat denatured (pH 3) S-ovalbumin	34.5 ± 0.2	–	–
Ovalbumin + 0.2 M Gdn-HCl	24.6 ± 0.2	–	–
S-ovalbumin + 0.2 M Gdn-HCl	25.5 ± 0.2	–	–

[a] Matsumoto and Inoue, 1991; [b] Matsumoto *et al.*, 1992; [c] From crystallografic data in Stein *et al.* (1991).

versely, Raman studies (Kint and Tomimatsu, 1979) suggest that there is a loss of α-helical structure, along with an increase of antiparallel β-sheet structures, when ovalbumin is converted to the S state. Little difference was noted between the CD spectra of both proteins (Nakamura *et al.*, 1980). The compactness, defined by Chan and Dill (1990) as the ratio of the number of intrachain contacts to the maximum possible number of such contacts, is inversely related to the radius of gyration. Values of R_g (Table I) for ovalbumin (23.9 ± 0.2 Å) and S-ovalbumin (24.8 ± 0.2 Å) show ovalbumin as more compact respect to its S state.

Crystallographic studies indicate that the structure of the ovalbumin molecule can be approximated by an ellipsoidal particle with dimensions of 70 x 45 x 50 Å.The R_g inferred from this data is 20.9 Å. Conversely, the molecule in solution seems almost spherical such elsewhere reported (Matsumoto and Inoue, 1993). Our values for the shape factor f (about 1.1 for ovalbumin and 1.0 for S-ovalbumin) show that both native proteins in solution confirm this assumption. Unfortunately, we cannot compare the crystal structures of native ovalbumin and S-ovalbumin. While the structure of native ovalbumin has been determined at 1.95 Å resolution (Stein *et al.*, 1991), the crystallographic structure of S-ovalbumin is still unknown. Our results show the importance of studying protein structure and conformational changes directly in solution. In fact the crystal packing forces and crystallization conditions may stabilize a particular conformation of the molecule, unrelated to its native conformation. It could be interesting to evaluate solution scattering data given the crystallographic atomic coordinates by using a method recently proposed (Svergun *et al.*, 1995) that takes into account the hydration shell.

Further information inferred from the scattering plots concerns the different change of the radii of gyration of the heat denatured proteins. Table I shows an increase of R_g for the heat denatured proteins equal to ΔR_g = 15.5 Å for ovalbumin and ΔR_g = 9.7 Å for S-ovalbumin. Nevertheless, the observed increase of R_g cannot be used to distinguish between an unfolding and an association process. A comparison of the I_o values for the native proteins and the heat denatured samples, at the same concentration, shows a difference which suggests a change in the conformation or density of protein monomers, or, conversely, an aggregation process. Our findings indicate an increase of I_o by a factor 2 for the heat denatured samples of ovalbumin, which could be related to an association of monomers in dimers. It has been proposed that the globular molecules aggregate to form a string of spheres (Matsumoto and Inoue, 1991). The increase of I_o is smaller for S-ovalbumin.

Additional information is provided by Kratky plots. As described in the previous section, Kratky plots can be used to distinguish between coil-like and globular scatterers. Compactness or globularity is one of the key structural characteristics to describe folded or molten globule states. Fig. 1 and Fig. 2 show that the heat denatured proteins and the molecule denatured by 0.2 M Gdn-HCl are globular. The experimental evidence is that the partially denatured or unfolded molecules are globular intermediates of the unfolding pathway. This conclusion is in agreement with the assumption that the unfolded states are quite compact in comparison to the random coil molecules, and

therefore have several hydrophobic contacts. Conformational entropy, heat capacity, and solvent exposure of non polar residues of the unfolded state are very different compared to the random coil (Dill *et al.*, 1989). Conversely, Kratky plots of the samples denatured with 6 M Gdn-HCl show that the proteins are not globular but fully unfolded due to the denaturing effect of Gdn-HCl.

In order to understand the different conformations of ovalbumin and S-ovalbumin it is necessary to recall some previous results. The hydrophobic coefficient of S-ovalbumin is larger than that of ovalbumin (Nakamura and Ishimaru, 1981). Moreover, the surface charges of the two proteins differ from each other. Since most of the hydrophobic amino acid residues are usually buried in the interior of the molecule, the exposure of internal hydrophobic amino acid residues might be related with changes of the protein surface charges. It has been shown (Nakamura *et al.*, 1981) by means of titration curves that the conversion of ovalbumin to S-ovalbumin is related to the release of carboxyl groups.

It is interesting to notice that, in the conversion process from ovalbumin to S-ovalbumin, the metastable state, namely ovalbumin, has a higher order structure and a greater compactness with respect to the final state. On the other hand, the final state, S-ovalbumin, has some properties characteristic of a metastable state , such as a greater hydrophobicity of the surface, a compactness intermediate between that of the native state and the unfolded state (the radius of gyration is slightly larger than that of the native state), but is a more stable form and cannot refold back to ovalbumin.

Our results support the assumption (Smith and Back, 1965) that S-ovalbumin is a native protein and cannot be considered as a denatured ovalbumin. We underline that the activation energy for transformation of ovalbumin to S-ovalbumin has been found equal to 15–25 kcal/mol or 0.5 cal/g, a value that is appreciably smaller than the typical activation energy for complete denaturation of a protein (of the order of 5 cal/g).

Since it is likely that partial conversion of ovalbumin to S-ovalbumin occurs during the first days of incubation of fertile eggs, S-ovalbumin can be regarded as a naturally occurring protein (Smith and Back, 1965) related to a chemical modification of the environment and related to a possible involvement of hormonal substances determining the rate of protein tertiary structure change. It has been found that *in vivo* the pH value increases with the storage: at 37 °C pH=9 after 100 hours storage, and at 22 °C pH=9 after 200 hours storage (Donovan and Mapes, 1976). It has been shown by NMR (Vogel and Bridger, 1982) that the ovalbumin may undergo a pH-dependent conformational change in which SerP-68 (one of two phosphoserine residues located on the surface of the protein) becomes shielded at higher pH but is more exposed at lower pH.

Acknowledgements

We thank Dr. Patrice Vachette for help during the SAXS experiments. This work was supported by grants from INFM and GNCB-CNR Italy.

Bordas J., Koch M. H. J., Clout P. N., Dorrington E., Boulin C. and Gabriel A. (1980), A synchrotron radiation camera and data acquisition system for time resolved X-ray scattering studies. J. Phys. E: Sci. Instrum. **13**, 938–944.

Chan H. S and Dill K. A. (1990), Origins of structure in globular proteins. Proc. Natl. Acad. Sci. USA **87**, 6388–6392.

Dill K. A., Alonso D. O. V. and Hutchinson. K. (1989), Thermal stability of globular proteins. Biochemistry **28**, 5439–5449.

Depautex C., Desvignes C., Leboucher P., Lemonnier M., Dagneaux D., Benoit J. P. and Vachette P. (1987), The small angle x-ray scattering instrument D24. LURE Annual Report.

Donovan J. W. and Mapes C. J. (1976), A differential scanning calorimetric study of conversion of ovalbumin to S-ovalbumin in Eggs. J. Sci. Food Agric. **27**, 197–204.

Eliezer C. J., Chiba K., Tsuruta K., Doniach S., Hodgson K. O. and Kihara H., (1993), Evidence of an associative intermediate on the myoglobin refolding pathway. Biophys. J. **65**, 912–917.

Glatter O. and Kratky O. (1982), Small Angle X-ray Scattering. Academic Press, New York Guinier A. and Fournet G. (1955), Small Angle Scattering of X-rays. John Wiley & Sons, Inc., New York.

Honeycutt J. D. and Thirumalai D. (1992), The nature of folded states of globular proteins. Biopolymers **32**, 32695–709.

Kataoka M., Hagihara Y., Mihara K. and Goto Y. (1993), Molten globule of cytochrome *c* studied by small angle X-ray scattering. J. Mol. Biol. **229**, 591–596.

Kint, S. and Tomimatsu Y. (1979), Raman difference spectroscopic investigation of ovalbumin and S-ovalbumin. Biopolymers **18**, 1073–1079.

Hagihara Y., Aimoto S., Fink A. L. and Goto Y. (1993), Guanidine hydrochloride-induced folding of proteins. J. Mol. Biol. **231**, 80–184.

Matsumoto T. and Chiba. J . (1990), Rheological and small-angle X-ray scattering investigations on the shape and ordered arrangement of native ovalbumin molecules in aqueous colloids. J. Chem. Soc. Faraday Trans. **86**, 2877–2882.

Matsumoto T. and Inoue H. (1991), Effect of heat denaturation on the structure and rheological properties of ovalbumin aqueous colloids. J. Chem. Soc. Faraday Trans. **87**, 3385–3388.

Matsumoto T., Inoue H. and Chiba J. (1992), Microscopic surface structure of a globular molecule of ovalbumin in aqueous buffer solutions. J. Appl. Phys. **71**, 1020–1025.

Matsumoto T. and Inoue H. (1993), Association state, overall structure, and surface roughness of native ovalbumin molecules in aqueous solutions at various ionic concentrations. J. Coll. Interface Sci. **160**, 105–109.

Nakamura R., Hirai M. and Takemori Y. (1980), Some differences noted between the properties of ovalbumin and S-ovalbumin in native state. Agric. Biol. Chem. **44,** 149–153.

Nakamura R., Takemori Y. and Shitamori S. (1981), Liberation of carboxyl groups on conversion of ovalbumin to S-ovalbumin. Agric. Biol. Chem. **45**, 1653–1659.

Nakamura R. and Ishimaru M. (1981), Changes in the shape and surface hydrophobicity of ovalbumin during its transformation to S-ovalbumin. Agric. Biol. Chem. **45**, 2775–2780.

Sall A., Shakhnovich E. and Karplus. M. (1994), How does a protein fold? Nature **369**, 248–251.

Smith M. B. (1964). Studies on ovalbumin I. Denaturation by heat and the heterogeneity of ovalbumin. Aust. J. Biol. Sci. **17,** 261–270.

Smith M. B. and Back J. F. (1965), Studies on ovalbumin II. The formation and properties of S-ovalbumin, a more stable form of ovalbumin. Aust. J. Biol. Sci. **18**, 365–77.

Smith M. B and Back J. F. (1968), Studies on ovalbumin III. Denaturation of ovalbumin and S-ovalbumin. Aust. J. Biol. Sci. **21**, 539–48.

Smith M. B. and Back J. F. (1968), Studies on ovalbumin IV. Trypsin digestion and the cystine peptides of ovalbumin and S-ovalbumin. Aust. J. Biol. Sci. **21**, 549–58.

Stein P. E., Leslie A. G. W., Finch J. T. and Carrell R. (1991), Crystal structure of uncleaved ovalbumin at 1.95 Å resolution. J. Mol. Biol. **221,** 941–959.

Svergun D., Barberato C., Kozin M., Volkov V., Stuhrmann H. B. and Kock M. H. J. (1995), New methods for solution scattering data analysis and their applications to biopolymers. Book on 5th International Conference on Biophysics and Synchrotron radiation.

Taborsky G. (1974), Phosphoproteins. Adv. Protein Chem. **28**, 1–210.

Vogel H. J. and Bridger W. A. (1982), Phosphorous-31 nuclear magnetic resonance studies of the two phosphoserine residues of hen egg white ovalbumin. Biochemistry **21**, 5825–5831.