The folding of ovalbumin

Renaturation *in vitro* versus biosynthesis *in vitro*

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Hen ovalbumin, the major secretory product of oviduct cells, is a 43 000-dalton glycoprotein. Many studies have led to controversy over the question of whether ovalbumin (OA) can be fully renatured after chemical denaturation. We have studied the renaturation of OA after denaturation with guanidinium chloride, urea or alkaline pH. Denatured OA displays an intrinsic viscosity consistent with nearly complete unfolding of the protein. Removal of the denaturant results in a complete reversal of the changes in intrinsic viscosity. However, closer examination of the renatured protein reveals major differences from the native form. Renatured OA (OAR) can be completely separated from the native form (OAN) by affinity chromatography on phenyl-Sepharose. OAR displays altered tryptophan fluorescence, u.v.-absorption and c.d. spectra. Only OAR binds anilinonaphthalenesulphonate (as measured by fluorescence enhancement). OAR, but not OAN, binds about 2 mol of the covalent hydrophobic affinity probe phenyl isothiocyanate/mol. Renaturation, and the production of OAR, occurs regardless of the oxidation state of the disulphide bonds, of phosphorylation of the protein, and of the presence or the absence of the single carbohydrate chain. OAR may be either monomeric or an irreversible aggregate. Which of these two states is formed depends on the protein concentration during renaturation. Monomeric and aggregated OAR can be distinguished on the basis of some spectroscopic characteristics, but they share the essential hydrophobic characteristics that distinguish them from OAN. OAN and OAR do not spontaneously interconvert. Antibodies raised to each can be made monospecific by immunoabsorption. Thus two stable forms of OA can be obtained, one of which, OAR, displays hydrophobic characteristics. OAN, but not OAR, is formed when OA is synthesized *in vitro* in a translation system.

It is not yet known how proteins fold to attain their 'native' state during or after biosynthesis. Physical-chemical aspects of protein folding have generally been approached experimentally by the use of denaturants (Tanford, 1968). However, renaturation in the test tube after removal of the denaturant is often a slow and inefficient process, especially for proteins of high molecular weight. In contrast, the biosynthetic formation of native protein is rapid and highly efficient (Lapanje, 1978). This difference raises the question whether the pathway(s) em-

ployed *in vivo* for finding the native conformation of some proteins are peculiar to the biosynthetic process. There has been a controversy in the literature over the past 30 years concerning the ability to renature OA after chemical denaturation. Many authors have noted the difficulty in regaining native OA (OAN) (Simpson & Kauzmann, 1953; Schellman *et al.*, 1953; Imahori, 1960; Holt & Creeth, 1972), but others have claimed that OAN can be recovered (Ahmad & Salahuddin, 1976). The folding of OA is of some interest biologically because OA, although a secretory protein, has no classical *N*-terminal signal sequence, i.e. no hydrophobic part of the protein is cleaved off during or

Abbreviations used: OA, ovalbumin; OAN, ovalbumin, native; OAR, ovalbumin, renatured.

after secretion of the molecule across the rough endoplasmic reticulum. If hydrophobic leader sequences are indeed required to initiate translocation across lipid bilayers, how then is ovalbumin translocated? Lingappa *et al.* (1979) proposed that ovalbumin possesses an internal hydrophobic 'signal' sequence, located quite far from the *N*-terminal. According to their scheme, the first 200–250 amino acids are already synthesized (presumably accompanied by folding of the peptide chain) before appearance of the hydrophobic sequence, which mediates interaction of the chain with the appropriate membrane. Evidence has since been presented that the *N*-terminus of the nascent OA chain acts as the leader sequence (Meek *et al.*, 1980; Braell & Lodish, 1982). Furthermore, we have shown (Kempf *et al.*, 1982) that the functional leader sequence of OA is located near the *N*-terminus but that this region gets sequestered as the protein becomes fully synthesized. Native OA does not possess an exposed leader sequence, and thus such a sequence must ultimately be folded into the interior of the molecule in the completed protein (Lingappa *et al.*, 1979). These authors claimed that such a sequence could be exposed on denaturation with urea.

Because of these considerations with respect to the biosynthetic folding of OA, we were intrigued by the consistent finding of relatively water-insoluble (hydrophobic) forms of the protein on spontaneous refolding from the denatured state. In the present study we report that renaturation of OA from the denatured state invariably results in the formation of a stable but altered form of the protein, 'renatured ovalbumin' (OAR), and that this form of the protein can be distinguished from the native form by a variety of physical-chemical criteria.

Materials and methods

Protein

OA was obtained from Miles Laboratories (Elkhart, IN, U.S.A.) and purified on concanavalin A–Sephadex 4B (Pharmacia, Piscataway, NJ, U.S.A.). The protein was then free of any detectable contaminant, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and by Ouchterlony immunoprecipitation with rabbit anti-OA antibody. All experiments described were done with OA purified in this way.

Denaturation and renaturation

Solutions of native ovalbumin (OAN) were made up fresh from freeze-dried desalted protein preparations that were kept at -20°C . Usually (unless specified otherwise in the text or legends to Figures) the protein was dissolved to a concentration of up to 20 mg/ml in phosphate-buffered saline (134 mM-

NaCl/3 mM-KCl/6.7 mM-sodium phosphate buffer, pH 7.2). Such solutions were found to remain stable for weeks without any detectable change in physical properties.

For denaturation of OA, an amount of freeze-dried protein was added to a solution of either 8 M-urea or 6 M-guanidinium chloride (both from Bethesda Research Laboratories, Bethesda, MD, U.S.A.), each in phosphate-buffered saline. All denaturant solutions were prefiltered through 0.45 μm Nalgene filters. The urea solutions were made up fresh each day. In general, the protein was kept in the denaturant for 30–60 min before renaturation was started.

Renaturation was performed by using a variety of techniques. Most frequently, the solution of denatured protein was placed inside Spectrapor no. 3 dialysis tubing (M_r cut-off 3000–8000) and dialysed against 3 vol. of a 500-fold excess of phosphate-buffered saline, at room temperature. The dialysis tubing was boiled in buffer before use. Renaturation through rate-controlled removal of the denaturant was accomplished by dialysis against a solution in which the guanidinium chloride concentration was gradually and linearly lowered to less than 10 mM by adding phosphate-buffered saline at any desired rate (over periods of minutes or less to up to 3 days), while removing the overflow from the dialysis vessel. No differences in OAR yield or characteristics as a function of the speed of dialysis were observed. OAR was also produced by rapid dilution of the OA solution in guanidinium chloride into phosphate-buffered saline, and by passing denatured OA over a Sephadex G-25 (medium grade) column with phosphate-buffered saline as elution buffer.

Spectroscopy

All fluorescence measurements were made with a Perkin–Elmer (Norwalk, CT, U.S.A.) model MPF-44B spectrofluorimeter interfaced to a Bascom–Turner (Newton, MA, U.S.A.) microcomputer. Corrected spectra were obtained by using a Rhodamine B standard. Quantum yields were determined relative to quinine sulphate (quantum yield 0.54; $\lambda_{\text{exc.}}$ 352 nm) in 0.05 M- H_2SO_4 . Fluorescence as a function of temperature was studied with the use of a circulating water bath. The temperature was monitored with a digital temperature probe (Bailey, Saddlebrook, NJ, U.S.A.). Absorbance measurements were performed on a Gilford spectrophotometer. All c.d. measurements were made with a Jasco 500-A (Japan Spectroscopy, Easton, MD, U.S.A.) spectropolarimeter, at 25°C . A circular quartz cell with a 1 mm path-length was used.

Probes and labelling

8-Anilino-naphthalene-1-sulphonate (Kodak, Rochester, NY, U.S.A.) was dissolved in 100% ethanol to a concentration of 100 mM and kept protected from

light. 8-Anilino-naphthalene-1-sulphonate was added to protein solutions either directly from the 100 mM stock solution or from dilutions thereof. The maximum ethanol concentration in the protein solution was always lower than 0.66% (v/v); protein samples without 8-anilino-naphthalene-1-sulphonate but with ethanol served as controls. The fluorescence of 8-anilino-naphthalene-1-sulphonate was measured with excitation at either 290 nm or 375 nm. Emission spectra were obtained by scanning over the range 300–600 nm.

Phenyl isothio[^{14}C]cyanate was obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.), and had a specific radioactivity of 7.1 Ci/mol. Phenyl isothio[^{14}C]cyanate (0.1 M in ethanol) was added to the protein solution (0.5 mg/ml in phosphate-buffered saline) to a final concentration of 1 mM. The reaction mixture was then incubated for 1 h at 37°C under continuous stirring. Excess phenyl isothio[^{14}C]cyanate was removed either by precipitating the protein with 10% (w/v) trichloroacetic acid and washing with ethanol, or by extensive dialysis against phosphate-buffered saline containing SM-2 Bio-Beads (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The amount of phenyl isothiocyanate incorporated per mg of protein was determined by counting the ^{14}C radioactivity.

Viscosity measurements

Concanavalin A–Sepharose-purified OAN was denatured in 8 M-guanidinium chloride. A portion was renatured to OAR at a concentration of 0.1 mg/ml by dialysis (in Spectrapor no. 1 tubing) against phosphate-buffered saline containing 1 mM- NaN_3 and then concentrated to 1 mg/ml. OAN was dialysed in a second sac in the same bottle of dialysis medium. The final guanidinium chloride concentration was 1 M. A portion of the OAR was further dialysed to a guanidinium chloride concentration of 8 mM.

Measurements were made with an Ostwald viscometer whose time of fall for distilled water (t_0) was 225.6 s. The temperature was maintained at $25.0 \pm 0.1^\circ\text{C}$ by a thermostatically controlled water bath. Before use solutions were de-gassed under vacuum and centrifuged briefly at low speed to remove dust and large particles.

Separation of differently phosphorylated OAN species

The major species (OA1) in natural OA has two phosphorylated serine residues. Singly phosphorylated (OA2) and non-phosphorylated (OA3) proteins are minor species accounting for approx. 20% of the total OA. The three were separated by elution on a CM-cellulose column, essentially as described by Rhodes *et al.* (1958). The CM-cellulose (Cellex-CM, H^+ form; Bio-Rad Laboratories) was hydrated in

0.1 M-sodium acetate buffer, pH 4.0, and poured to form a 2.6 cm $\times$ 15 cm column. After the column had been washed with 4 vol. of acetate buffer, a 150 mg sample of OAN was added. Gradient elution was performed with 2 litres each of 0.1 M-sodium acetate buffer at pH 4 and pH 7.

Antibody preparations

Antisera were raised in two rabbits (nos. 845 and 847) by subcutaneous injection of OAN (0.5 mg in 1.5 ml) with complete Freund's adjuvant. Antisera were similarly raised in rabbits nos. 844 and 846 by injection of OAR (0.5 mg in 1.5 ml) together with the adjuvant. The OAR used had been renatured at 0.4 mg/ml, and therefore consisted of a mixture of monomeric and irreversibly aggregated forms (see below). Then 3 weeks later all animals were boosted with identical concentrations of antigen in 1.5 ml of incomplete Freund's adjuvant. Animals were bled 1 week after the boosting. Immunoglobulin G fractions were obtained from the sera by affinity chromatography on protein A–Sepharose CL-48 (Pharmacia, Piscataway, NJ, U.S.A.). The immunoglobulin G was eluted with 0.1 M-acetic acid, titrated to pH 7.4, and dialysed against phosphate-buffered saline containing 1 mM- NaN_3 .

Immunoabsorption of anti-OAR antibody with OAN

OAN was cross-linked to CNBr-activated Sepharose 4B (Pharmacia) essentially by the method described in the Pharmacia booklet *Affinity Chromatography, Principles and Methods* (1974). A 30 mg portion of OAN (not affinity-purified on concanavalin A–Sepharose) was dissolved in 30 ml of a 0.1 M- NaHCO_3 (pH 8.3) coupling buffer containing 0.5 M- NaCl . CNBr-activated Sepharose (3.2 g) was washed with 200 ml of 1 M-HCl and added. Coupling was allowed to proceed for 18 h at 2°C with end-over-end mixing. Any remaining coupling sites were filled by further incubation for 3 h with 1 M-glycine at pH 9. After three cycles of washing with coupling buffer and 0.5 M- NaCl /0.1 M-sodium acetate buffer, pH 4, the gel was resuspended in phosphate-buffered saline containing 1 mM- NaN_3 . Of the original OAN, 81% became coupled. Experiments with 8-anilino-naphthalene-1-sulphonate indicated no appreciable OAR-like hydrophobicity of the bound protein (see the Results and discussion section).

Protein A–Sepharose-purified rabbit anti-OAR antibody (1.8 mg in 0.2 ml) was eluted with phosphate-buffered saline on a 10 mm $\times$ 60 mm column of the OAN–Sepharose. The immunoglobulin G-containing fractions (anti-OAR_{abs} antibody) were pooled and concentrated to 32 mg/ml in an ultrafiltration apparatus (UH 100/2a; Schleicher and Schuell), with 25 000- M_r -cut-off membrane.

An attempt at similar absorption of anti-OAN antibody on an OAR–Sephacrose column resulted in loss of almost all reactivity towards OAN or OAR. The OAR used in coupling was probably contaminated with enough OAN to absorb the antibody.

Ouchterlony double-diffusion assay

Gels were made from 1.5% agarose (type A45; Pharmindustrial, Clichy, France) in a barbital buffer, pH 8.3. After addition of antibody and antigens, diffusion was allowed to proceed at room temperature overnight. The precipitin lines were checked with Coomassie Blue staining (not shown).

Cell-free translation system

Translation of ovalbumin mRNA (kindly given by Dr. Meek and Dr. Palmiter, University of Washington, Seattle, WA, U.S.A.) was conducted in a rabbit reticulocyte-lysate cell-free translation system (New England Nuclear, Boston, MA, U.S.A.) essentially as described in the booklet that comes with it.

Immunoprecipitation of ovalbumin synthesized in vitro

Appropriate dilutions of antibody and 60 min product (consisting almost entirely of completed ovalbumin chains, as indicated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) were incubated together on ice for 3 h in phosphate-buffered saline. A binding excess of fixed *Staphylococcus aureus* A (Pansorbin; Calbiochem, San Diego, CA, U.S.A.) was added to the incubation mixture. After 90 min on ice for binding, the Pansorbin was washed twice with 10 mM-Tris/HCl buffer, pH 7.2, containing 0.15 M-NaCl, 1 mg of methionine/ml and 0.25% Nonidet P-40 detergent. The Pansorbin was then resuspended in 0.5 ml of buffer for counting of ^{35}S radioactivity.

Other methods

Protein was determined in the presence of 0.1% sodium dodecyl sulphate by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by using the system of Laemmli (1970).

Results and discussion

It has been observed previously that OA fails to reassume its native conformation when allowed to renature from a fully denatured state (Simpson & Kauzmann, 1953; Imahori, 1960). In the present study we confirm the observation and characterize the renatured form of the protein, using the following parameters: intrinsic viscosity, tryptophan, fluor-

escence, accessibility of tryptophan to aqueous collisional quenchers, u.v. absorption, affinity for various labels, affinity for hydrophobic resins, sedimentation behaviour, effect of post-translational modifications (carbohydrate, phosphate), and antigenic properties.

Intrinsic viscosity

Measurements of viscosity have been used extensively to monitor the dramatic hydrodynamic changes that accompany denaturation and refolding of globular proteins. The literature on OA contains claims that it does (Ahmad & Salahuddin, 1976) or does not (Holt & Creeth, 1972) regain its original viscosity after denaturation–renaturation cycles with guanidinium chloride. We repeated these measurements to determine whether our preparations would appear, by this rough measure, to have renatured reversibly.

Reduced viscosity ($\eta_{\text{red.}}$) can be calculated from the expression (Tanford, 1955):

$$\eta_{\text{red.}} = \eta(t - t_0)/t_0c + (1 - \bar{v}\rho_0)/\rho_0 \quad (1)$$

where c is the concentration in g/ml, t and t_0 are the times of fall for sample and solvent respectively, $\bar{v}$ is the partial specific volume of the protein and ρ_0 is the density of the solvent. Values of t and t_0 were taken as the averages from at least six experiments. However, given reasonable approximations to $\bar{v}$ and ρ_0 , the second term in eqn. (1) contributes no more than a few percent and hence was neglected in the calculation of $\eta_{\text{red.}}$. Then the intrinsic viscosity $[\eta]$ was approximated from iterative solutions of the expression:

$$\eta_{\text{red.}} = [\eta] + k[\eta]^2c \quad (2)$$

where k is the Huggins constant. For denatured OA we used the value $k = 0.31$ found by Ahmad & Salahuddin (1974) for denatured OA in 6 M-guanidinium chloride and 0.1 M-2-mercaptoethanol. For OAN and for OAR, the term $k[\eta]^2c$ is negligible, since the protein concentration in the experiments was 1 mg/ml. The results of these studies are shown in Table 1. The values obtained for both OAN and denatured OA are in excellent agreement with literature values, and reveal the expected dramatic unfolding of the protein. When the guanidinium chloride concentration in the solution of denatured OA is lowered to 1 M by dialysis (see the Materials and methods section), an intrinsic viscosity similar to that of the native protein is found. Further lowering of the guanidinium chloride concentration to 8 mM yields no further discernible change in viscosity. It appears, then, that OAN unfolds within a guanidinium chloride concentration range of 1–6 M and regains its original hydrodynamic properties as the guanidinium chloride concentration is returned to 1 M or below.

Table 1. *Determination of the intrinsic viscosities of native, denatured and renatured OA*
Values are given as means $\pm$ S.E.M. for at least six experimental determinations.

	t_0	t	$\eta_{red.}$	$[\eta]$	
OA, denatured	441.28 $\pm$ 0.10	456.03 $\pm$ 0.33	32.1 $\pm$ 0.73	31.8 $\pm$ 0.73	(27.0*)
OAN (1 M-guanidinium chloride)	234.36 $\pm$ 0.10	235.33 $\pm$ 0.10	4.0 $\pm$ 0.57	4.0 $\pm$ 0.57	(3.9*)
OAR (1 M-guanidinium chloride)	234.36 $\pm$ 0.10	234.97 $\pm$ 0.10	3.7 $\pm$ 0.83	3.7 $\pm$ 0.83	
OAR (8 mM-guanidinium hydrochloride)	231.14 $\pm$ 0.09	232.16 $\pm$ 0.23	4.1 $\pm$ 0.98	4.1 $\pm$ 0.98	

* Literature values taken from Ahmad & Salahuddin (1974).

Ahmad & Salahuddin (1976) have explained the discrepancy in viscosities obtained for renatured OA in terms of microheterogeneity of the protein. They suggest that higher viscosities may be obtained after renaturation if non-phosphorylated and singly phosphorylated OA are not first separated from the major (doubly phosphorylated) species. We find no evidence for anomalously high viscosities in the mixture. In the following sections we show that, despite the roughly identical values of intrinsic viscosity obtained for the two different states of the protein, OAN and OAR differ markedly in other physical-chemical properties, including their apparent hydrophobicity and their state of aggregation.

Hydrophobic affinity chromatography

OAN was found to bind to phenyl-Sepharose in phosphate-buffered saline. Less than 5% of the applied protein mass was recovered from the column, even after 20 column volumes of buffer had been passed through the bed. When the salt concentration of the elution buffer was lowered 10-fold, 98% of the OAN could be recovered from the column. OAR, on the other hand, was entirely (>98%) retained on the column under these conditions. To test whether this difference in affinity could reliably be used to quantify the amount of OAN or OAR in a solution of ovalbumin, we mixed known quantities of OAN with OAR at ratios ranging from 1:10 to 2:1 (OAN/OAR). With any of these mixtures all (>95%) of the amount of applied OAN was recovered from the column. There was no evidence of any OAR being eluted from the gel, as judged by the spectroscopic criteria described below. We were unable to elute OAR from the column with either water or 50% (v/v) ethylene glycol in water. Elution with 2% (w/v) sodium dodecyl sulphate resulted in the recovery of about 30% of the protein, and if 8 M-urea was included with the detergent then between 40% and 60% could be recovered. From the results of these experiments we calculated that the OAR preparation contained less than 2% OAN. The 'yield' of OAR on renaturation was always greater than 98%, irrespective of the concentration of OA during renaturation (range 1 mg/ml to 10 ng/ml).

Tryptophan studies

Examination of the fluorescence properties of the three tryptophan residues within the OA molecule reveals a number of differences between the native and the renatured form. The emission and excitation spectra of OAR differ slightly from those of OAN. With the use of quinine sulphate as a standard and with correction of all spectra with a Rhodamine B standard, the average quantum yield of each tryptophan residue in OAN is found to be 0.18, whereas for OAR it is only 0.14. The quenching of tryptophan fluorescence by the negatively charged aqueous collisional quencher I^- is much more pronounced with OAR than with OAN (Fig. 1). The latter result suggests that in OAR one or more of its tryptophan residues are more accessible to the quencher as a result of changes in the three-dimensional structure of the protein.

Labelling of OAR and OAN

The fluorescent probe 8-anilino-naphthalene-1-sulphonate binds to many proteins. This binding is accompanied by an increase in fluorescence quantum yield of the probe when compared with that of the probe in water. The hydrophobic nature of the binding sites for 8-anilino-naphthalene-1-sulphonate is documented by an increased quantum yield of the probe and a blue-shift in its emission spectrum. In contrast with many highly hydrophilic proteins, OAN causes essentially no alteration of fluorescence when compared with aqueous buffer alone (in aqueous solution 8-anilino-naphthalene-1-sulphonate fluoresces only very weakly, with an emission maximum at 520–530 nm). If 8-anilino-naphthalene-1-sulphonate is mixed with an aqueous solution of OAR, however, a large increase in the 8-anilino-naphthalene-1-sulphonate fluorescence intensity and a blue-shift of its emission maximum to 480 nm are observed. The data shown in Fig. 2 were obtained at a probe/protein molar ratio of 2:1. When we measure the 8-anilino-naphthalene-1-sulphonate fluorescence intensity as a function of the probe/protein molar ratio, the intensity is found to increase until a probe/protein molar ratio of 2:1 is reached. A greater ratio does not result in a further

Fig. 1. Quenching of tryptophan fluorescence of OA by KI

Fluorescence intensity was measured by exciting at 280 nm and measuring the integrated emission from 310 nm to 400 nm. Each addition of KI represented a 1:150 volume dilution. □, OAN (1 mg/ml); Δ, OAR (0.1 mg/ml); ○, OAR (1 mg/ml) (concentrations of OAR represent concentrations during renaturation).

Table 2. Spectroscopic differences between OAN and OAR

	OAN	OAR
Tryptophan fluorescence		
Quantum yield	0.18	0.14
Emission maximum (nm)	337	341.5
Excitation spectrum (I_{280}/I_{290})	1.09	1.27
Absorption		
ϵ_{280} ($M^{-1} \cdot cm^{-1}$)	30.9×10^3	29.6×10^3
A_{275}/A_{290} ratio	1.49	1.81
Circular dichroism		
$[\theta]_M$ (222 nm)	-8960	-9296
$[\theta]_M$ (208 nm)	-7420	-10864
8-Anilidonaphthalene-1-sulphonate binding (mol/mol of protein)	Not detectable	2.0

increase in fluorescence intensity. This suggests that a maximum of two molecules of 8-anilidonaphthalene-1-sulphonate bind per molecule of OAR. The 8-anilidonaphthalene-1-sulphonate fluorescence effect is independent of the OAR concentration; at a given probe/protein molar ratio (<2:1) the 8-anilidonaphthalene-1-sulphonate fluor-

Fig. 2. 8-Anilidonaphthalene-1-sulphonate fluorescence at 480 nm (excitation at 290 nm) as a function of probe/protein molar ratio

The 8-anilidonaphthalene-1-sulphonate/ethanol solution was diluted 200-fold into the protein solution. 8-Anilidonaphthalene-1-sulphonate fluorescence at 480 nm in the absence of protein was subtracted for each point.

escence varies linearly with the OAR concentration over a 100-fold concentration range. When OAR and OAN are mixed in equimolar amounts and subsequently labelled with 8-anilidonaphthalene-1-sulphonate (at a probe/total OA molar ratio of 4:1), the fluorescence obtained is exactly one-half of that seen if the same amount of protein consists solely of OAR. We use the highly reproducible high quantum yield of 8-anilidonaphthalene-1-sulphonate in the presence of OAR, and its linear dependence on the OAR concentration as a routine check for possible cross-contamination of OAN with OAR.

The 8-anilidonaphthalene-1-sulphonate binding studies complement the hydrophobic affinity chromatography. Both strongly suggest that one or more hydrophobic sites are exposed in OAR but not in OAN.

Phenyl isothiocyanate, a hydrophobic label for membrane-buried protein segments (Kempf *et al.*, 1981), reacts with nucleophilic groups only in their deprotonated form (Drobnica *et al.*, 1977). At pH 7.2 all nucleophilic groups of amino acid side chains in a protein are protonated unless they are located in a hydrophobic environment. They are therefore not accessible to aqueous solution and protonation. Ovalbumin in its native form binds only an insignificant amount of phenyl isothiocyanate (0.3 ± 0.2 mol/mol). In contrast, OAR binds

2.0 ± 0.3 mol of phenyl isothiocyanate/mol of protein. The most straightforward interpretation of this finding is as follows. During denaturation and subsequent renaturation the protein undergoes a conformational change in a way such that some nucleophilic groups exist in a deprotonated and therefore reactive form. Such groups, in a deprotonated state, would most probably constitute a hydrophobic region. The specific labelling of each OAR molecule with two phenyl isothiocyanate moieties provides the possibility of locating specific hydrophobic sites newly 'exposed' in OAR.

Work by Braell & Lodish (1982) as well as data from our own laboratory (Kempf *et al.*, 1982) have corroborated the evidence obtained by Meek *et al.* (1980), that the *N*-terminal region of the ovalbumin molecule acts as the functional leader sequence during biosynthesis. We have also obtained results pointing to the possibility that in OAR leader-sequence-type activity is 'exposed' (Kempf *et al.*, 1982): (1) OAR, but not OAN, binds to rough endoplasmic reticulum, whereas no significant binding of either type of OA to other cellular membranes is seen; (2) OAR, but not OAN or bovine serum albumin (either native or renatured), blocks the binding of a pre-protein (pre- κ -light chain) to rough endoplasmic reticulum. At present, we cannot indicate whether this leader-sequence-type activity 'exposed' in OAR is, in fact, located in the *N*-terminal region of the protein.

Aggregation of OAR

It has been noted previously that OA tends to aggregate during renaturation (Gottschalk, 1972). We were concerned that the many differences thus far described between OAR and OAN were due to the tendency of ovalbumin to form aggregates during renaturation. We therefore examined the state of aggregation of OAN and OAR by using molecular-sieve chromatography on Sephacryl S-300 (Pharmacia) columns. The results of these studies are shown in Fig. 3. OAN showed no tendency to form aggregates at any concentration studied (from 0.1 to 10 mg/ml). Equilibrium sedimentation measurements (kindly performed by Dr. J. Osborne) also showed a homogeneous protein of M_r 43000. On the other hand, when OA was renatured at a protein concentration of 1 mg/ml, chromatography on Sephacryl showed virtually all of the protein to be present in aggregates (Fig. 3). This result was confirmed by ultracentrifugation. When we made a 10-fold dilution of the OAR formed at 1 mg/ml, there was no change in the chromatographic profile. This finding again indicated that the aggregation was irreversible. Formation of those aggregates turned out to be critically and reproducibly dependent on the protein concentration during renaturation. When OA was

Fig. 3. Sephacryl S-300 gel chromatography of OAN and OAR

OAN at a concentration of 1 mg/ml (---○), OAR renatured at 0.1 mg/ml (---△) and OAR renatured at 1 mg/ml (—○) were, each in 2 ml of phosphate-buffered saline, applied to a 1.5 cm × 50 cm column of Sephacryl S-300, eluted with phosphate-buffered saline and collected in 2 ml fractions. The absorbance at 280 nm was monitored and is shown as the normalized absorbance (highest value for each sample = 1.0). Protein concentration measured by the Lowry *et al.* (1951) assay method showed that more than 90% of each sample was recovered.

renatured at concentrations of 0.1 mg/ml or less, virtually 100% of the protein was monomeric (Fig. 3). No aggregation was observed when the protein was subsequently concentrated 10-fold to 1 mg/ml. Renaturation at intermediate concentrations of protein produced mixes of monomeric and aggregated protein in variable ratios. At 0.5 mg/ml, for example, about 50% of the renatured protein was monomeric. Interestingly, the extent of aggregation seen at 1 mg/ml appeared to be independent of the speed of renaturation.

Characteristics of monomeric and aggregated OAR

Monomeric OAR was indistinguishable from aggregated OAR, as determined by phenyl-Sepharose affinity chromatography. We compared other physical-chemical characteristics of monomeric OAR (formed at 0.1 mg/ml) and aggregated OAR (formed at 1 mg/ml). The quantum yields of intrinsic tryptophan fluorescence are identical, as are the absorption coefficients at either 280 or 290 nm. Both forms of OAR show increased collisional quenching constants with I^- , but, as shown in Fig. 1, aggregated OAR is somewhat more readily quenched. It is noteworthy that virtually all of the labelling techniques used to distinguish OAR from OAN give identical results for aggregated and

monomeric OAR. One difference between monomeric and aggregated OAR is seen with 8-anilino-naphthalene-1-sulphonate fluorescence. At probe/protein molar ratios of 2:1 monomeric OAR displays markedly enhanced 8-anilino-naphthalene-1-sulphonate fluorescence when compared with OAN. However, the fluorescence intensity is only 80% of that seen with aggregated OAR.

Influence of disulphide bonds on the formation of OAR

Disulphide bonds play a significant role in maintaining the structure of many proteins. Reducing the disulphide groups in OAN with either 2-mercaptoethanol or dithiothreitol did not result in the appearance of any of the characteristics of OAR. Furthermore, the presence of glutathione during renaturation of OA from guanidinium chloride did not inhibit the formation of OAR. Similarly, when OA was denatured in guanidinium chloride in the presence of dithiothreitol, followed by removal of dithiothreitol (dialysis against 8 M-guanidinium chloride) and renaturation in the presence of either O₂ or glutathione, formation of OAR was not inhibited.

These observations lead to two conclusions: first, the transition of OAN to OAR (as defined by the criteria presented here) occurs irrespective of the presence of intact or broken disulphide bonds within the protein molecule during renaturation, and, secondly, if denatured OA with intact disulphide groups is conformationally different from denatured OA with reduced disulphide groups, then the folding pathway taken from either configuration apparently leads to molecules with the properties of OAR.

Effect of the proteins' carbohydrate and phosphate residues on formation of OAR

After formation of its peptide backbone *in vivo*, ovalbumin is covalently modified in two ways. A polymannose side chain is attached to an asparagine residue; this carbohydrate moiety is structurally polymorphous and consists of five or six mannose residues (Marshall & Neuberger, 1972). We treated OAN with a mixture of endoglycosidases H and D and jack-bean mannosidase. The effectiveness of demannosylation was assessed by using a coupled enzymic assay for free mannose residues (Finch *et al.*, 1969). We were able to remove 3–4 mannose residues, on the average, per protein molecule. This degree of carbohydrate stripping of the native molecule had absolutely no effect on the formation of OAR, as judged by 8-anilino-naphthalene-1-sulphonate binding, tryptophan fluorescence and interaction with phenyl-Sepharose.

Ovalbumin species with different degrees of phosphorylation separated from each other on a

CM-cellulose column as described by Rhodes *et al.* (1958) all showed the same renaturation behaviour.

Immunological distinction between OAR and OAN

Differences in the secondary, tertiary or quaternary structure of proteins can lead to different immunological reactivities. We raised rabbit antisera to OAN and OAR and used them to probe structural differences between the two. Immunoglobulin G fractions were obtained by affinity chromatography on protein A-Sepharose and tested by Ouchterlony double-diffusion analysis. These fractions gave qualitatively the same precipitation patterns as did the whole antisera, and were used in all of the experiments reported here. Unless otherwise stated, the OAR had been renatured at 0.1–0.2 mg/ml and was therefore principally monomeric. All OA used for immunological studies (except that used for the immunizations themselves) was concanavalin A-Sepharose-purified. Non-purified OAN showed the same reactivity and one additional faint precipitin line. One rabbit (no. 845) immunized with OAN produced antibody strongly reactive to OAN but with very little reactivity to OAR (Fig. 4a). This striking difference suggests that the antibody is directed to a quite restricted set of determinants on the OAN. The pre-immunization serum showed no discernible reaction towards either OAN or OAR (Fig. 4b). A second animal (no. 847) produced antibody reactive towards OAN and, to a smaller extent, to OAR. Spur formation indicated determinants on OAN not expressed on OAR, but not the reverse (gel not shown). One antiserum (no. 846) raised to OAR contained antibody poorly reactive towards either form of OA; a second (no. 844) reacted strongly to both, as shown in Figs. 4(c) and 4(e). Fig. 4(d) shows the results with anti-OAR_{abs.} antibody. Since the concentrations of relevant antibody in each preparation are not known, direct comparison of Figs. 4(c) and 4(d) does not conclusively prove that the absorption enriched the preparation with antibody reactive towards determinants exclusively on OAR. However, gels similar to that shown in Fig. 4(c) but with progressively less antibody proved the point: at no concentration did the reaction towards OAR predominate as it does with the absorbed antibody.

We tested whether the completed product of translation *in vitro* resembles OAR, OAN or neither in its immunological characteristics. Fig. 5 shows the results obtained with anti-OAN antibody and anti-OAR_{abs.} antibody (i.e. an anti-OAR antibody preparation absorbed on OAN-Sepharose). Both antibodies had been purified on protein A. On the basis of total immunoglobulin G present, the anti-OAN antibody precipitated the 60 min product at more than three orders of magnitude lower

Fig. 4. Ouchterlony double-diffusion analysis of anti-OA antibody preparations

(a) Anti-OAN antibody (no. 847) interacts strongly with OAN (N) but not with OAR (R). (b) The corresponding pre-immunization immunoglobulin G (IgG) does not react visibly with either antigen. (c) Anti-OAR antibody (no. 844) reacts strongly with both OAR and OAN; spurs indicate that determinants recognized on OAR are not present on OAN. (d) Anti-OAR antibody (no. 844) absorbed with OAN-Sepharose (anti-OAN_{abs.} antibody) reacts with OAR but not with OAN. (e) Anti-OAR antibody (no. 844) gives no precipitin line with aggregated OAR (R'), either because the aggregated form differs antigenically or because it fails to enter the gel. Quantities of antibody and antigen: (a) 50 μ g of total immunoglobulin G, 1.5 μ g of antigen; (b) 42 μ g of total immunoglobulin G, 1.5 μ g of antigen; (c) 135 μ g of total immunoglobulin G, 4 μ g of antigen; (d) 480 μ g of total immunoglobulin G, 4 μ g of antigen; (e) 87 μ g of total immunoglobulin G, 1 μ g of antigen. Aggregated OAR was dialysed at 1.0 mg/ml rather than at 0.1 mg/ml.

Fig. 5. Immunoprecipitation of 60 min OA-mRNA translation product by anti-OAN antibody (O) and anti-OAR_{abs.} antibody (Δ)

The translation product was immunoprecipitated with fixed *Staphylococcus aureus* A as described in the Materials and methods section.

precipitate the product. Since both the anti-OAN antibody and the anti-OAR_{abs.} antibody gave precipitin lines on Ouchterlony plates, it seemed highly unlikely that differences in content of relevant antibody could account for the full three-order-of-magnitude difference. Competition studies demonstrated the point in that OAN was over an order of magnitude more effective than OAR at competing with translation product for reaction with anti-OAN antibody.

These findings suggest two major conclusions. (i) The product of translation *in vitro* can fold rapidly into something resembling the mature OAN molecule in the character of its exposed determinants. These observations suggest that formation of OAN requires sequential folding during translation. (ii) Microsomal fraction is not required for this folding. One might have supposed that membrane-associated machinery, chemical constituents inside the saccule or perhaps geometric consequences of passage across the membrane would be necessary. None of these seems to be.

concentration than did the anti-OAR_{abs.} antibody. Anti-OAR_{abs.} antibody did not differ greatly from normal rabbit immunoglobulin G in its ability to

Further observations on formation and stability of OAR

Renaturation of OA from urea resulted in the formation of OAR, according to the criteria already described. We needed 8 M-urea to obtain complete denaturation (with guanidinium chloride we needed only 5 M). Treatment of OAN with NaOH (to pH 10.8) for 5 min at 25°C, with subsequent back-titration to neutral pH (7.4), resulted in the formation of OAR. In contrast, treatment with HCl (pH < 2; 5 min at 25°C) did not yield OAR on back-titration.

Formation of OAR (from guanidinium chloride-denatured OA) occurred equally well at 4, 25 or 37°C. Its formation was not influenced by the presence of 10 mM-Ca²⁺, and it was independent of ionic strength from 0.0001 M- to 0.2 M-NaCl. Furthermore, when either OAN or OAR in buffer was incubated at 4, 25 or 42°C, there was no evidence of interconversion of forms over several weeks. Neither freeze-drying nor freezing changed OAN into OAR or vice versa.

Concluding remark

Remaining is the question: how does ovalbumin actually attain its native state? From the present study it seems clear that, if one allows ovalbumin to fold from a random-coil state, the resulting conformation is always that of OAR. This implies that biosynthetic folding follows a 'preferred' pathway, which leads to a 'unique' native state. The steps of this pathway are presumably linked to the process of biosynthesis itself in a highly sequential manner.

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References

- Ahmad, F. & Salahuddin, A. (1974) *Biochemistry* **13**, 245–249
- Ahmad, F. & Salahuddin, A. (1976) *Biochemistry* **15**, 5168–5175
- Braell, W. A. & Lodish, H. F. (1982) *J. Biol. Chem.* **257**, 4578–4582
- Drobnica, L., Kristian, P. & Augustin, J. (1977) in *The Chemistry of Cyanates and their Thioderivatives* (Patai, S., ed.), part 2, pp. 1002–1222, John Wiley and Sons, New York
- Finch, P. R., Yuen, R., Schachter, H. & Moscarello, M. A. (1969) *Anal. Biochem.* **31**, 296–305
- Holt, J. C. & Creeth, J. M. (1972) *Biochem. J.* **129**, 665–676
- Imahori, K. (1960) *Biochim. Biophys. Acta* **37**, 336–341
- Kempf, C., Brock, C., Sigrist, H., Tanner, M. J. A. & Zahler, P. (1981) *Biochim. Biophys. Acta* **641**, 88–98
- Kempf, C., Van Renswoude, J., Weinstein, J. N., Blumenthal, R. & Klausner, R. D. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 1191
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–682
- Lapanje, S. (1978) *Physicochemical Aspects of Protein Denaturation*, John Wiley and Sons, New York
- Lingappa, V. R., Lingappa, J. R. & Blobel, G. (1979) *Nature (London)* **281**, 117–121
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Marshall, R. D. & Neuberger, A. (1972) in *Glycoproteins* (Gottschalk, A., ed.), vol. 5, part B, pp. 732–757, Elsevier, Amsterdam
- Meek, R. L., Walsh, K. A. & Palmiter, R. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 1867
- Rhodes, M. B., Azari, P. R. & Feeney, R. E. (1958) *J. Biol. Chem.* **230**, 399–408
- Schellman, J., Simpson, R. B. & Kauzmann, W. (1953) *J. Am. Chem. Soc.* **75**, 5152–5154
- Simpson, R. B. & Kauzmann, W. (1953) *J. Am. Chem. Soc.* **75**, 5139–5152
- Tanford, C. (1955) *J. Phys. Chem.* **59**, 798–799
- Tanford, C. (1968) *Adv. Protein Chem.* **23**, 121–282