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A comparison of the immunogenicity of the native and denatured forms of a protein

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The effect of heat denaturation on the physicochemical and immunological properties of a model protein, ovalbumin, and its formaldehyde/lysine-treated form was investigated. Polyacrylamide gel electrophoresis and gel filtration showed that heat denaturation converted ovalbumin to high Mr polymers, whereas formaldehyde/lysine-treated ovalbumin remained monomeric with only a small proportion forming oligomers. NMR analysis demonstrated that non-denatured structures could easily be differentiated from the denatured structures. Intraperitoneal immunization of rabbits and mice showed that both native and denatured forms of ovalbumin induced an immune response, but denatured forms of ovalbumin were found to be less immunogenic and to have a lower epitope density than native ovalbumin. Analysis of the antisera in crossed immunoelectrophoresis showed that they were specific for either native or denatured forms of ovalbumin. These findings were further investigated by ELISA and immunoaffinity chromatography, and the high specificity and low cross-reactivity was confirmed. We conclude that the immunogenic epitopes on denatured ovalbumin are different from those on ovalbumin, and that these epitopes reflect a continuum of denatured conformations.

Key words: Immunogenicity; antigens; native; denatured; structure; ovalbumin.

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The immunological properties of denatured proteins have been the focus of much attention lately because of the relatively new concept of neoantigens and neoepitopes. Such structures may evolve during heat treatment or other manipulations of plasma proteins as an antivirus safety procedure before the proteins are used for therapy. It is important to be able to monitor such structural changes, and there is a need for relevant tools – *e.g.* antibodies – which will specifically detect neoepitopes. However, despite the theoretical and practical importance of neoantigens, the properties of neoepitopes have only been studied to a limited degree.

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Furthermore, a model system with the same polypeptide in its native and its denatured/unfolded conformation provides an ideal opportunity for characterizing immunological cross-reactivity at the molecular level. One aspect of this is a comparison of the reactivity of antibodies to supposedly sequence specific epitopes on a denatured protein with the reactivity against the same protein in its native conformation.

With the purpose of using antibodies to study the structure of denatured/unfolded proteins we have analysed the structure and immunogenicity of various denatured forms of ovalbumin and the specificity of the antibodies obtained. The immunogenicity of native and heat-denatured ovalbumin was investigated as well as the effect of prior formaldehyde/lysine treatment. Form-

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aldehyde/lysine treatment was chosen as it is a widely used method for inactivating toxic vaccine components and is highly effective in preventing precipitation during heat denaturation.

Ovalbumin was chosen as the model protein for these studies as it is a well-characterized single domain protein. Moreover, the native form of ovalbumin has been used as a model system for numerous immunological studies, and some of its B- and T-cell epitopes have been characterized (*Johnsen & Elsayed* 1990; *Elsayed et al.* 1988; *Falk et al.* 1993; *Rötzshke et al.* 1991; *Shastri & Gonzalez* 1993; *Carbone & Bevan 1989; Buus et al.* 1986). In contrast, the immunological properties of denatured ovalbumin have been less thoroughly studied. *Sette et al.* (1989) showed that denatured proteins are capable of direct interaction with empty MHC molecules without prior uptake and intracellular processing. *Ikura et al.* (1992) defined some epitopes on denatured ovalbumin by peptide mapping. *Mowat* (1985) and *Chesnut et al.* (1980) investigated B- and T-cell cross-reactivity of native and denatured ovalbumin. *Elsayed et al.* (1988) and *Kilshaw et al.* (1986) studied the reactivity of human sera against native and denatured ovalbumin, and found the highest reactivity against native ovalbumin.

MATERIALS AND METHODS

Chemicals

Ovalbumin, bovine serum albumin (BSA), lysine, sodium dodecylsulfate (SDS), tris(hydroxymethylamino)methane (Tris), N,N,N′,N′-tetramethylethylenediamine, glycerol, mercaptoethanol, urea, biotin-*N*-hydroxysuccinimide ester and Coomassie Brilliant Blue R-250 were from Sigma (St. Louis, USA). Aquous $Al(OH)_3$ gel (6 mg/ml) (Alhydrogel) was from Superfos (Vedbæk, Denmark). Formaldehyde (35%), Triton X-100, Tween-20, NaCl, KCl, NaOH, NaH_2PO_4, Na_2HPO_4, Na_2CO_3, $NaHCO_3$, acetic acid, propionic acid, citric acid, glycine, ethylene glycol, *N,N*-dimethylformamide, 5,5-diethylbarbituric acid and ethylenediaminetetraacetic acid (EDTA) were from Merck (Darmstadt, Germany). Acrylamide, bisacrylamide, ammonium persulfate, bromophenol blue, and molecular weight standard proteins were from Bio-Rad (Richmond, USA). CNBr-Sepharose, CH-Sepharose, Mono Q and Superose 12 columns were from Pharmacia (Uppsala, Sweden). *o*-phenylenediamine and streptavidin were from Kem-En-Tek (Copenhagen, Denmark). H_2O_2 was from Struers (Copenhagen, Denmark). H_2SO_4 and Freund's complete and incomplete adjuvants were from Statens Seruminstitut (Copenhagen, Denmark). Peroxidase conjugated rabbit immunoglobulins against mouse immunoglobulins (RaM^P) and peroxidase conjugated swine immunoglobulins against rabbit immunoglobulins (SaR^P) were from DAKO (Copenhagen, Denmark). Ethanol was from De Danske Spritfabrikker (Copenhagen, Denmark). Merthiolate was from Nomeco (Copenhagen, Denmark). Ethanolamine was from BDH Chemicals (Poole, England). D_2O was from Stohler (Germany). Dialysis bags were from Medicell (London, England). Maxisorb ELISA plates were from NUNC (Roskilde, Denmark). Filters (0.22 μm pore size) and Milli Q plus apparatus for water purification were from Millipore (Boston, USA).

Animals

Mice (strain AJxBALB/c or CF1xBALB/c) and rabbits (strain SSc:CPH) were from Statens Seruminstitut, Copenhagen, Denmark.

Buffers

Phosphate buffer (PB) was prepared by mixing 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 to a final pH of 7.4. Phosphate buffered saline (PBS) was prepared by adding NaCl to 8 mM phosphate buffer to a final concentration of 0.9%. Physiological saline was 0.9% NaCl. Citrate phosphate buffer was made by adjusting the pH of a 0.1 M citric acid, 0.5 M NaCl solution to 2.8 by the addition of solid Na_2HPO_4. Washing buffer for immunoassays consisted of 8 mM phosphate buffer, 0.5 M NaCl, 3 mM KCl, 1% Triton X-100, pH 7.2. Incubation buffer for immunoassays was made from washing buffer by adding BSA to 1%, Tween 20 to 0.05%, and finally adjusting pH to 7.2 with NaOH. Electrophoresis buffer for crossed immunoelectrophoresis (CIE) consisted of 20 mM 5,5-diethylbarbituric acid, 40 mM Tris, 0.5 mM calcium acetate, 3 mM sodium azide, pH 8.6.

Preparation of antigens.

Native ovalbumin (N) was dissolved in PBS and passed through 0.22 μm pore size filters before use. Heat-denatured ovalbumin (D) was prepared by dissolving ovalbumin in PB and autoclaving at 110°C for 1 h. After centrifugation (10,000 *g*, 30 min) the supernatant was dialyzed against PBS (3×1 L) and passed through a 0.22 μm pore size filter. Formaldehyde/lysine-treated ovalbumin (NF) was prepared by dissolving ovalbumin in PB followed by addition of 35% formaldehyde to a concentration of 0.025 M and lysine to a concentration of 0.025 M. The solution was incubated at 35°C with end-over-end agitation for 2 weeks. Finally the solution was centrifuged (10,000 g, 30 min), dialyzed against PB5 (3×1 L) and passed through a 0.22 μm pore-size filter. Heat-denatured formaldehyde/lysine-treated ovalbumin (DF) was prepared by autoclaving (110°C, 1 h) formaldehyde/lysine-treated ovalbumin before centrifugation

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and dialysis. Urea-denatured ovalbumin (UD) was prepared by dissolving ovalbumin at 1 mg/ml in 8 M urea in PB. After 1 h at room temperature the solution was dialyzed against increasing volumes of PBS, starting with one volume and finishing with 1 L. Biotinylation of antigens was carried out by mixing 0.5 ml antigen in PBS (2 mg/ml) with 0.5 ml 0.5 M $NaHCO_3$ and adding 10 µl biotin-*N*-hydroxysuccinimide ester in *N,N*-dimethylformamide (40 mg/ml). After 2 h incubation at room temperature with gentle agitation, the reaction mixture was dialyzed against PBS.

Immunizations of mice and rabbits

Antigens at the appropriate concentration were diluted with 0.9% NaCl, and $Al(OH)_3$ gel was added to a final concentration of 2 mg/ml followed by merthiolate to 0.02%. The vaccines were kept at 4°C. For intraperitoneal immunization mice received 0.5 ml vaccine at intervals of 14 days. For intravenous immunization 0.2 ml antigen in PBS was injected into a tail vein. At 10 days after each injection 0.2 ml of blood was taken from the lateral eye vein and collected in an Eppendorf tube containing EDTA. The blood was centrifuged for 5 min in an Eppendorf centrifuge (2000 *g*), and the plasma was collected and kept at −20 °C.

Rabbits were injected subcutaneously at 14- day intervals with vaccines prepared as described above and mixed with Freund's complete adjuvant (1:1) for the first injection and with Freund's incomplete adjuvant for the following injections. The rabbits were bled from the marginal ear vein 10 days after each injection, and the blood was allowed to stand overnight at 4°C, whereafter the serum was decanted and centrifuged (2000 *g*, 15 min). The supernatant was collected and stored at −20°C.

The titre of a serum is defined as the dilution factor giving half maximal response.

Enzyme-linked immunosorbent assay (ELISA)

Two types of assays were used. For antibody capture assays the antigens were coated directly onto the surface of the wells of microtitre plates, using 0.05 M carbonate buffer, pH 9.3, as coating buffer (3 µg/ml, 100 µl per well). For antigen capture assays the capturing antibody or streptavidin was coated onto the wells using the same carbonate buffer as above. Concentration of antibody was 10 µg/ml, whereas that of streptavidin was 0.3 µg/ml. Coatings were carried out overnight at 4°C or at room temperature for 2 h. After coating, the plates were washed three times in washing buffer. All subsequent incubations with antibodies, antigens or biotinylated antigens diluted in incubation buffer were carried out for 1 h at room temperature on a shaking table and were followed by three washes in washing buffer. Concentration of antigen was 0.05 µg/ml, both for biotinylated and for non-labelled antigen. The final incubation was with peroxidase-conjugated rabbit anti mouse immunoglobulins or swine anti-rabbit immunoglobulins diluted 1:1000. Bound antibodies were quantitated using 0.066 M Na_2HPO_4, 0.035 M citric acid, pH 5.0, with 0.4 µl 35% H_2O_2 and 0.4 mg *o*-phenylenediamine per ml as staining solution. After 30 min, 150 µl 1 M H_2SO_4 was added and the absorbance was measured (490 nm–650 nm). Absorbance measurements were done on a Thermomax microtitre plate reader (Molecular Devices, Menlo Park, CA, USA). Background was in all experiments <0.030 , which was subtracted from the values.

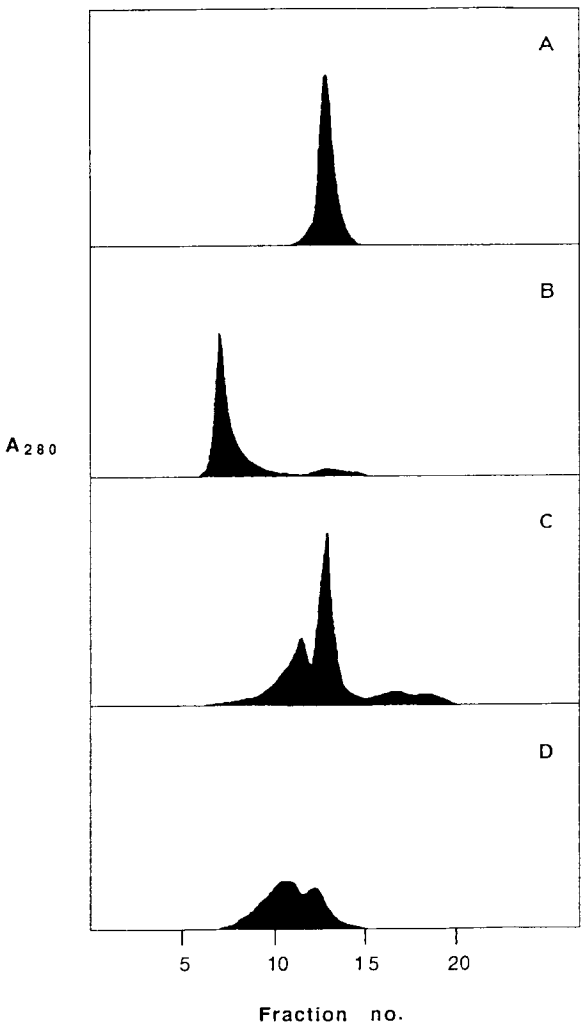

Fig. 1. Gel filtration analysis of ovalbumin forms. A. Native ovalbumin (N). B. Heat-denatured ovalbumin (D). C. Formaldehyde/lysine-treated ovalbumin (NF). D. Formaldehyde/lysine-treated and heat-denatured ovalbumin (DF). The samples were analysed on a Superose 12 column eluted with PBS at a flow rate of 0.5 ml/min.

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Gel filtration chromatography

Gel filtration chromatography was carried out on a Superose 12 column, using an FPLC system (Pharmacia, Uppsala, Sweden), in PBS at a flow rate of 0.5 ml/min.

Immunoaffinity chromatography

Denatured or native antigens were immobilized on CNBr activated Sepharose essentially as described by the manufacturer. In brief, CNBr-activated Sepharose was washed with 10 mM HCl and then with PBS, 0.5 M NaCl. Antigens were used at a concentration of 2.5 mg/ml and were added at a ratio of 1:1 (v:v) to the settled washed matrix. The coupling was allowed to proceed for 2 h at 4°C with end-over-end agitation. The antigen solution was then removed and the matrix was incubated overnight at 4°C with 0.1 M ethanolamine, pH 8.0. The matrix was washed successively with citrate phosphate buffer, 30% ethylene glycol, pH 11.5, and PBS containing 0.5 M NaCl. For affinity chromatography the sera were diluted 1:1 in PBS and passed through the column at a flow rate of 20 ml/h. The column was washed with PBS, 0.5 M NaCl until the absorbance at 280 nm reached baseline value, and then eluted with citrate phosphate buffer, washed with PBS, eluted with 30% ethylene glycol, pH 11.5, washed with PBS and finally eluted with 1 M propionic acid. The column was washed with PBS following elution and the eluates were dialyzed against PBS.

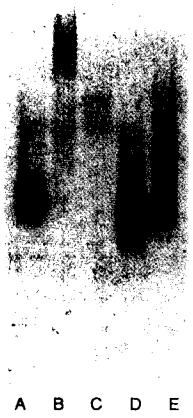

Fig. 2. Analysis of ovalbumin forms by PAGE. A. Native ovalbumin. B. Heat-denatured ovalbumin. C. Urea-denatured ovalbumin. D. Formaldehyde/lysine-treated ovalbumin. E. Formaldehyde/lysine-treated and heat-denatured ovalbumin. Sample (40 µl) was applied per lane and subjected to electrophoresis at 200 V in CIE buffer. Proteins were stained with Coomassie Brilliant Blue.

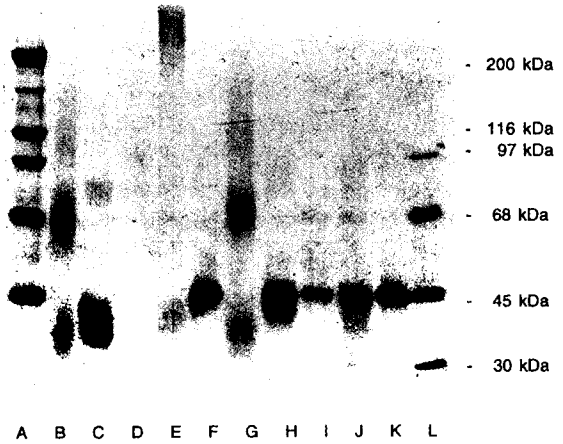

Fig. 3. Analysis of ovalbumin forms by SDS-PAGE. A. High molecular weight markers. L. Low molecular weight markers. B-F. Non-reducing conditions. G-K. Reducing conditions. B,G. DF. C,H. NF. D,I. UD. E, J. D. F,K. N. Samples were boiled 1:1 in sample buffer and 25 µl was loaded per lane. Electrophoresis was carried out at 200 V and proteins were stained with Coomassie Brilliant Blue.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed in 7% gels, cast and run in CIE buffer. Antigen (20 µg) was applied per lane and electrophoresis was carried out at 40 mA per gel for 2 h. Gels were stained with 0.1% Coomassie Brilliant Blue in 10% CH_3COOH, 20% EtOH, and destained with 10% CH_3COOH. SDS-PAGE was performed as described by *Laemmli* (1970) using 7% gels. Antigen (20 µg) was applied per lane and electrophoresis was carried out at 40 V. Gels were stained and destained as for PAGE gels. Crossed immunoelectrophoresis (CIE) was carried out as described in *Axelsen et al.* (1983). The first electrophoresis was performed in 1% agarose gels in CIE buffer for 1 h at 10 V/cm on 10 x 10 cm glass plates. Electrophoresis in the second dimension was carried out in the same type of gels, with antisera added, at 2 V/cm overnight.

Amino acid analysis

Amino acid analysis was done as described by *Barkholt & Jensen* (1989).

NMR spectroscopy

NMR spectra were recorded on a Bruker model AMX-600 600 MHz instrument. Approximately 20 mg of each of the different ovalbumin forms was dissolved in 1 ml 50% D_2O.

RESULTS

Native ovalbumin (N and four different forms of modified ovalbumin were prepared: heat-denatured ovalbumin (D), formaldehyde/lysine-treated ovalbumin (NF), formaldehyde/lysine-treated heat-denatured ovalbumin (DF) and urea-denatured ovalbumin (UD). All forms of ovalbumin were soluble at 1 mg/ml in water or PBS, except urea-denatured ovalbumin, which had a tendency to aggregate. Amino acid analy-

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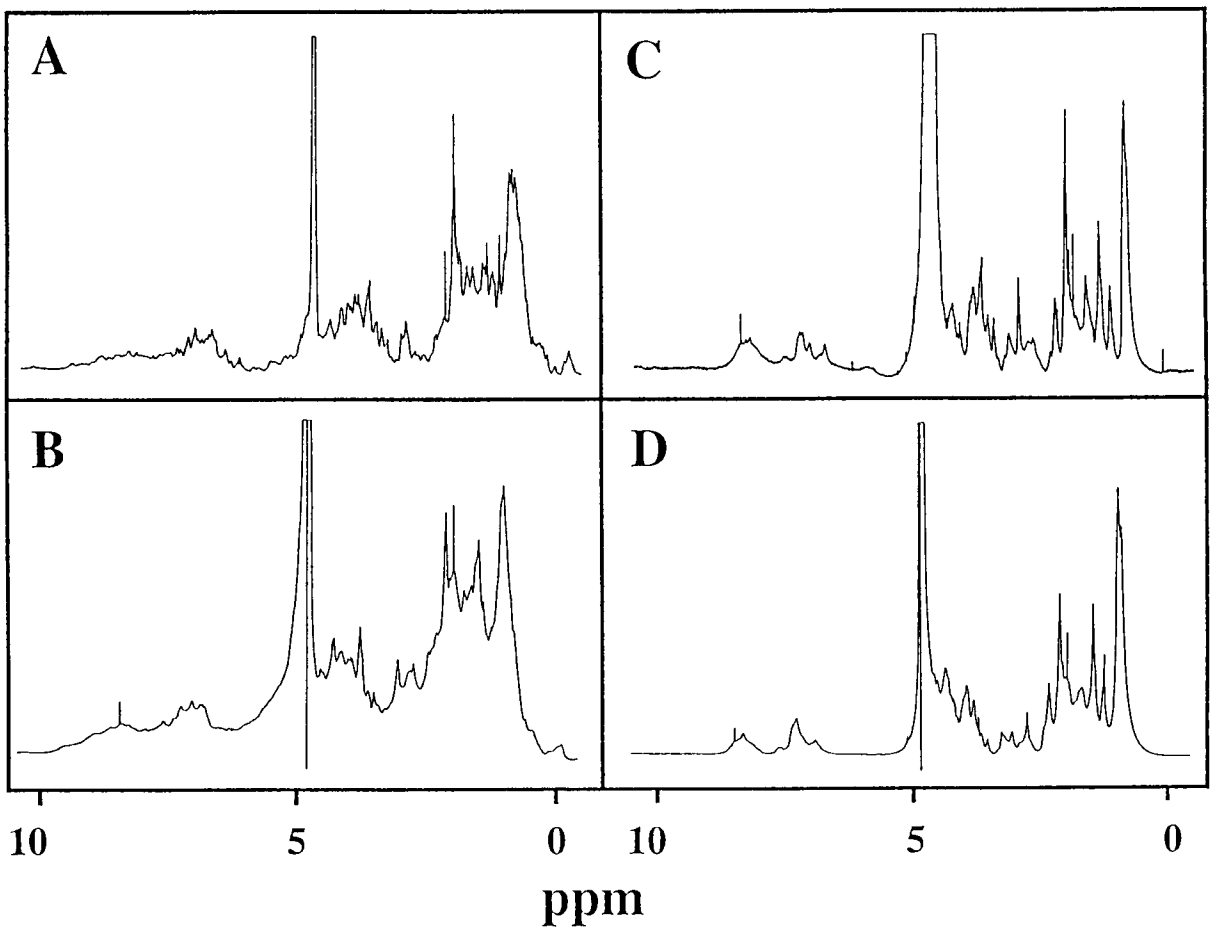

Fig. 4. NMR spectrograms of ovalbumin forms. A. Native ovalbumin. B. Formaldehyde/lysine-treated ovalbumin. C. Heat-denatured ovalbumin. D. Formaldehyde/lysine-treated and heat-denatured ovalbumin.

sis of NF (data not given) showed an average of 8 extra lysines per ovalbumin molecule.

The effect on ovalbumin of heat denaturation with or without prior formaldehyde/ lysine treatment was investigated employing gel filtration, PAGE, SDS-PAGE and NMR spectroscopy. Fig. 1 shows gel filtration profiles of the various ovalbumin derivatives. Heat-denatured ovalbumin is eluted at a position corresponding to a high molecular weight polymer. The formaldehyde/lysine-treated ovalbumin is eluted with two major peaks, one at the position of ovalbumin. The elution profile of heat-denatured formaldehyde/lysine-treated ovalbumin resembles that of formaldehyde/lysine-treated ovalbumin, but is shifted towards a higher molecular weight.

Fig. 2 shows the results of electrophoretic analysis of the same antigens using PAGE. Native ovalbumin forms a fairly homogeneous band, whereas the D form is seen as a more heterogeneous and high Mr band. The UD form hardly enters the gel except for a faint band with a mobility corresponding to the heat-denatured form. The UD form seems to be homogeneous as native ovalbumin, but has greater mobility owing to a greater net negative charge (elimination of lysine charges). The DF form does not form high Mr polymers/aggregates, but is more heterogeneous and of lower mobility than NF. These results are in complete agreement with the results from gel filtration analysis.

Fig. 3 shows analysis of the ovalbumin forms by SDS-PAGE. When they were analysed under non-reducing conditions, the antigens behaved essentially the same as in native PAGE. Under reducing conditions N and DF had the same mobility as under non-reducing conditions. The mobility of NF was slightly reduced, but re-

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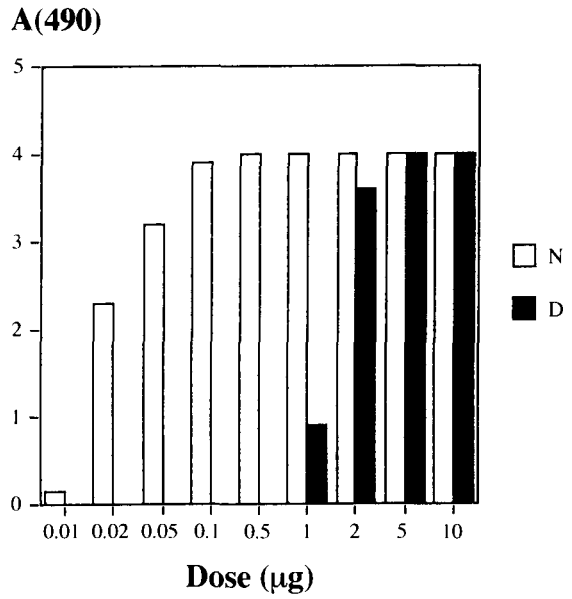

Fig. 5. Immunization experiments in AJxbALB/c mice. Open bars: Native ovalbumin. Blocked bars: Heat-denatured ovalbumin.

vealed itself as two closely spaced bands, and D and UD were both reduced to monomers behaving as the native molecule. These results show that intermolecular disulfide bond rearrangement is involved in the formation of the higher molecular weight D, DF and UD forms of ovalbumin, and that the polymers thus formed are considerably larger in UD.

NMR spectra of the various ovalbumin forms provided further structural information on their similarity. As seen in Fig. 4, NF has an NMR spectrum very similar to that of N, without evidence of denaturation. D shows a "simpler" spectrum than N, owing to the greater average distance and consequently fewer "through space" couplings between side chains, and as is characteristic for denatured proteins, there are no signals in the very low and very high field region. The spectrum of DF resembles that of D, with the main difference being more "heterogeneity". In conclusion, it can be seen that the formaldehyde/lysine treatment does not have a major impact on the conformation of the non-denatured antigens, and that the denatured forms also show similar patterns.

Immunization and dose response experiments are illustrated in Fig. 5 and in Table 1. The figure shows the results of dose-response experiments in AJxBALB/c mice with native and denatured ovalbumin as antigen, and $Al(OH)_3$ as adjuvant. Even the lowest dose (0.01 μg) of native ovalbumin induced an antibody response. This response was detectable after three injections, and reached titres of 1:32.000 after five injections. The highest doses induced a rapid response, with titres reaching 1:500,000 after five injections. Immunization with heat-denatured ovalbumin also induced a rapid response of high titre when high doses were used, and the response level was comparable to that produced by native ovalbumin. However, low doses were not immunogenic or only induced a limited response of low titre.

Table 1 summarizes threshold antigen doses for the various forms of ovalbumin. In CF1xBALB/c mice the same pattern of immunogenicity was observed as for

TABLE 1. *Threshold immunogenic doses (μg)[a] for a positive response to intraperitoneal immunization of mice with forms of ovalbumin using $Al(OH)_3$ gel as adjuvant.*

Mouse strain	Ovalbumin derivative			
	N	D	NF	DF
AJ×BALB/c	0.01	1	nd	1
CF1×BALB/c	0.005	0.5	1	0.5

[a] Defined as the minimum dose that induces a response to the immunizing antigen after five injections.
nd=not determined.

TABLE 2. *Cross-reactivity of rabbit antisera subjected to affinity chromatography on columns with immobilized N (for rabbit anti-DF) and immobilized DF (for rabbit anti-N). Sera and fractions were analysed for reactivity against biotinylated antigens immobilized on streptavidin-coated plates. Cross-reactivity is expressed as the ratio between the antigen concentrations giving an absorbance of 1 when tested on homologous and heterologous antigen*

Antiserum	Cross-reactivities (%)			
	rabbit anti-N		rabbit anti-DF	
Whole serum	7.5		1.3	
Flow through	0.2		0.3	
Eluate	110		2.3	
	Recoveries (%) of reactivity with			
	N	D	N	D
Flow through	71	1.5	6.2	33
Eluate	1.8	20	59	34
Total	72.8	21.5	65.2	67

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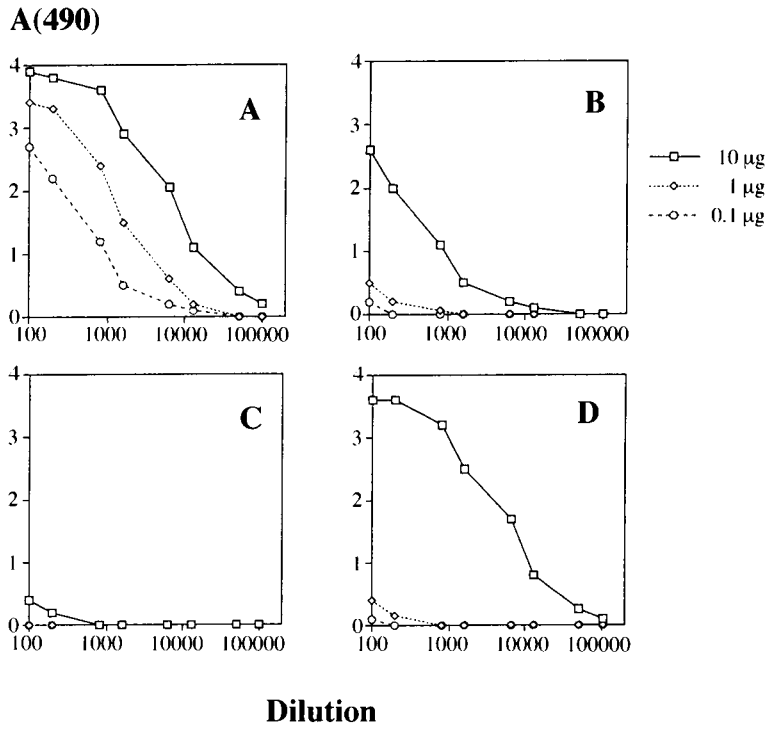

Fig. 6. Analysis of mouse sera by ELISA. Plates were coated with streptavidin and biotinylated antigens were applied. Sera from the fifth bleed were analysed from a starting dilution of 1:100.
A. Mouse anti-N on biotinylated N.
B. Mouse anti-N on biotinylated D.
C. Mouse anti-DF on biotinylated N.
D. Mouse anti-DF on biotinylated D.

AJxBALB/c mice, with the exception that the threshold values for immunogenicity were lower for both native and denatured forms.

The specificity and cross-reactivity of the sera obtained were analysed by ELISA. Fig. 6 shows the results of testing mouse anti-N and mouse anti-DF on biotinylated antigens bound to streptavidin-coated plates. This approach eliminates errors due to the denaturation of native ovalbumin on the surface of the ELISA plates. The antisera were highly specific for either the denatured or native form of ovalbumin, and only a low level of cross-reacting antibodies was present. Sera from mice immunized with 10 µg N had titres up to 1: 100,000 when tested on biotinylated N, whereas the titres against biotinylated D were only 1: 200. Thus the level of cross-reacting antibodies remained low even at the highest immunogenic dose. As shown previously, the immunogenicity of denatured forms was substantially lower than that of the native forms. However, sera from DF-immunized mice showed good reactivity against biotinylated D at the highest immunization dose, whereas the lower doses were essentially non-immunogenic. To obtain a quantitative measure of cross-reactivity the ratios of dilution factors giving an absorbance of 1 when tested on the immunizing (homologous) antigen and on the cross-reacting (heterologous) antigen were determined. At the highest immunization dose (10 µg) the cross-reactivity of anti-N against biotinylated D was 4.2%, whereas the cross-reactivity of anti-DF against biotinylated N was undetectable. At the lower immunization doses no cross-reactivity could be observed. Analyses of the same sera in an antibody capture ELISA with rabbit polyclonal anti-N, -D, -NF or -DF as coatings for catching the relevant antigens, or in ELISA with antigens coated directly on the plates, gave essentially the same results (data not shown). Finally, we performed inhibition experiments, where reactivities were measured in excess of the non-homologous antigen. Degree of inhibition was identical to that seen by direct titrations (data not shown).

The results obtained by immunizing mice were substantiated by experiments in which rabbits were immunized with the various ovalbumin forms. When analysed in CIE (Fig. 7), the mobility of the antigen in the first dimension reflected the behaviour observed in PAGE, and the sera were found to be highly specific for the native or denatured forms. The highest cross-reactivity was found between rabbit anti-N and D (Fig. 7-J), whereas anti-DF did not cross-re-

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act significantly with N (Fig. 7-C). The cross-reactivity of the sera was analysed in ELISA against biotinylated antigens as for the mouse sera using the dilution factors giving an absorbance of 1 when tested on homologous and heterologous antigen. The cross-reactivities thus found for rabbit anti-N against biotinylated D were 6.9% and 0.6% for rabbit anti-DF against biotinylated N.

To further quantitate the level of cross-reacting antibodies, rabbit antisera to N or DF were chromatographed on columns of immobilized DF or N respectively. As seen in Table 2, the cross-reactivities of the sera were in agreement with the previously obtained ELISA values, whereas the cross-reactivities of the specifically eluted antibodies were close to 100% for the DF-bound antibodies of rabbit anti-N against biotinylated D, but only 2.3% for the N-bound antibodies of rabbit anti-D against biotinylated N.

DISCUSSION

Ovalbumin is a protein of Mr 45,000, and the complete primary and tertiary structures including the posttranslational modifications are known (*Nisbet et al.* 1981; *Stein et al.* 1990; *Potempa et al.* 1994; *Fothergill & Fothergill* 1970; *Thompson & Fisher* 1978). Ovalbumin is a serpin and as such, it is a single domain protein with approximately 40% β-sheet and 35% α-helix (*Batra et al.* 1989a; *Batra et al.* 1989b; *Carrell & Evans* 1992; *Huber & Carrell* 1989). Four out of six cysteines are free, and this has important consequences for its properties, At higher temperatures, ovalbumin unfolds readily and preferentially forms a heat-stabilized form, S-ovalbumin (*Donovan & Mapes* 1976; *Smith & Back* 1965), presumably by loop-sheet polymerization, as has been described for another serpin, α-protease inhibitor (*Lomas et al.* 1992). When denatured by heat, it forms disulphide-bonded high Mr polymers (*Fukuda et al.* 1991).

The physicochemical studies were aimed at analysing the degree of change in molecular structure imposed by heat denaturation, and also the effect of treatment with formaldehyde and lysine. Heat denaturation is known to cause precipitation of most proteins, owing to intermolecular interactions. Such interactions are both non-covalent and due to formation of intermolecular disulfide bridges.

The reason for including formaldehyde/lysine treatment was our experience that such treatment prevents precipitation after denaturation, most likely owing to the proteins gaining an overall negative charge, which will subsequently partially limit intermolecular interactions.

Gel filtration (Fig. 1) demonstrated that formaldehyde/lysine treatment reduced the tendency to polymerization (compare B and D in Fig. 1), but also that formaldehyde treatment of native ovalbumin led to a certain degree of polymerization, probably by dimerization. These findings were confirmed by PAGE analysis (Fig. 2), which also demonstrates the overall effect of formaldehyde/lysine treatment on net charge (lane D). SDS-PAGE without and with reduction of disulfide bridges (Fig. 3) demonstrates the degree of disulfide bridge-dependent aggregation and confirms that formaldehyde/lysine treatment reduces this kind of aggregation during denaturation.

Finally, NMR spectra (Fig 4) show that native ovalbumin with or without formaldehyde/lysine treatment has a similar (native) structure, and that denaturation of both untreated and formaldehyde/lysine-treated ovalbumin leads to similar overall structural changes. The physicochemical analysis of the antigens also confirmed that the heat-denatured ovalbumins do not contain detectable amounts of native ovalbumin. This is essential for interpreting the cross-react-

Fig. 7. Analysis of antisera in CIE. The antigen analysed and the specificity of the antisera used were as follows. *A–D.* N as antigen, rabbit anti-N in the upper gel, and in the intermediary gel: *A*, control without serum, *B*, rabbit anti-N, *C*, rabbit anti-DF, *D*, rabbit anti-D. *E–H.* NF as antigen, rabbit anti-NF in the upper gel, and in the intermediary gel: *E*, control, *F*, rabbit anti-N, *G*, rabbit anti-DF, *H*, rabbit anti-D. *I–L.* D as antigen, rabbit anti-D in the upper gel, and in the intermediary gel: *I*, control, *J*, rabbit anti-N, K, rabbit anti-DF, *L*, rabbit anti-D. *M–P.* DF as antigen, rabbit anti-DF in the upper gel, and in the intermediary gel: *M*, control, *N*, rabbit anti-N, *O*, rabbit anti-DF, *P*, rabbit anti-D.

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ivities of the sera obtained after immunization with either native or denatured proteins.

Our data show that native ovalbumin was most immunogenic, as the threshold immunizing dose for obtaining an antibody response was 100 times lower than that of denatured ovalbumin. However, it is important to stress that the level of antibodies to the two structures was similar, provided enough antigen was used for immunization. We also demonstrated that formaldehyde treatment with the doses of formaldehyde used did not influence the immunogenicity/antigenicity. This is in agreement with the physicochemical data.

ELISA-based titrations with biotin-labelled antigens and data from CIE with rabbit antisera demonstrate a high specificity and low cross-reactivity. The low cross-reactivity suggests that the lower immunogenicity of denatured ovalbumin is not due to a faster clearance of denatured ovalbumin from the circulation, but rather reflects a difference between the structure and the immunogenicity of native and denatured proteins. In intraperitoneal immunization experiments both native and denatured ovalbumin will be taken up, processed and presented by antigen presenting cells, and thus both forms of ovalbumin will induce T-cell help. Antibody response to both native and denatured ovalbumin is supposed to be dependent on T cells, but we have not formally shown whether identical T-cell epitopes are used by both structures. We are presently investigating this question, and likewise we are analysing the subclass specificity of the sera to see whether the antigens employ the same T_H1/T_H2 pathway.

The state of denatured proteins is often described as a random coil, implying an absence of well-defined energetically favoured structures. This description may be appropriate for proteins in solutions such as 8 M urea or 6 M guanidine, but is probably not adequate for a denatured protein in water. A random coil structure would be expected to be of very low immunogenicity, simply because encountering lymphocytes with a recognizable conformation would be a very infrequent event, and antibodies to the random coil structure would be expected to be highly sequence directed. Thus, the finding that specific antibodies are induced suggests that a limited set of conformations are present in the denatured state. In CIE, the denatured ovalbumins show the characteristic asymmetrical precipitin patterns often seen with denatured proteins (*Koch et al.* unpublished observations). This indicates the existence of a multitude of denatured forms with overlapping epitopes. A rough estimate of the number of conformations in the denatured state would be around 100 as judged by the different threshold values for immunogenicity. Thus the immunological data suggest that the denatured state of proteins is most appropriately described as a set of states, possibly in equilibrium with each other:

$$N \leftrightarrow D_1 \leftrightarrow D_2 \leftrightarrow D_n$$

The cross-reactivities of the mouse and rabbit antisera were found to be highest for anti-N, and this may be taken as further evidence for the existence of a multitude of denatured conformations in equilibrium with the native conformation. Antibodies to N may be able to "select" cross-reactive conformations from the denatured set of conformations, whereas antibodies to denatured conformations cannot select cross-reactive conformations from the more rigid native conformations and will only cross-react if they are able to recognize a linear surface-exposed epitope.

The description of the unfolded state as a limited set of conformations also suggests that the random coil description of denatured proteins may not be appropriate. The relation of this set of conformations to the molten globule state of proteins cannot be assessed as this state has not been defined in molecular terms. However, as the molten globule has been described as having significant secondary structure, stabilized by non-specific hydrophobic interactions, but does not undergo cooperative unfolding transitions (*Murphy & Freire* 1992; *Kim & Baldwin* 1990), this hypothetical state may bear some resemblance to the immunogenic structures of the denatured state.

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