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Ovalbumin and angiotensinogen lack serpin S–R conformational change

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Cleavage of ovalbumin and angiotensinogen at sites homologous to the reactive centre loop of α_1-antitrypsin is not accompanied by the increase in heat-stability associated with the transition from the native stressed (S) structure to a cleaved relaxed (R) form that is typical of other serpins. Failure to undergo the S–R change in ovalbumin is not due to phosphorylation of Ser-344 near the sites of cleavage on the loop. The suggested explanation is the unique presence of bulky side chains at the P_{10}–P_{12} site in ovalbumin and angiotensinogen.

INTRODUCTION

Ovalbumin [1] and angiotensinogen [2] are members of the serine-proteinase inhibitor (serpin) superfamily, showing primary sequence homologies of 30% and 21% respectively with the archetype of the family, α_1-antitrypsin. Sequence matching based on the crystallographic structure of α_1-antitrypsin [3] that has been catalytically cleaved at its reactive centre implies that the serpins share a common secondary and tertiary structure. In the tertiary structure of cleaved α_1-antitrypsin the two amino acid residues forming the reactive-site peptide bond are found at opposite poles of the molecule separated by a distance of 6.7 nm (67 Å), indicating that cleavage is accompanied by a major conformational change (see diagrammatic representation in Fig. 1). The structure of α_1-antitrypsin in its native intact form is unknown. However, it has been predicted that the A4 peptide strand, the central strand of the major (A) pleated sheet in the structure of the cleaved molecule, forms, in native α_1-antitrypsin, an external stretched peptide loop that exposes the reactive centre and causes the native inhibitor to exist in a stressed and relatively unstable form. Experimental evidence based on both inhibitor [4–6] and non-inhibitor [7] members of the serpin family supports this model. The native serpins are susceptible to proteolytic cleavage in the peptide sequence corresponding to strand A4, and this modification is associated with a marked increase in thermal stability compatible with the transition from the stressed (S) structure to a more ordered relaxed (R) form, as predicted from the model.

We have demonstrated failure of the S–R transition to occur in ovalbumin and angiotensinogen and consider possible structural explanations and the evolutionary significance of this finding.

MATERIALS AND METHODS

Protein preparation

Three preparations of ovalbumin were studied. (*a*) Commercially prepared ovalbumin was obtained from Sigma Chemical Co. (grade VI). (*b*) Ovalbumin was purified from newly laid hen eggs by $(NH_4)_2SO_4$ fractionation and precipitation at pH 4.6 as previously described [8] followed by DEAE-Sephadex chromatography. A 1.5 g portion of protein in 50 mM-Tris/HCl buffer, pH 7.5, was applied to a 2.5 cm × 50 cm DEAE-Sephadex A-50 column previously equilibrated with the same buffer. Ovalbumin was eluted with a linear gradient of 0–0.3 M-NaCl in 50 mM-Tris/HCl buffer, pH 7.5 (1.5 litres total volume), at 75 ml/h. Purity was assessed by SDS/polyacrylamide-gel electrophoresis. (*c*) Ovalbumin purified as above (*b*) was dephosphorylated [9] by using alkaline phosphatase type 1 from bovine (calf) intestine (Sigma Chemical Co.). Ovalbumin (60 μM) was incubated with phosphatase (2.5 mg/ml; 1–3 units/mg) at pH 5.5 in the presence of 1 mM-$MgSO_4$ for 15 h at 37 °C. Approx. 80% of the original phosphate was removed as estimated from measurements of P_i after precipitation of protein with trichloroacetic acid. Altered mobility of the dephosphorylated protein was demonstrated by isoelectric focusing in a 5% (v/v) polyacrylamide gel containing 3.3% (v/v) carrier ampholytes (Pharmacia) of pH 4.0–6.5.

Angiotensinogen was prepared from human plasma as previously described [10].

Proteolytic cleavage

Each preparation of ovalbumin was incubated with subtilisin Carlsberg (Sigma Chemical Co.) or pig pancreatic elastase type IV (Sigma Chemical Co.) at a molar ratio of 70:1 in 50 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-$CaCl_2$ for 5 h at 37 °C [11]. Cleavage was demonstrated by SDS/polyacrylamide-gel electrophoresis under reducing conditions. Angiotensinogen was incubated with human neutrophil elastase (a gift from Dr. M. Visser, Christchurch Clinical School, University of Otago, Christchurch, New Zealand) at a molar ratio of 10:1 in 5 mM-potassium phosphate buffer, pH 7.4, for 80 min at 37 °C. Portions of the incubation mixture were removed at timed intervals for analysis by SDS/polyacrylamide-gel electrophoresis under non-reducing conditions.

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Identification of cleavage sites in angiotensinogen

A neutrophil-elastase digest of angiotensinogen incubated for 45 min was analysed by reverse-phase h.p.l.c. on a PLRP-S 30 nm-pore-size 8 μm-particle-size 250 mm × 4.6 mm column (Polymer Laboratories, Shropshire, U.K.). Fragments were eluted with a linear gradient of 5–80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, with monitoring at 214 nm, and peaks were collected separately. A portion of each peak was dried under vacuum for analysis by SDS/polyacrylamide-gel electrophoresis under non-reducing conditions, with the use of a silver stain to identify protein bands. Portions of protein-containing peaks were sequenced through nine *N*-terminal amino acid residues by the gas-phase method.

Heat-stability of ovalbumin and angiotensinogen in native and cleaved forms

Portions of each native and cleaved protein (3–5 μM) in 75 mM-Tris/75 mM-glycine/75 mM-NaH_2PO_4 buffer, pH 7.5, were heated at constant temperatures between 40 and 100 °C for 2 h, then centrifuged, and residual protein was determined in the supernatant by the electroimmunoassay, with sheep antiserum to chicken ovalbumin (The Binding Site Ltd., Birmingham, U.K.) or rabbit antiserum to human angiotensinogen as appropriate.

RESULTS AND DISCUSSION

Previous studies have demonstrated that ovalbumin is susceptible to proteolytic cleavage by subtilisin [12] and pancreatic elastase [11] in a region of sequence aligned with the reactive site of α_1-antitrypsin (Fig. 1). SDS/polyacrylamide-gel electrophoresis of the cleavage products of ovalbumin obtained with each enzyme is shown (Fig. 2).

Digestion of angiotensinogen by neutrophil elastase has not been described before this study. SDS/polyacrylamide-gel electrophoresis (Fig. 3) showed altered mobility of the major angiotensinogen band after 15 min incubation, indicating that proteolytic cleavage had occurred, although a band corresponding to the released low-molecular-mass peptide was not identified. Gradual disappearance of the major angiotensinogen fragment with time suggested that this initial cleavage was followed by more extensive degradation.

The limited angiotensinogen digest after 45 min incubation was analysed by h.p.l.c. Two protein-containing peaks were resolved and sequenced. The first peak gave two overlapping sequences: the major sequence corresponded exactly to that of the *N*-terminus of intact human angiotensinogen [13], and a minor sequence was consistent with cleavage of angiotensinogen between Val-3 and Tyr-4. The second peak contained two peptides derived from the *C*-terminus of angiotensinogen. The major sequence was consistent with cleavage between Thr-413 and Gln-414 and a minor overlapping sequence was consistent with cleavage between Leu-416 and Asn-417.

Thus angiotensinogen, as well as ovalbumin, is susceptible to proteolytic cleavage at sites homologous to the exposed peptide loop of α_1-antitrypsin (Fig. 1). However, examination of the heat-stabilities of native and cleaved forms of each protein yielded unexpected results. Native and cleaved forms of ovalbumin showed almost identical heat-stabilities (Fig. 4*a*), and cleaved angiotensinogen was slightly less stable than the intact protein (Fig. 5). These results are in marked contrast with those described for all other members of the serpin family so far investigated [4,5,7]. The heat-stability profiles of native and cleaved forms of thyroxine-binding globulin are shown for comparison (Fig. 6), illustrating the dramatic increase in stability that typically

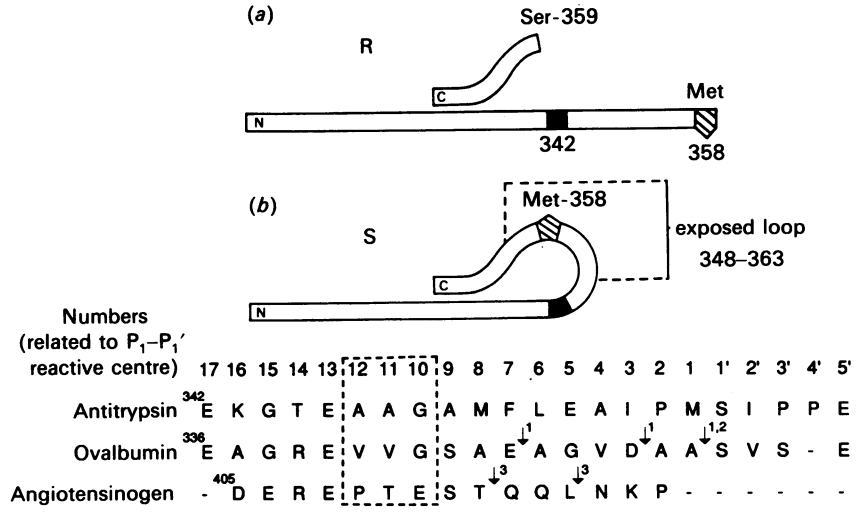

Fig. 1. Sites of cleavage of ovalbumin and angiotensinogen

The structure of the serpins is based on that of the cleaved relaxed (R) form of α_1-antitrypsin shown diagrammatically (*a*). The native stressed (S) form may be reconstructed by a deduced exposed loop that hinges near residue 342 (*b*). Alignment of the sequences of the exposed loop [19] shows, arrowed, the sites of cleavage of ovalbumin by subtilisin[1] and pancreatic elastase[2] [11,12] and of angiotensinogen by neutrophil elastase[3]. There is conservation of small side chains at P_{10}–P_{12} (boxed) in the serpins with the exception of ovalbumin and angiotensinogen.

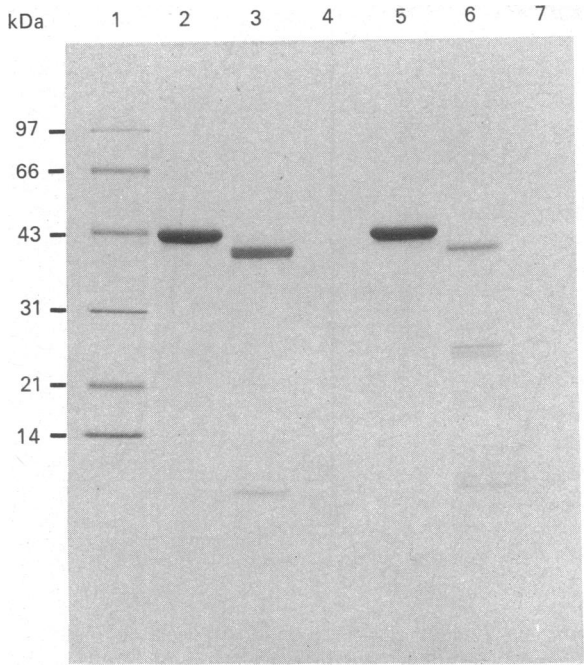

Fig. 2. Cleavage of ovalbumin by pancreatic elastase and subtilisin

Samples were electrophoresed in an SDS/10–20%-polyacrylamide-gel gradient under reducing conditions. Lane 1, markers [phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), bovine carbonic anhydrase (31 kDa), soya-bean trypsin inhibitor (21 kDa) and lysosyme (14 kDa)]; lanes 2 and 5, intact ovalbumin; lane 3, pancreatic-elastase-cleaved ovalbumin; lane 6, subtilisin-cleaved ovalbumin (as described in the Materials and methods section); lane 4, pancreatic elastase; lane 7, subtilisin (Coomassie Blue stain).

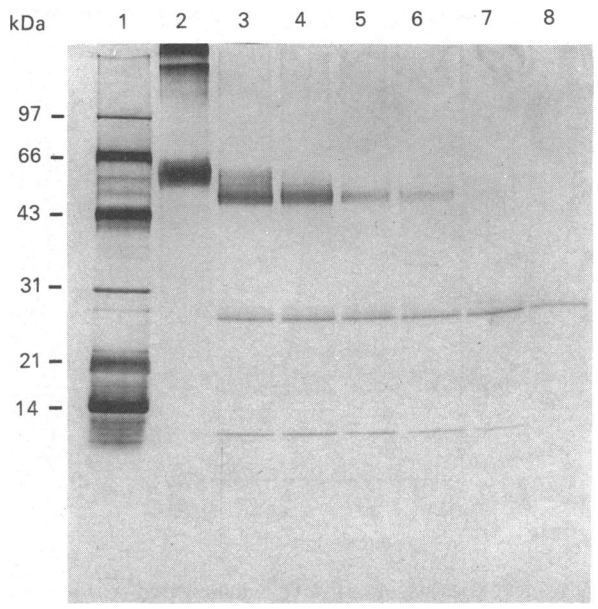

Fig. 3. Cleavage of angiotensinogen by neutrophil elastase

Samples were electrophoresed in an SDS/10–20%-polyacrylamide-gel gradient under non-reducing conditions. Lane 1, markers (as Fig. 2); lane 2, intact angiotensinogen; lanes 3–7, portions of the incubation mixture (as described in the Materials and methods section) taken after 15, 30, 45, 60 and 80 min; lane 8, neutrophil elastase (silver stain).

accompanies cleavage of the exposed loop region of a native serpin, compatible with the S–R conformational charge. Our finding, that the heat-stabilities of native and modified forms of ovalbumin and angiotensinogen are similar, suggests that these two proteins do not undergo the typical serpin S–R transition. Recent work [14] has confirmed that proteolysis of the reactive-site loop of several inhibitory serpins is accompanied by a major conformational change, as indicated by differences in c.d. and fluorescence spectra, and with increased resistance to denaturation by guanidinium chloride. However, in agreement with our results, ovalbumin showed neither spectral changes nor altered stability upon cleavage. Thus the earlier inference [4] from crystallization behaviour [12] that cleavage of ovalbumin would be accompanied by the S–R change is incorrect.

A poorly characterized conformation variant of ovalbumin, designated S (stable)-ovalbumin, has been described [15] that is distinguished from the usual form of the protein by its relative resistance to heat denaturation. The proportion of the S-ovalbumin fraction in an ovalbumin preparation appears to be directly related to the length of time for which eggs are stored before purification of the protein. Therefore in our study ovalbumin prepared from newly laid eggs was tested as well as the commercial product in order to exclude an artifact due to the presence of S-ovalbumin. The heat-stability profiles of both ovalbumin preparations were identical (Figs. 4*a* and 4*b*).

Despite failure to undergo the S–R change, other structural evidence supports the prediction that ovalbumin and angiotensinogen do in fact share the tertiary structure of α_1-antitrypsin. Potential glycosylation and phosphorylation sites in ovalbumin and angiotensinogen are all externally situated in the predicted structure. Ovalbumin is known to have a single disulphide bond between residues 73 and 120 [16]. The separation of the aligned residues of α_1-antitrypsin (90 and 133) is reported [3] to be compatible with a disulphide bond, assuming that the insertion in ovalbumin brings residue 90 closer to 133. Angiotensinogen has four thiol groups, but the position of any disulphide bonds has not been established.

On the basis that angiotensinogen and ovalbumin have typical serpin tertiary structures, it follows that there must be a structural constraint preventing the S–R transition on cleavage of the exposed peptide loop. Ovalbumin is phosphorylated at serine residues 68 and 344 [17]. The phosphate group at Ser-344 is situated on the exposed loop and might have the potential to interfere with a major structural transition. This phosphate group is completely hydrolysed under conditions similar to those used for dephosphorylation in this study [18], whereas the phosphate group at Ser-68 is less susceptible to degradation and probably accounts for the residual phosphate content of our dephosphorylated preparation. However, we found that the heat-stabilities of intact and cleaved forms of dephosphorylated ovalbumin were identical (Fig. 4*c*), indicating that the phosphate group on the exposed loop is not an important factor preventing the S–R change.

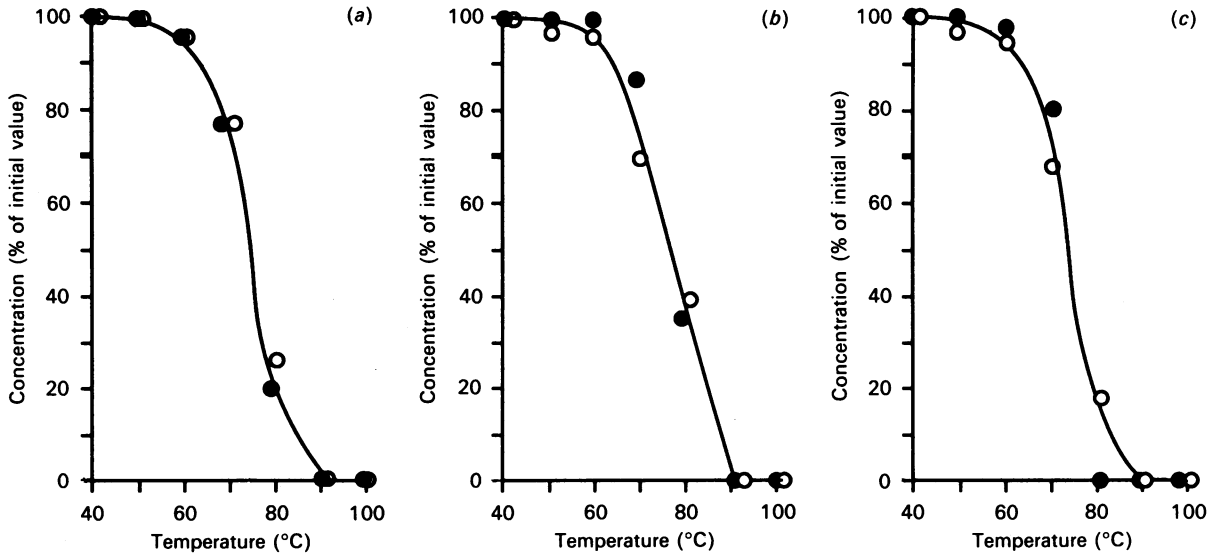

Fig. 4. Heat-stability of native (●) and cleaved (○) ovalbumin

(*a*) Commercially prepared ovalbumin; (*b*) ovalbumin purified from newly laid eggs; (*c*) dephosphorylated ovalbumin. Experimental details are described in the Materials and methods section. Residual protein concentration after heating is expressed as a percentage of the initial (unheated) concentration.

In an endeavour to explain these unexpected results we have examined the aligned amino acid sequences of 14 serpins [19]. The alignments with the exposed loop region of a α_1-antitrypsin reveals a conserved site at P_{10}–P_{12} present in all members of the family with the exception of ovalbumin and angiotensinogen. Whereas the P_{10}–P_{12} site in the other serpins is occupied by residues with small side chains (predominantly alanine), in ovalbumin there are two large valine residues at P_{11} and P_{12}, and in angiotensinogen there is, uniquely, the imino acid proline at P_{12} with a large polar glutamate at P_{10} (Fig. 1). In order to bring about the S–R change on cleavage of the exposed loop of the native serpin, the model requires folding of the A4 strand into the major pleated sheet. In the majority of serpins, the presence of small residues at P_{10}–P_{12} may be a necessary spatial requirement. The alanine at P_{12} fits tightly into a conserved pocket formed by phenylalanine residues (residues 51, 190 and 384 of α_1-antitrypsin) and a methionine residue (residue 374). Thus, in ovalbumin and angiotensinogen, the presence of large residues at P_{10}–P_{12} will predictably prevent movement of the cleaved A4 strand into the sheet.

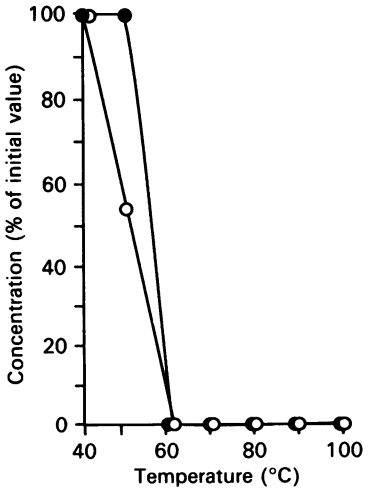

Fig. 5. Heat-stability of native (●) and cleaved (○) angiotensinogen

Experimental details are described in the Materials and methods section. Residual protein concentration after heating is expressed as a percentage of the initial (unheated) concentration.

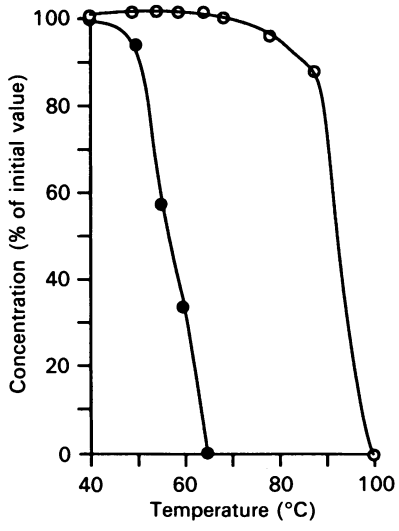

Fig. 6. Heat-stability of native (●) and cleaved (○) thyroxine-binding globulin

A marked increase in stability follows cleavage of thyroxine-binding protein by neutrophil elastase in a region of sequence aligned with the exposed loop of α_1-antitrypsin [7].

In those serpins with inhibitory functions the S–R change is a constant finding and likely to be consequent to the need for stress in the reactive-site loop. During evolution some members of the serpin family appear to have lost their role as proteinase inhibitors. In certain non-inhibitory serpins the S–R change has been utilized to modulate a different property of the protein, such as hormone transport in cortisol-binding globulin [7], but in others, including ovalbumin and angiotensinogen, the S–R transition may have served no useful purpose and therefore has been lost by evolutionary change.

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