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Conformational Properties of Disulfide-Free Recombinant Chicken Ovalbumin

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Chicken egg ovalbumin is a non-inhibitory member of the serpin (serine protease inhibitors) family whose members share a common tertiary fold. In the present study, we succeeded in high-level production of a disulfide-free form of refolded recombinant ovalbumin. Conformational characterization of the recombinant ovalbumim revealed that it is well-folded, following two-state unfolding transition with the midpoint of transition at 4.7 M at 25°C. This value is very close to that of the reduced form of authentic ovalbumin. The recombinant ovalbumin can serve as a model molecule of non-inhibitory serpins in comparative studies with inhibitory members of the serpin family.

Keywords: Conformational stability, High-level expression, Ovalbumin.

Introduction

The serine protease inhibitor (serpin) superfamily includes not only protease inhibitors such as α_1-antitrypsin (α_1AT), antithrombin, α_1-antichymotrypsin, and plasminogen activator inhibitors, but also non-inhibitory members such as ovalbumin and angiotensinogen (Huber and Carrell, 1989). In human plasma, inhibitory serpins play an important role in inflammation, as well as in controlling the activities of proteases in coagulation, connective tissue turnover, fibrinolytic, complement, and kinin cascades (Heimark *et al.*, 1980; Carrell and Owen, 1985; Cugno *et al.*, 1997). Several members of the superfamily are endowed with novel functions in various biological processes including hormone delivery (Pemberton *et al.*, 1988), tumor suppression (Zou *et al.*, 1994), and apoptosis (Vaux *et al.*, 1994). Functions of some serpins like chicken ovalbumin (OVA) and angiotensinogen are yet to be defined. Serpins share a common tertiary structure composed of three β-sheets and several α-helices (Huber and Carrell, 1989). One of the intriguing aspects of serpin structure is that the native conformation of the serpins is strained (Huber and Carrell, 1989). The native strain of serpins is considered to be crucial to their physiological functions, including plasma protease inhibition (Stein and Carrell, 1995), hormone delivery (Pemberton *et al.*, 1988), Alzheimer filament assembly (Abraham *et al.*, 1988; Ma *et al.*, 1994; Eriksson *et al.*, 1995), and extracellular matrix remodeling (Stefansson and Lawrence, 1996), but not for tumor suppression (Pemberton *et al.*, 1995). The reactive center loop of serpins is exposed at one end of the molecule (Carrell *et al.*, 1994; Schreuder *et al.*, 1994; Wei *et al.*, 1994; Song *et al.*, 1995) but its cleavage accompanies a drastic conformational change that allows the insertion of the reactive center loop into the central β-sheet, the A sheet (Loebermann *et al.*, 1984; Baumann *et al.*, 1991), with concomitant release of the strain (Bruch *et al.*, 1988; Munch *et al.*, 1991).

It is thought that execution of functions like protease binding also requires such conformational change (Wright and Scarsdale, 1995). In OVA, however, cleavage of the loop does not induce a drastic conformational switch as in inhibitory serpins (Gettins, 1989; Stein *et al.*, 1990; Wright *et al.*, 1990). The ability of loop insertion of serpins thus appears to be very critical in relating the native strain of serpins to their biological functions. We have studied the native strain of human α_1AT to understand its structural basis in relation to serpin function (Lee and Yu, 1989; Kim *et al.*, 1990; Kwon *et al.*, 1994; Lee *et al.*, 1996). Since OVA does not have inhibitory activity and does not undergo the conformational change, comparative studies between ovalbumin and α_1AT on various physico-biochemical aspects will lead to understanding of the factors driving the conformational switch. In the present study, we set up a system for high-level expression of a recombinant form of OVA in *Escherichia coil* (*E. coli*). We

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constructed a disulfide-free OVA to minimize possible complications during the refolding process. Conformational characterization of the recombinant OVA suggests that the molecule is suitable for comparative studies with inhibitory serpins such as α_1AT.

Materials and Methods

Bacterial strains *E. coli* BL21[F^-, *ompT, hsd S, gal* (λc *I875, ind1, Sam7, nin5, lacUV5-T7gene1), dcm(DE3)*] was used to express recombinant ovalbumin. *E. coli* CJ236[*dut1, ung1, thi-1, relA1/pCJ105* (F^- *cam*^{r})]was used to make single-stranded DNA with a helper phage M13K07 for site-directed mutagenesis. For the propagation of plasmids, *E. coli* DH5α[*supE44, ΔlacU169*(ϕ*80 lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1*] was used as a bacterial host.

Materials T4 polynucleotide kinase, T4 DNA polymerase, and T4 DNA ligase were obtained from New England Biolabs (Cambridge, USA). T7 RNA polymerase, restriction endonucleases, and ultra-pure urea were purchased from Promega (Madison, USA). Porcine pancreatic elastase, bovine pancreatic trypsin, and chicken ovalbumin were purchased from Sigma (St. Louis, USA). Chicken ovalbumin was further purified by Mono-Q column chromatography. [^{35}S]methionine and [^{35}S]dATP were purchased from Amersham (Buckinghamshire, England). All other chemicals were of reagent grade.

Plasmid construction and protein expression The plasmid pFETOVA for expression of OVA was constructed by substituting α_1AT cDNA in pFEAT30 (Lee, 1995) with the cDNA of OVA from pYOV5, a generous gift from Dr. H. J. Kim. A unique *Nco*I site in the cDNA sequence was removed without changing the amino acid sequence by oligonucleotide-directed mutagenesis (Kunkel, 1985), as the change was reported to improve the expression level of OVA cDNA in *E. coli* (Takahashi *et al.*, 1995). For overexpression, the nucleotide sequence of OVA cDNA encoding the amino terminus of OVA was modified by oligonucleotide-directed mutagenesis (Kunkel, 1985) to yield pFETOVA-N2. The plasmid pF(BGL)OVA for *in vitro* translation of OVA was constructed by replacing α_1AT cDNA in pF(BGL)AT (Yu *et al.*, 1995) with OVA cDNA from pYOV5. In the pF(BGL)AT vector, α_1AT cDNA was connected downstream of the T7 promoter and the 5′-untranslated region of the rabbit β-globin gene. In all cases, cysteine at 120 was replaced with alanine by site-directed mutagenesis to remove a unique disulfide bond between Cys73 and Cys120 in the molecule. Most OVA expressed in *E. coli* accumulated as inclusion bodies. Cells were disrupted by sonication in Buffer A (50 mM Tris·HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol). Inclusion bodies were collected by centrifugation, washed twice with buffer A containing 0.5% Triton X-100, and were solubilized in 10 M urea in buffer A at a protein concentration of 2 mg/ml by the aid of sonication. Refolding was carried out by diluting the sample 20-fold with buffer B (10 mM phosphate buffer, pH 6.5, 1 mM EDTA, 1 mM β-mercaptoethanol). The refolded protein was dialyzed against buffer B. The dialysate was applied to a DEAE-Sephacel column equilibrated with buffer B and proteins were eluted with a linear gradient of 0–0.6 M NaCl in buffer B. The purification steps were assessed on 12% SDS-polyacrylamide gel electrophoresis or native gel electrophoresis in the absence of SDS and β-mercaptoethanol.

***In vitro* translation** *In vitro* transcription was performed with the SP6/T7 transcription system (Boehringer-Mannheim, Mannheim, Germany) at 37°C for 1 h. *In vitro* translation was performed with a rabbit reticulocyte lysate system (Promega, Madison, USA) according to the manufacturer's instruction. A typical translation reaction mixture (final volume of 25 μl) contained 20 μCi [^{35}S]methionine, 0.5 μg mRNA, and 17.5 μl of reticulocyte lysate that was treated with micrococcal nuclease. The labeling reaction was carried out during translation at 30°C for 1 h, which was followed by a chase with 5 mM unlabeled methionine.

Urea-induced equilibrium unfolding transition Equilibrium unfolding as a function of urea concentration was monitored by fluorescence spectroscopy, details of which were described previously (Kwon *et al.*, 1994; Lee *et al.*, 1996). The buffer used for folding experiments was 10 mM phosphate, 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 6.5, unless described otherwise. The native protein was incubated in the folding buffer containing various concentrations of urea at 25°C for 8 h. The protein concentration for the unfolding transition was 7–20 μg/ml. Experimental data of the fluorescence measurements were fitted to a two-state unfolding model, as previously described (Kwon *et al.*, 1994; Lee *et al.*, 1996).

Transverse urea gradient gel electrophoresis Gels were prepared with a gradient of 0 to 8 M urea perpendicular to the direction of electrophoresis with an opposing gradient of acrylamide from 15% to 11% (Goldenberg, 1989). Four slab gels (100 × 80 mm) were prepared simultaneously in a multigel caster by using a gradient maker and a single-channel peristaltic pump. The electrode buffer was 50 mM Tris-acetate, 1 mM EDTA, pH 7.5. The native protein (20 μg in 100 μl), the protein unfolded in 8 M urea (10 min at room temperature), or the *in vitro* translation product was applied across the top of the gel. The gels were run at a constant current of 6 mA for 3 h at a controlled temperature of 25°C. The protein bands were visualized either by Coomassie Brilliant Blue staining or by autoradiography.

Protease digestion Various forms of OVA were digested with porcine pancreatic elastase and trypsin. *In vitro* translation products (3 μl) or purified OVA (3 μl, 1 mg/ml) were digested in 10 mM sodium phosphate, pH 6.5, 1 mM EDTA, 1 mM β-mercaptoethanol, 50 mM NaCl, and 1 μl of elastase (0.01 μg/μl) or 1 μl of trypsin (0.01 μg/μl) for 20 min at room temperature. Digestion was stopped by adding 1 μl of PMSF (0.2 M). The cleavage products were separated on 12% SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography.

Results and Discussion

High-level expression of recombinant chicken OVA A previous study reported that chicken OVA cDNA could be expressed under the control of phage T7 promoter in *E. coli* only by mutating the unique *Nco*I site in the cDNA

sequence and without adding IPTG to induce the protein expression (Takahashi *et al.*, 1995). However, we could not reproduce the result of overexpressing OVA in *E. coli* by a similar vector, pFETOVA. We reasoned that the secondary structure of the 5′-end of mRNA encoded by the original OVA cDNA could form a stable hairpin structure in the translation initiation site (Fig. 1, wt; $\Delta G^{25°C}$ = -6.5 kcal mol^{-1}), which might interfere with translation in the prokaryotic system. We modified the cDNA sequence encoding the 5′-end of mRNA to decrease the stability of the presumed hairpin structure without changing amino acid sequences (Fig. 1, OVA-N2; $\Delta G^{25°C}$ = -1.9 kcal mol^{-1}). When the expression of OVA from the resulting plasmid, pFETOVA-N2, was induced in *E. coli* by IPTG, the expression level of OVA was about 30% of the total cellular proteins (Fig. 2A, lane 2).

The OVA polypeptides accumulated mainly as inclusion bodies (Fig. 2A, lane 3) and had to be refolded. Refolding was carried out by direct 20-fold dilution of the sample solution containing 10 M urea into a refolding buffer.

The refolding yield of the wild-type recombinant OVA was extremely low and most of the polypeptides were recovered in aggregated forms. We reasoned that much of the aggregation during refolding resulted from non-native disulfide formation. OVA contains one disulfide bond between Cys 73 and Cys 120, which is not present in the prototype inhibitory serpin, α_1AT (Huber and Carrell, 1989). We constructed a disulfide-free OVA by substituting Cys 120 with alanine (C120A) to minimize possible complications during the refolding process, especially disulfide reshuffling (Chang, 1997; Onda *et al.*, 1997). Over 50% of the C120A polypeptides were recovered in the soluble monomeric fraction. Refolded OVA, designated rf-OVA, was purified by DEAE column chromatography (Fig. 2A, lane 4). About 10 mg of the purified C120A rf-OVA was obtained from the 1L culture. Due to the lack of post-translational modifications such as phosphorylation and glycosylation, the rf-OVA migrated faster than the authentic form (Fig. 2A, lane 5) on SDS-polyacrylamide gel electrophoresis. N-terminal sequencing of the rf-OVA revealed that the N-terminus was blocked. The rf-OVA showed a single band in native gel electrophoresis (Fig. 2B, lane 2) and a single peak at a position estimated to be 40 kDa in mass on gel filtration chromatography (Fig. 3). These results indicated that C120A rf-OVA was in a soluble and monomeric state.

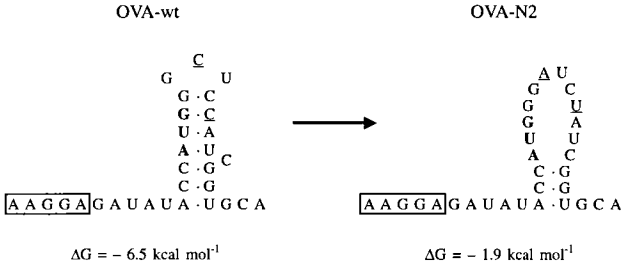

Fig. 1. Predicted secondary structures for the 5'-end region of ovalbumin mRNAs. Hairpin structures were generated by the RNA folding program PC/Gene for mRNA sequences encoded by pFETOVA (OVA-wt) and pFETOVA-N2 (OVA-N2) vectors. The ribosome binding site is indicated by boxes. Bold letters indicate the initiation codon. The nucleotide sequences changed in OVA-N2 are underlined. The estimated stabilities of the predicted secondary structures at 25°C are also shown.

Conformational properties of C120A rf-OVA It was reported that the conformational state of the reduced form of authentic ovalbumin was almost indistinguishable from that of the disulfide-bonded form, as revealed by CD spectra (Takahashi and Hirose, 1992; Onda *et al.*, 1997). The only difference was that the oxidized form showed a slightly higher unfolding temperature than the reduced form (78°C vs 71°C, Onda *et al.*, 1997). Conformational stability of rf-OVA carrying C120A was examined by equilibrium unfolding analysis. Excitation of the native rf-OVA at 280 nm produced an emission maximum at 338 nm (Fig. 4, insert). The presence of 8 M urea in solution induced a decrease in fluorescence intensity (about 40% of native rf-OVA) and a shift in emission maximum from 338 nm to 352 nm, indicating that tryptophan residues were exposed to a more polar environment after unfolding

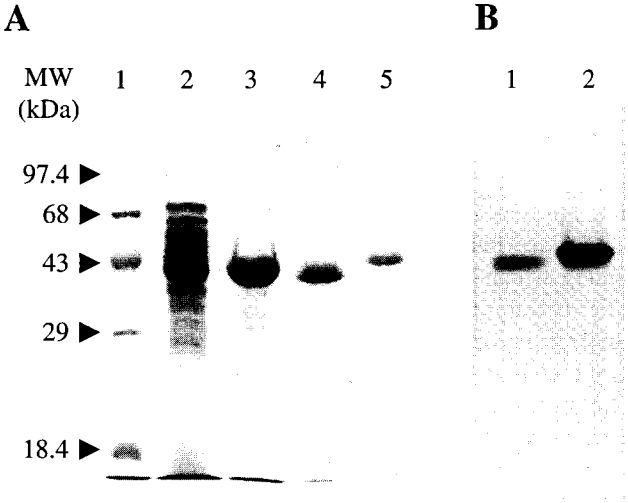

Fig. 2. Expression and purification of recombinant ovalbumin produced in *E. coli*. A. SDS-polyacrylamide gel electrophoresis of purified OVA. Lane 1, molecular mass markers; lane 2, cell lysate; lane 3, inclusion bodies; lane 4, purified refolded OVA carrying C120A substitution; and lane 5, purified chicken OVA. B. Native polyacrylamide gel electrophoresis of purified OVA. Lane 1, chicken OVA; and lane 2, refolded C120A OVA. The conditions for the native gel were the same as SDS-polyacrylamide gel electrophoresis except that the gel did not contain SDS and β-mercaptoethanol.

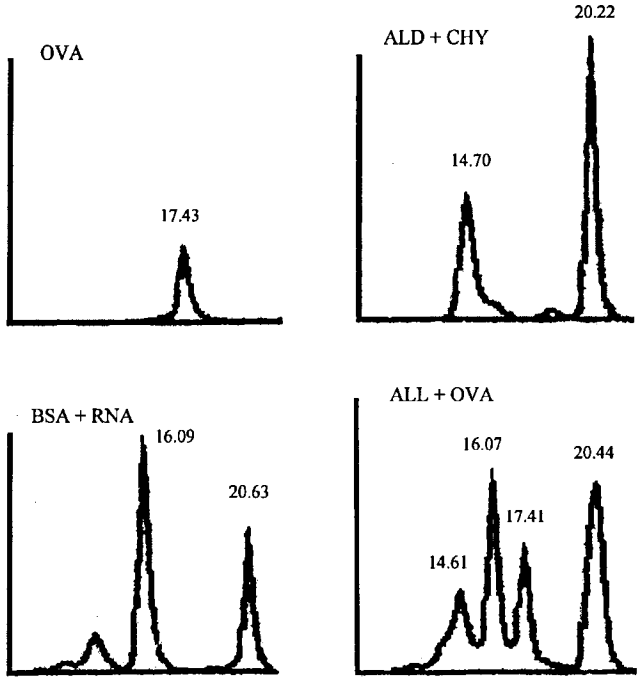

Fig. 3. Gel-filtration chromatography of refolded ovalbumin. From the top-left: purified C120A rf-OVA (OVA); mixture of bovine serum albumin (BSA, 66 kDa) and ribonuclease A (RNA, 14 kDa); mixture of aldolase (ADL, 158 kDa) and α-chymotrypsin (CHY, 25 kDa); and mixture of all the molecular weight markers and C120A rf-OVA. The elution time of each protein is also shown. The peak eluted earlier than BSA in the second profile is the aggregated form of BSA. The column was Protein Pak 300SW (Waters) and the elution buffer was 10 mM phosphate buffer, pH 6.5, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.3 M NaCl, and 1 mM NaN_3.

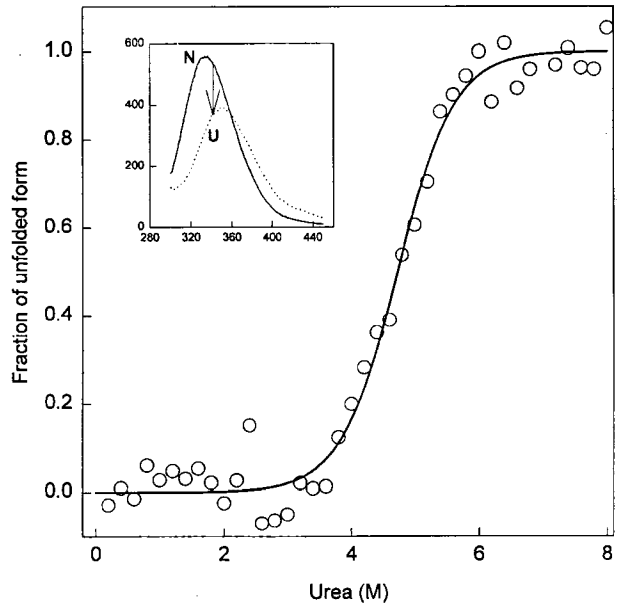

Fig. 4. Conformational stability of ovalbumin carrying C120A substitution. Samples of C120A OVA were equilibrated in a buffer (10 mM sodium phosphate, 1 mM EDTA, 1 mM β-mercaptoethanol, 50 mM NaCl, pH 6.5) containing various concentrations of urea for 16 h at 25°C. Fluorescence emission intensity was measured at 340 nm (λ_{em} = 280 nm, excitation and emission slit widths = 5 nm for both). The concentration of protein was 7 μg/ml. The *Cm* was 4.7 (± 0.1) M as determined by fitting the experimental data to a two-state unfolding model. The insert is of the fluorescence spectra of refolded C120A OVA (20 μg/ml) in the absence of urea (N, native state) and in the presence of 8 M urea (U, unfolded state).

in the presence of urea. OVA has three tryptophan residues at residues number 160 (hF), 194 (s3A), and 275 (hH), among which Trp 194 was completely buried, while the other two tryptophans were partially exposed (Stein *et al.*, 1990). It is likely that the buried Trp 194 contributes most to the change in fluorescence intensity during unfolding. The urea-induced equilibrium unfolding of rf-OVA carrying C120A was monitored by fluorescence intensity change at 340 nm. The fractions of unfolded molecules at various urea concentrations were determined by fitting the experimental data to a two-state unfolding model (Fig. 4). The midpoint of unfolding transition (*Cm*) of the C120A rf-OVA was 4.7 M. A similar value of unfolding *Cm* (4.8 M) was reported with a reduced authentic OVA, obtained from CD (circular dichroism) spectroscopy (Takahashi and Hirose, 1992). The results indicate that the conformation of rf-OVA is similar to that of the authentic form and that the amino acid substitution of C120A itself does not have any significant effect on the conformational stability of the molecule. The equilibrium unfolding of rf-OVA showed a cooperative transition and fitted well to a two-state transition. The results suggest that rf-OVA is folded well and follows a normal unfolding conformational change that is observed in regular globular proteins.

Conformational stability of rf-OVA was further examined by transverse urea gradient gel electrophoresis. Refolded OVA carrying C120A showed an unfolding transition at about 5 M urea (Fig. 5, rf-OVA), which is consistent with the *Cm* value of equilibrium unfolding monitored by fluorescence probe (Fig. 4). In addition, any intermediate having a different hydrodynamic property than either the native or the unfolded form was not observed during the unfolding transition. This is quite a contrast to the unfolding pattern of α_1AT in which a compact equilibrium unfolding intermediate was observed in urea gradient gel (Kim *et al.*, 1995). These results are consistent with previous reports that authentic OVA undergoes two-state unfolding transition as revealed by various physico-chemical probes, including circular dichroism and viscosity (Ahmad and Salahuddin, 1976; Bruch *et al.*, 1988; Takahashi and Hirose, 1992).

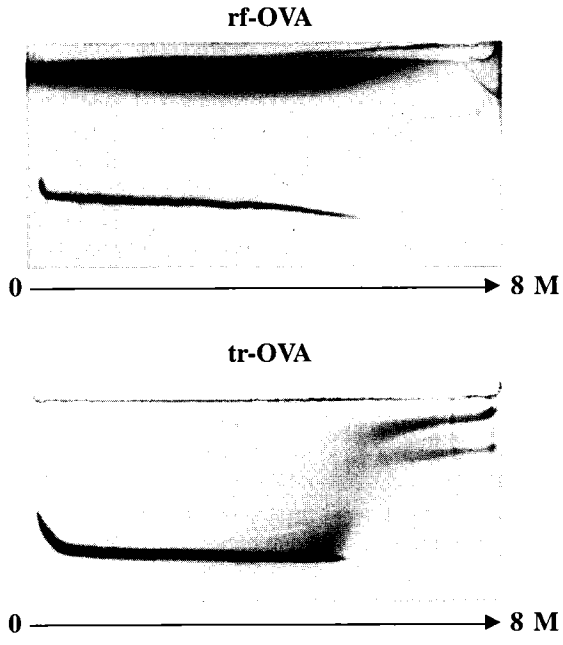

Fig. 5. Stability of ovalbumin on transverse urea gradient gel electrophoresis. The purified refolded OVA (rf-OVA) or *in vitro* translational product of pF(BGL)OVA (tr-OVA) was applied at the top of the gels. Both OVA samples carried the C120A substitution. Electrophoresis was carried out at 6–10 mA of constant current for 3 h at 25°C. Protein bands were visualized either by staining with Coomassie Brilliant Blue (rf-OVA) or by autoradiography (tr-OVA).

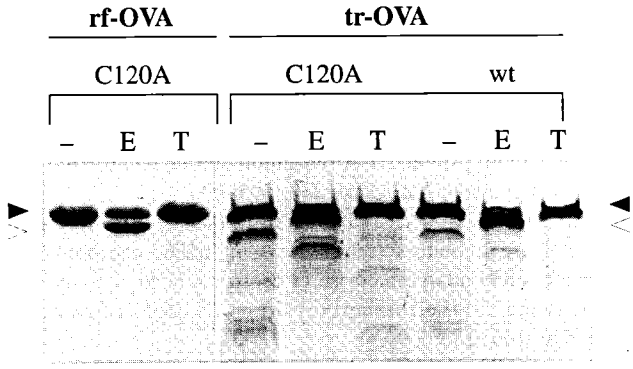

Fig. 6. Conformational probing of ovalbumin by limited proteolysis. Ovalbumin was incubated with 1 μl of elastase (0.01 μg/μl) or trypsin (0.01 μg/μl) for 20 min at 25°C. The reaction was terminated by adding 1 ml of PMSF (0.2 M). The rf-OVA, refolded-OVA; tr-OVA, *in vitro* translational products of pF(BGL)OVA plasmid. Filled arrow heads indicate the intact form of OVA and empty arrow heads indicate the proteolytic product that was cleaved, presumably at the reactive center loop. The elastase attack of tr-OVA yielded an additional smaller polypeptide, which appears to be produced from an unidentified protein band contained in the original translational products. The amount of OVA used was 3 μg of purified rf-OVA or 3 μl of tr-OVA that was estimated to contain 10–30 ng/μl of OVA and various other translational components. The products were analyzed on 12% SDS-polyacrylamide gel electrophoresis. Protein bands were visualized either by staining with Coomassie Brilliant Blue (rf-OVA) or by autoradiography (tr-OVA).

Finally, the conformational state of rf-OVA was compared with that of OVA translated *in vitro*. It was reported previously that refolded OVA is different from the *in vitro* translation product (Klausner *et al.*, 1983). Analyses on urea gradient gel electrophoresis showed, however, that the unfolding transition of C120A OVA translated *in vitro* (tr-OVA) occurred at a similar urea concentration as that of C120A rf-OVA (Fig. 5). The conformation of each form of OVA was further probed by limited proteolysis. It was reported that chicken egg OVA is not an inhibitor of elastase but rather serves as a substrate, where the reactive center loop of OVA is cleaved by elastase (Wright, 1984). Figure 6 showed that rf-OVA and tr-OVA were both cleaved by elastase, yielding a 36 kDa polypeptide on SDS polyacrylamide gel electrophoresis (indicated by empty arrow heads). Both forms of OVA, however, were resistant to the proteolytic attack by trypsin (Fig. 6). The elastase attack of tr-OVA yielded an additional smaller polypeptide, which was presumably produced from a contaminated protein originally contained in the translational products. The results suggest that both rf-OVA and tr-OVA are likely to have a similar conformation.

In summary, the following criteria strongly support that the conformation of rf-OVA produced in the present study is very similar to authentic OVA besides the lack of post-translational modifications. First, the refolded form is soluble and monomeric, eluting on gel filtration chromatography at the position expected for a molecular mass of 40 kDa (Fig. 3). Second, conformational stability probed by equilibrium unfolding (Figs. 4 and 5) is very similar to that of authentic OVA reported previously (Takahashi and Hirose, 1992). Third, consistent with the two-state unfolding of authentic OVA, the unfolding of rf-OVA did not yield any detectable equilibrium intermediates (Figs. 4 and 5). Finally, rf-OVA is very similar to the form of OVA produced during *in vitro* translation, as judged by urea-induced unfolding (Fig. 5) and limited proteolysis (Fig. 6). Being available in a large quantity, the rf-OVA carrying C120A can now serve as a model molecule of non-inhibitory serpins in comparative folding studies with inhibitory members, which should eventually elucidate the molecular basis of the conformational switch that is related to serpin function.

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