Serpin Conformational Change in Ovalbumin. Enhanced Reactive Center Loop Insertion through Hinge Region Mutations†

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ABSTRACT: Ovalbumin is a noninhibitory member of the serpin superfamily that does not spontaneously undergo the loop-to-sheet conformational change upon cleavage of its reactive center that is characteristic of inhibitory serpins. We tested the hypothesis that ovalbumin could be turned into a proteinase inhibitor by increasing the rate of loop insertion through hinge region mutations alone. We found that none of the three variants examined showed any detectable proteinase inhibitory properties. However, replacement of the P14 arginine residue of ovalbumin by serine, either alone or in combination with changes of P12−P10 to alanine, resulted in a large increase in the rate of loop insertion into β-sheet A following cleavage at the P1−P1′ bond by porcine pancreatic elastase (PPE), as shown by the spontaneous formation of a loop-inserted form upon cleavage that has increased the thermal stability. From the magnitude of the increase in stability of the cleaved, loop-inserted forms of the P14 ovalbumin variants, as well as the accessibility of the P1−P1′-cleaved reactive center loop to further proteolysis at P8−P7, we concluded that the reactive center loop can only partially insert into β-sheet A and therefore that ovalbumin is also defective in the ability of β-sheet A to expand to fully accommodate the whole of the reactive center loop. This defect, through its effect on the extent and/or rate of loop insertion, is likely to be a principal reason for ovalbumin not being a proteinase inhibitor.

Ovalbumin is a noninhibitory member of the serpin superfamily that does not spontaneously undergo the loop-to-sheet conformational change upon reaction with proteinases that is considered to be necessary for inhibition of the proteinase by the serpin kinetic trapping mechanism (Gettins et al., 1993; Wright, 1996). This conformational change involves integration of all or part of the previously exposed reactive center region into β-sheet A as a central strand (Figure 1) and has been proposed to result in a kinetic trapping of the covalent acyl−ester intermediate formed between the serpin and the proteinase through a translocation of the proteinase from one end of the serpin to the other (Wright & Scarsdale, 1995; Gettins et al., 1996). The hinge point for this insertion is at P15−P14,[1] with the side chain of P14 becoming buried in the hydrophobic interior of the serpin upon insertion of the reactive center loop. In inhibitory serpins, the P14 residue has a small uncharged side chain such as serine, threonine, or valine (Figure 1). On the basis of X-ray structures of native (Stein et al., 1991) and cleaved (Wright et al., 1990) ovalbumin, it was originally hypothesized that the presence of a charged residue, arginine, at position P14 in ovalbumin made loop insertion thermodynamically prohibitive and thus explained the inability of ovalbumin to inhibit proteinases by completely blocking loop insertion (Wright et al., 1990). However, it was subsequently shown that a P14 arginine variant of the inhibitory serpin

	P16	P15	P14	P13	P12	P11	P10	P9	P8
Inhibitory Consensus:	Glu	Gly	Thr	Glu	Ala	Ala	Ala	Ala	Thr
% Identity:	38	98	80	85	98	75	65	75	83
Ovalbumin	Ala	Gly	Arg	Glu	Val	Val	Gly	Ser	Ala
P12-P10	Ala	Gly	Arg	Glu	**Ala**	**Ala**	**Ala**	Ser	Ala
P14	Ala	Gly	**Ser**	Glu	Val	Val	Gly	Ser	Ala
P14, P12-P10	Ala	Gly	**Ser**	Glu	**Ala**	**Ala**	**Ala**	Ser	Ala
Secondary Structure									
Ovalbumin	C	C	C	C	C	C	C	α	α
Ovalbumin*	C	C	C	C	C	C	C	C	C
P14, P12-P10*	β	β	β	β	β	β	β	?	C
α_1-PI	C	C	C	C	C	C	C	C	β
α_1-PI*	β	β	β	β	β	β	β	β	β

FIGURE 1: Primary and secondary structure of the reactive center loop of ovalbumins and other serpins and the consensus sequence for inhibitory serpins. (Top) Comparison of the primary structures of wild type and the three variant ovalbumins with the consensus sequence of inhibitory serpins for the distal part of the reactive center region. Residues in ovalbumin that were changed in this study are shown in bold. (Bottom) secondary structure of this region for cleaved (*) and uncleaved ovalbumins and cleaved (*) and uncleaved α_1-PIs, together with the present proposal for secondary structure of the P1−P1′-cleaved P14,P12−P10 ovalbumin variant. Note the transformation of unstructured reactive center loop residues into β-strand upon cleavage for α_1-PI. The secondary structures were obtained from the published structures of ovalbumin (Stein et al., 1991), cleaved ovalbumin (Wright et al., 1990), α_1-proteinase inhibitor (Elliott et al., 1996), and cleaved α_1-proteinase inhibitor (Löbermann et al., 1984). α represents α-helix, β β-sheet, and C nonordered structure.

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[1] The nomenclature of Schechter and Berger (1967) is used to designate residues on a proteinase substrate close to the peptide bond that is cleaved. The residues that form the scissile bond are designated P1 and P1′. Residues N-terminal to P1 are designated P2, P3, ..., etc., and those C-terminal to P1′ are designated P2′, P3′, ..., etc.

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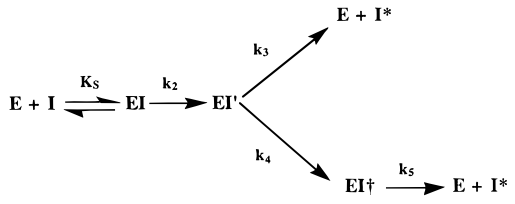
Scheme 1



α_1-proteinase inhibitor (α_1-PI)[2] was still an inhibitor of trypsin and elastase, although with significantly reduced efficiency (Hood et al., 1994). In addition, native ovalbumin can very slowly undergo partial insertion of the reactive center loop to give a more stable form (*S*-ovalbumin), though with a high activation energy for insertion (Donovan & Mapes, 1976; Huntington et al., 1995). Reactive center-cleaved chicken ovalbumin can also be very slowly converted to a loop-inserted form as indicated by its enhanced thermal stability (Shitamori & Nakamura, 1983; Huntington et al., 1995). A recent X-ray structure of a P14 arginine variant of α_1-antichymotrypsin provided a structural explanation for how arginine at this position can be compatible with loop insertion by showing that the reactive center loop inserted from P13 to P3, with exclusion of the P14 arginine side chain from the sheet (Lukacs et al., 1996). In light of these findings, the question still remains of why ovalbumin is not an inhibitory serpin, since the presence of arginine at P14 is not *per se* sufficient to preclude either inhibition or loop insertion.

A more plausible explanation for the absence of inhibitory properties in ovalbumin is that it is the slow *rate* of loop insertion that determines the outcome of the encounter between ovalbumin and a potential target proteinase and that this rate is principally determined by residues in the hinge region. This follows directly from the branched pathway suicide substrate inhibition mechanism of serpins (Patston et al., 1994) (Scheme 1). In this scheme, EI′ represents the acyl enzyme intermediate, which can be deacylated by the normal substrate pathway with rate constant k_3 or form a stable complex, EI†, with rate constant k_4, as a result of loop insertion trapping the complex. Thus, it is plausible that loop insertion in ovalbumin, while thermodynamically favorable, is kinetically disfavored to such an extent that the substrate branch of the pathway (k_3) is very much faster than the inhibitory branch (k_4).

We describe here the creation and characterization of three hinge region variants of ovalbumin that were designed to test whether changes in this region alone can transform ovalbumin into a proteinase inhibitor by increasing the rate of loop insertion. The mutations involved (i) replacement of the P14 arginine by serine and (ii) replacement of the P12−P10 Val-Val-Gly residues by the consensus sequence Ala-Ala-Ala and (iii) a combination of all four mutations (Figure 1). We found that none of the variants showed any detectable proteinase inhibitory properties. However, replacement of the arginine at position P14 by serine, either alone or in combination with the P12−P10 changes, did greatly increase the rate of loop insertion following reaction with elastase. Comparison of the stabilities of spontaneously loop-inserted forms of these cleaved ovalbumins, as well as the continued accessibility of part of the loop to further proteolysis, indicated that only partial loop insertion probably occurred. This suggests that additional structural defects exist in ovalbumin that prevent the sufficiently extensive and/or fast loop insertion required to bring about proteinase inhibition. It is likely that these defects involve regions outside of the reactive center loop itself, most probably helices B and C, which underlie β-sheet A and may therefore control its expansion.

[2] Abbreviations: α_1-PI, α_1-proteinase inhibitor; CD, circular dichroism; PPE, porcine pancreatic elastase; SI, stoichiometry of inhibition; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Production and Isolation of Recombinant Ovalbumins. Chicken ovalbumin cDNA was excised from plasmid pOV230 (McReynolds et al., 1978) (a gift from B. W. O'Malley, Baylor College of Medicine) by digestion with *Taq*I and *Xba*I and ligated into pBluescript (Stratagene) using the complementary 3′ *Cla*I and 5′ *Xba*I sites (Krieg et al., 1982). Cleavage of this construct by *Sal*I, immediately upstream of the *Cla*I/*Taq*I site, and by *Xba*I gave an ovalbumin cDNA-containing fragment which was inserted into pAlter-1 (Promega) in the antisense orientation. All mutagenesis was carried out in this vector according to the protocol supplied by the manufacturer, using alternating rounds of selection with ampicillin and tetracycline in JM 109 cells. An internal *Nco*I site at position 181 was disrupted by a silent mutation as previously described (Takahashi et al., 1995), which permitted subsequent ligation into the pET3d (Novagen, Madison, WI) expression plasmid. The P14 arginine to serine mutation was accomplished using the antisense oligonucleotide 5′-CC TAC CAC CTC **G**CT GCC TGC TTC-3′, thus changing AGA to AGC on the coding strand. The valine-valine-glycine tripeptide at P12−P10 was mutated to alanine-alanine-alanine using the oligonucleotide 5′-GC CTC TGC TGA C**G**C T**G**C C**G**C CTC TCT GCC TGC-3′. The variant containing both sets of mutations, at P14 and at P12−P10, was made using the template of the P14S mutant and the oligonucleotide 5′-GC CTC TGC TGA C**G**C T**G**C C**G**C CTC GCT GCC TGC-3′. Ovalbumin constructs were excised by *Nco*I and *Bam*HI and ligated into pET3d. Competent BL21 (DE3) cells (Novagen) were transformed with the ovalbumin cDNA-containing pET3d plasmids following the protocol provided by the manufacturer. Transformants were selected on ampicillin-containing (50 mg/L) LB plates. Expression and purification of recombinant ovalbumin were accomplished essentially as was described previously (Takahashi et al., 1995). A single colony was grown to saturation in 500 mL of LBA (100 mg/L), and cells were collected by centrifugation and resuspended in 25 mL of 5 mM sodium phosphate buffer (pH 6.0) to which had been added 0.5 mL of 250 mM PMSF, and 0.2 mL of 500 mM EDTA to inhibit proteolysis. The cells were sonicated on ice two times for 2 min. The soluble fraction was collected after spinning in 15 mL falcon tubes for 10 min at 10 000 rpm at 4 °C and dialyzed against 2 L of 5 mM sodium phosphate buffer (pH 6.0) overnight at 4 °C. Expression was checked by radial immunodiffusion assay, with plates made according to the procedure of The Binding Site using an anti-ovalbumin antibody from Calbiochem. The soluble fraction typically contained ∼40 mg of ovalbumin per liter of culture medium. After dialysis, the sample was split into three 10 mL fractions which were separately filtered through a 0.2 μm filter and loaded onto a

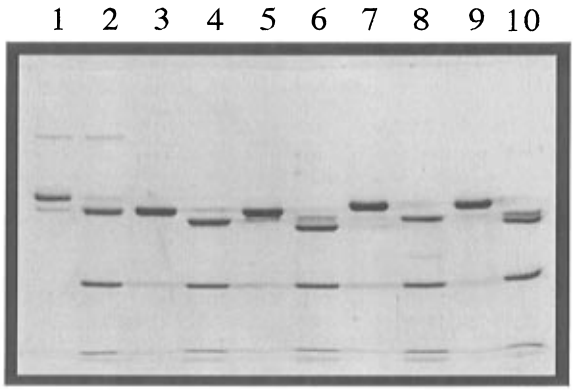


FIGURE 2: Specific cleavage of recombinant ovalbumins by PPE. A 10% polyacrylamide−SDS gel of native and PPE-reacted chicken ovalbumin (lanes 1 and 2), recombinant wild-type ovalbumin (lanes 3 and 4), the P12−P10 variant (lanes 5 and 6), the P14S variant (lanes 7 and 8), and the P14S,P12−P10 combination variant (lanes 9 and 10). Ovalbumin (2 μg) was loaded in each lane. A 1:2 molar ratio of ovalbumin and PPE was used for all reactions (2.6 μg of PPE) in order to demonstrate the selectivity of the proteinase for the reactive center loop of ovalbumin, and to allow for distinction of any potential complex bands. After reaction for 20 min at room temperature, no bands other than that corresponding to the reactive center-cleaved product were observed. The band in the middle of the gel corresponds to the ovalbumin species; the upper band in lanes 1 and 2 is a contaminant from the commercial chicken ovalbumin, and the lower band is PPE. The band running with the dye front is the C-terminal fragment (MW of 3740) which is released upon cleavage. Note that both the P12−P10 and P14,-P12−P10 variants show two cleavage products.

Mono Q column (Pharmacia). Ovalbumin eluted halfway through a gradient from 5 to 150 mM sodium phosphate (pH 6.0) at a flow rate of 1 mL/min. The column was regenerated between applications as recommended by Pharmacia. The middle of the ovalbumin peak was collected for each application, and these fractions were pooled, dialyzed against 5 mM sodium phosphate (pH 6.0), and re-run on the Mono Q column. The resulting fractions yielded ~8 mg of ovalbumin and were >95% pure by SDS−PAGE.

Cleavage and Purification of Ovalbumins. Ovalbumin variants which contained the P12−P10 AAA mutations were susceptible to further cleavage within the reactive center loop in a sequential manner, as shown by both SDS−PAGE (see Figure 2) and mass spectrometric analysis (see Table 1). Cleavage could be limited to the P1−P1′ position, however, by use of a higher ovalbumin:PPE ratio (200:1) and a shorter reaction time (30 min) at room temperature. Reactions were stopped with a large excess of PMSF and subsequent dilution into 5 mM sodium phosphate (pH 6.0) running buffer. Completion of the reaction was verified by SDS−PAGE and resulted in greater than 80% singly cleaved variants. In all cases, PPE was separated from the cleaved ovalbumins via Mono Q chromatography with the same gradient used for initial purification of ovalbumin. This method was sensitive enough to separate uncleaved and multiply cleaved ovalbumins as well and resulted in relatively pure P1−P1′-cleaved species.

Electrospray Mass Spectrometric Analyses. Mass spectra were recorded using a Finnigan TSQ 700 (San Jose, CA) triple-quadrupole mass spectrometer equipped with a Finnigan electrospray ion source, as described previously for examination of a serpin−proteinase complex (Strömqvist et al., 1996). Immediately before the spectra were recorded, a buffer exchange of the proteins was performed on a Bio-Spin 6 column (Bio Rad Laboratories, Hercules, CA) equilibrated in 1% acetic acid/water (v/v). The sample was infused at 1 μL/min and mixed at 3 μL/min with a modifier solution [30% acetonitrile/water (v/v)] directly in the spray needle using two Harvard Syringe pumps (Harvard Apparatus, South Natick, MA). The capillary temperature was 200 °C and the spray voltage 3.5 kV. The mass spectrometer was calibrated in the high-MW range using myoglobin and PEG 2000. The uncertainty of the mass derived from EC-MS is less than 1 unit of mass for the low-MW peptide and ±50 for the high-MW species. All protein samples were run twice.

Table 1: Electrospray Mass Spectrometric Analysis of Recombinant Ovalbumins and Their Elastase Cleavage Products

	intact or large fragment		small fragment	
ovalbumin	theoretical	experiment[a]	theoretical[b]	experiment
uncleaved				
wild type	42 885	42 898	n/a[c]	
P12−P10	42 843	42 858	n/a	
P14	42 816	42 820	n/a	
P14,P12−P10	42 774	42 789	n/a	
cleaved				
wild type	39 163[d]	39 193	3740	3740
P12−P10	38 349[e]	38 341	3740	3740
P14	39 094[d]	39 095	3740	3740
P14,P12−P10	39 052[d]	39 050	3740	3740
P14,P12−P10	38 280[e]	38 297	3740	3740

[a] Although the error in the determination of the mass of the small fragment is small, the error for the large fragment in the cleaved species may be as high as ±50 due to some heterogeneity of the main charged fragments. However, this uncertainty is very much less than the mass change resulting from more N-terminal cleavage. [b] For cleavage at P1−P1′. If additional cleavages occur N-terminal to this, the 3740 fragment will still be the only detectable fragment in this size range. [c] n/a, not applicable. [d] Calculated for cleavage at P1−P1′. [e] Calculated for major new cleavage at P10−P9, in addition to P1−P1′. Also evidence for a minor cleavage product at P9−P8. This form corresponds to the second P14,P12−P10 form listed in Table 2.

Circular Dichroism Spectra. CD spectra were recorded on a Jasco 710 spectropolarimeter. Ovalbumin samples were approximately 0.5 mg/mL in 20 mM sodium phosphate buffer (pH 7.0). Spectra were recorded from 260 to 200 nm using a 1 mm jacketed quartz cell at 25 °C, a scan rate of 5 nm/min, a response time of 8 s, a band width of 1 nm, and a resolution of 0.5 nm. Each spectrum represents the average of two scans. Spectra reported here are the average of two such spectra. The data were smoothed and normalized to the concentration of peptide bonds and are reported as mean residual differential extinction coefficients, $\Delta\epsilon$, using a molecular weight and number of peptide bonds of 42 700 and 385, respectively. Thermal denaturation was followed by monitoring the CD signal at 222 nm as a function of temperature, with an 8 s response time and a 2 nm band width. A jacketed 1 mm cell was heated at 0.5 °C/min, and data were collected at 0.5 °C intervals. Data were smoothed, and the first derivative was taken with a step of two data points. The first derivative results in a negative peak at the position of the inflection point corresponding to the midpoint of thermal denaturation and is a better way of determining the midpoint of the transition than that from the raw denaturation traces. Each temperature scan was repeated at least two times.

Kinetics of Cleavage of the P14,P12−P10 Variant by Subtilisin Carlsberg. The time course of cleavage of the

P14,P12−P10 ovalbumin variant by subtilisin Carlsberg, starting with either intact ovalbumin or ovalbumin that had been previously cleaved specifically at P1−P1′ by PPE and subsequently purified, was followed by SDS−PAGE of reactions of 5.3 μM P14,P12−P10 variant ovalbumin with 5.3 nM subtilisin Carlsberg at room temperature. Reactions were stopped at different times by addition of PMSF to a final concentration of 900 μM, followed by addition of a sample buffer containing dithiothreitol and boiling. Protein bands were visualized by staining with Coomassie brilliant blue and quantitated by scanning on an HP Deskscan II apparatus at a resolution of 600 dpi and analysis using the program Scan Analysis (Specom Research). Band intensities were corrected for background contributions and normalized to the total ovalbumin species present in each lane. The resulting concentrations of each ovalbumin species were then fitted simultaneously, using Scientist for Windows, to a model for sequential reactions for the reaction that started with intact ovalbumin and proceded to P1−P1′-cleaved ovalbumin and then to P8−P7-cleaved ovalbumin [it has previously been well documented that the second cleavage site at P8−P7 only becomes available after cleavage at P1−P1′ (Ottesen, 1958; Ottesen & Wollenberger, 1956)]. For the reaction that started with P1−P1′-cleaved variant, the data were fitted to a single pseudo-first-order reaction.

Analysis of the Ability of Ovalbumin To Inhibit Porcine Pancreatic Elastase. The ability of recombinant ovalbumins to form SDS stable complex with porcine pancreatic elastase (PPE) was assessed by running reaction mixtures of ovalbumin and PPE, at a high ratio of serpin to proteinase, on SDS−PAGE. Two identical 10% polyacrylamide gels were run. One was stained with Coomassie brilliant blue, and the other was used for transfer to nitrocellulose for Western blot analysis with a primary antibody for ovalbumin (Cal-BioChem) and a secondary anti-rabbit antibody. Ovalbumin (10 μg) was incubated for 30 min with 1 μg of PPE (molar ratio of 5.9:1). The reaction was stopped by addition of a sample buffer containing dithiothreitol and by boiling for 5 min. The ability of recombinant ovalbumins to inhibit PPE was also assayed in solution using a chromogenic substrate for PPE, *N*-succinyl-$(Ala)_3$-*p*-nitroanilide (Sigma). After 5, 15, and 60 min incubations at room temperature of 20 μL of 100 nM PPE with 12.4 μM ovalbumin, in a buffer containing 20 mM sodium phosphate, 100 mM NaCl, 0.1 mM EDTA, and 0.1% PEG 8000 (pH 7.4), residual PPE activity was determined by adding 180 μL of 500 μM *N*-succinyl-$(Ala)_3$-*p*-nitroanilide and following the change in absorbance at 405 nm on a Shimadzu spectrophotometer. A stoichiometry of inhibition (SI) of 1 was found for the reaction of α_1-PI with PPE under identical conditions.

RESULTS

Correct Folding of Recombinant Ovalbumins. Prior to any functional characterization of the recombinant ovalbumins, it was necessary to demonstrate that we had produced full length and correctly folded proteins. Correct size was demonstrated by mobility on an SDS gel run under reducing conditions, which gave a single band for each ovalbumin (Figure 2, lanes 3, 5, 7, and 9) that migrated slightly faster than glycosylated ovalbumin from chicken eggs (Figure 2, lane 1). A small difference in mobility was consistently seen for the two variants that contained R → S mutations at P14, which must result from a sequence specific change in SDS solvation. Electrospray mass spectrometry gave masses for all four recombinant ovalbumins consistent with the primary structure (Table 1). PAGE run under nondenaturing conditions showed that all four recombinant ovalbumins ran as a single band corresponding to native monomer (data not shown), rather than the dimers or higher-order aggregates that some serpins form (Lomas et al., 1992; Patston et al., 1995).

Correct folding of the monomeric ovalbumins was examined by two approaches. PPE cleaves chicken ovalbumin specifically and only within the exposed reactive center at the P1−P1′ bond (Wright, 1984), despite many potential cleavage sites in the primary structure. Such specific cleavage by PPE and release of the correct size fragments is thus an indication of correct folding of ovalbumin. A 20 min incubation at room temperature of the four ovalbumins with PPE at a 1:2 ratio showed (Figure 2) that all of the recombinant ovalbumins were correctly folded since they have the same cleavage that is limited to the reactive center (see also below) and which results in a small increase in mobility from loss of the C-terminal MW 3740 fragment.

Analysis of the conformation of the cleaved and uncleaved forms of chicken and recombinant ovalbumins was also performed using circular dichroism (CD) spectroscopy. CD spectra of chicken and native recombinant ovalbumins (Figure 3A) were all very similar in shape, indicating that the overall fold is like that of chicken ovalbumin. Although there are small differences in the magnitudes, they probably result from small differences in concentration. PPE cleavage did not result in any major change in CD spectra of either chicken or recombinant ovalbumins, consistent with specific cleavage in the reactive center loop of all of the ovalbumins and minimal change in the overall conformation of the protein as a result of this cleavage (Figure 3B).

Location of PPE Cleavage Sites within Recombinant Ovalbumins. Although PPE cleaves chicken ovalbumin specifically at P1−P1′ (Wright, 1984), it was necessary to determine the location of PPE cleavage sites in the recombinant ovalbumins, since it was possible that the mutations, particularly the replacement of the tripeptide VVG by AAA, had introduced additional sites of cleavage. SDS−PAGE of the recombinant ovalbumins that had been incubated with PPE at different molar ratios and/or for different times showed evidence of more than one cleavage site within the reactive center for the P12−P10 variant and the P14,P12−P10 variant, but not for the wild type or the P14 variant, from the presence of a band of mobility intermediate between that of uncleaved and that of fully reacted ovalbumin (see Figure 2).

Electrospray mass spectrometry confirmed that the P1−P1′ site remained a cleavage site in all cases, from the release of a MW 3740 fragment, corresponding exactly to the mass of the fragment from P1′ to the C terminus (Table 1). The masses of the large fragment (from the N terminus to the closest reactive center cleavage site) were as expected for a single cleavage at P1−P1′ for the P14 variant and for wild-type ovalbumins but indicated a second principal cleavage at P10−P9 (alanine-serine) in the P12−P10 and combination P14,P12−P10 variants (Table 1). When more limited reaction with PPE was carried out for the P12−P10 and P14,-P12−P10 combination variants, only the single cleavage at P1−P1′ was observed, as shown by lower mobility on SDS−PAGE (data not shown). For the P14,P12−P10 variant, mass

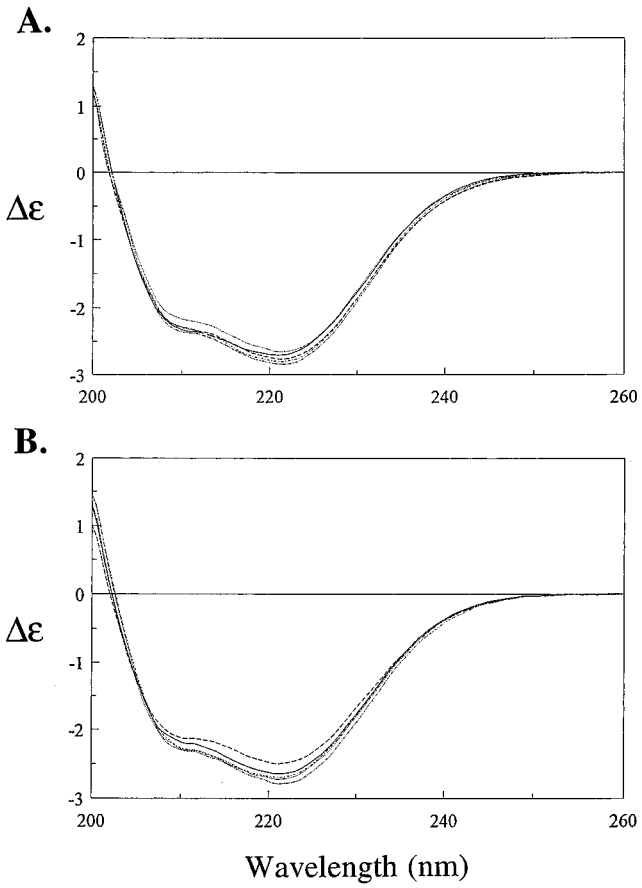


FIGURE 3: Correct folding of recombinant ovalbumins. CD spectra of native (A) and cleaved (B) ovalbumins are reported in mean residual differential extinction coefficients. For both native and cleaved species: chicken ovalbumin, solid line; recombinant wild-type ovalbumin, dashed line; the P12−P10 variant, dotted line; the P14 variant, dashed-dotted line; and the P14,P12−P10 variant, dashed-double-dotted line. The spectra shown are averages of at least two separately run spectra. Variability in CD intensities is due to errors in the concentration determinations, resulting from the low concentrations and small volumes of the samples.

spectrometry of this more restrictively cleaved species confirmed that cleavage was only at P1−P1′ (Table 1). The reactivity of the two sites was examined by SDS−PAGE of the P12−P10 variant incubated for different times with PPE. Cleavage at the P10−P9 site was found to be sequential and ∼17-fold slower than that at P1−P1′ (data not shown).

Stability of Cleaved Forms of Ovalbumin Variants. To determine whether any of the mutations had enhanced the ability of the reactive center loop to insert into β-sheet A of ovalbumin, we examined the thermal stability of the various reactive center-cleaved forms, using the change in CD ellipticity at 222 nm as an indicator of thermal denaturation. We found that the two variants in which P14 had been changed from arginine to serine had greatly increased stability, indicative of spontaneous loop insertion, whereas wild-type ovalbumin and the P12−P10 AAA variant (cleaved at P1−P1′) both showed the same 1 °C *decrease* in stability upon cleavage that is found for chicken ovalbumin (Figure 4 and Table 2). The P12−P10 variant cleaved at P10−P9 showed a slightly larger decrease in stability. Cleavage of the P14 R → S variant ovalbumin resulted in an 11 °C increase in thermal stability. Further replacement of the sequence VVG at P12−P10 with the sequence AAA gave an additional increase in stability of 5 °C, indicating that loop insertion must involve not only P14 but also some or all of residues P12−P10. The failure of the P14,P12−P10 variant to exhibit enhanced stability when cleaved at P10−P9 (Table 2) suggests that introduction of a new carboxyl on the P10 residue disfavors loop insertion and therefore that residues up to and perhaps including P10 are involved in insertion into β-sheet A.

Extent and Rate of Reactive Center Loop Insertion. To further examine the extent and rate of loop insertion, we exploited the fact that subtilisin Carlsberg cleaves ovalbumin first at P1−P1′ and subsequently at P8−P7 (Ottesen, 1958; Ottesen & Wollenberger, 1956) and determined whether the secondary proteinase cleavage site at P8−P7 was still accessible in the already loop-inserted form of the P1−P1′-cleaved P14,P12−P10 variant. A time course of reaction of both intact and P1−P1′-cleaved P14,P12−P10 variant ovalbumins with subtilisin Carlsberg was examined by SDS−PAGE. From the intensities of the bands corresponding to intact, P1−P1′-cleaved and P8−P7-cleaved species, the rate of the P8−P7 cleavage was determined for each starting ovalbumin (Figure 5). The P8−P7 bond remained accessible to subtilisin Carlsberg cleavage in the pre-loop-inserted P1−P1′-cleaved ovalbumin. However, the rate of cleavage by subtilisin Carlsberg was ∼36-fold lower than that for cleavage of the same site in P1−P1′-cleaved material generated *in situ* by starting with intact ovalbumin. Second-order rate constants for cleavage at P8−P7 were 2.5×10^4 and 6.9×10^2 M^{-1} s^{-1} for the faster- and slower-reacting species, respectively. This suggests continued, but more restricted, access to the P8−P7 bond in the precleaved and thus already loop-inserted species.

Interestingly, when the P1−P1′-cleaved species was reacted further, either at the same ovalbumin:proteinase ratio of 1000:1 for a longer time or at lower ovalbumin:proteinase ratios, it was apparent that, whereas ∼70% is slowly cleaved at P8−P7, the remaining 30% remains uncleaved. We know that the P1−P1′-cleaved starting material is chemically homogeneous from mass spectrometric analysis (Table 1) and from the presence of a single band by both SDS−PAGE and by nondenaturing PAGE. This suggests two different conformations for the P1−P1′-cleaved P14,P12−P10 variant in which the P8−P7 bond is protected to different degrees compared to uninserted ovalbumin. For the conformer present at ∼70%, the data are consistent with insertion from P14 no further than P10 or P9, such that the P8 side chain can be recognized by subtilisin Carlsberg. For the conformer present at ∼30%, insertion may also involve P9 and possibly P8.

These findings also allowed us to place limits on the rate of loop insertion. The rate constant of 2.5×10^4 M^{-1} s^{-1} for cleavage by subtilisin of the P14,P12−P10 variant at the P8−P7 position is that for the not-yet-inserted conformation. Therefore, following cleavage at P1−P1′, the loop must remain fully exposed long enough for cleavage at P8−P7 to occur (Figure 5). Thus, the rate of cleavage at the P8−P7 position in the exposed conformation places an upper limit on the rate of loop insertion. Using this second-order rate constant and a proteinase concentration of 5.3 nM, one obtains a first-order rate constant for loop insertion that must be less than 1.3×10^{-4} s^{-1}. This compares with a reported value for loop insertion in the inhibitory serpin plasminogen activator inhibitor 1 of 3.4 s^{-1} (Shore et al., 1995). Thus, even with the beneficial changes in the ovalbumin hinge

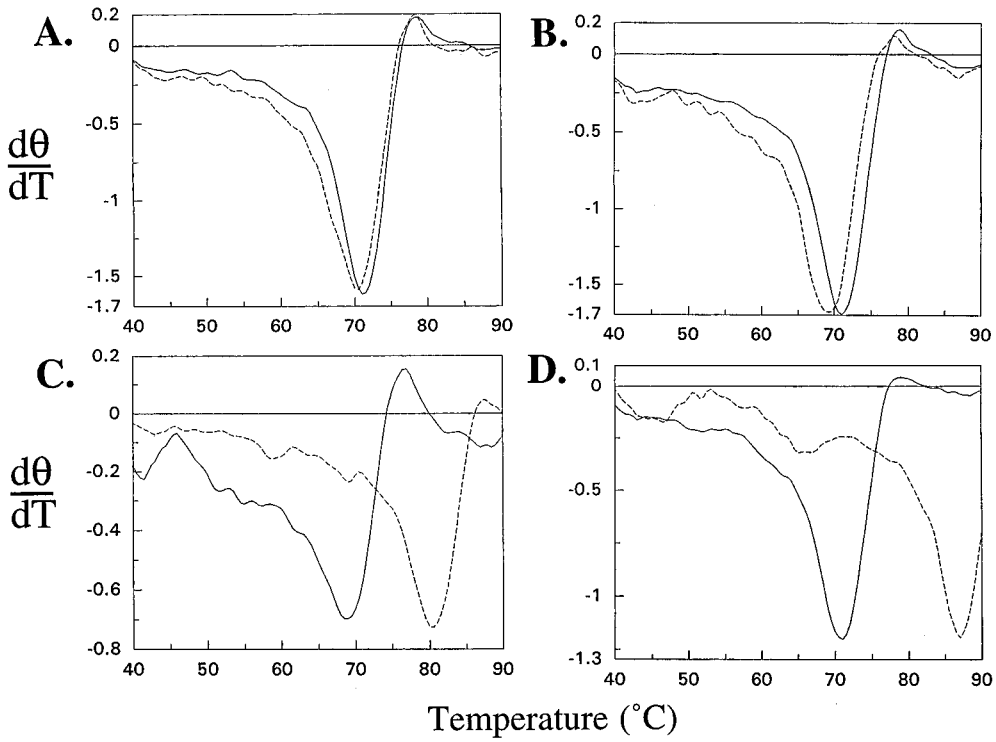


FIGURE 4: Increase in the thermal stability of P14 variants. Thermal denaturation profiles of native (solid lines) and P1−P1′-cleaved (dashed lines) ovalbumins followed by the change in CD ellipticity at 222 nm as a function of increasing sample temperature in a jacketed cell. The first derivatives of the ellipticity are reported here to make more clear the midpoint of the unfolding transition (T_m), which appears here as the negative peak. The T_ms for recombinant wild-type ovalbumin (A), the P12−P10 variant (B), and chicken ovalbumin (not shown) were all decreased by about 1 °C upon cleavage, whereas those for the P14 variant (C) and the P14,P12−P10 variant (D) showed increases of 11 and 16 °C, respectively, upon cleavage. The magnitudes of the derivatives for the cleaved ovalbumin species were normalized to make comparison to the uncleaved ovalbumins easier. For the P14,P12−P10 combination variant, an additional transition is visible at lower temperatures and almost certainly arises from the presence of a small amount of more extensively cleaved (cleaved at P10−P9) material, which is known, from the mass spectrometric analysis, to be present.

Table 2: Change in the Stability (Degrees Celsius) of Cleaved vs Native Forms of Chicken and Recombinant Ovalbumins

	chicken	wild type	P12−P10	P12−P10	P14	P14,P12−P10	P14,P12−P10
native	75.1 ± 0.1	71.1 ± 0.1	70.7 ± 0.4	70.7 ± 0.4	69.0 ± 0.3	71.1 ± 0.1	71.1 ± 0.1
cleaved	74.3 ± 0.1[a]	70.3 ± 0.4[a]	69.1 ± 0.1[a]	68.5[b]	80.4 ± 0.2[a]	87.0 ± 0.1[a]	65.7[b]
ΔT_m	−0.8	−0.8	−1.3	−2.2	+11.4	+15.9	−5.4

[a] Cleaved at P1−P1′. [b] This form is the more extensively cleaved form in which proteolysis at or close to P10−P9 has occurred in addition to cleavage at P1−P1′. The reactive center loop is thus greatly shortened, and a new carboxyl group is present on the P10 residue.

region at P14 and P12−P10 that permit more facile loop insertion, the rate of such insertion is still ~26000 times slower than in the inhibitory serpin plasminogen activator inhibitor 1. This is, however, an improvement on the rate of insertion for wild-type ovalbumin, which is estimated to be $\sim 5 \times 10^{-7}$ s^{-1}, based on the rate of conversion of ovalbumin into the partially loop-inserted conformer, *S*-ovalbumin (Donovan & Mapes, 1976). Although we did not determine rate constants for the sequential cleavages by PPE first at P1−P1′ and subsequently at P10−P9, we arrived at the same qualitative conclusion that loop insertion must be slow enough that the P10−P9 bond has not been buried in β-sheet A and remains accessible long enough for cleavage to go substantially to completion.

Inhibitory Properties of Hinge Region Variants of Ovalbumin. Although mutation of the P14 residue in ovalbumin from arginine to serine resulted in spontaneous loop insertion upon reactive center cleavage, this was not sufficient to confer proteinase inhibitory properties on the ovalbumin variants, as determined by the inability to form detectable covalent complex with PPE on SDS−PAGE (Figure 6) or to observe measurable inhibition of PPE in solution (data not shown). This means that, if proteinase inhibition did indeed occur, it was at a level below that detectable by these experiments. These two assays can therefore be used to place lower limits on the stoichiometry of inhibition for the reaction of ovalbumin with PPE. At an ovalbumin:PPE ratio of 5.9:1 (dictated by the need not to overload the gel), with 10 μg of ovalbumin loaded, no covalent complex was detectable on SDS−PAGE, analyzed either by Coomassie staining or by Western analysis, even though the reaction had proceeded to completion, as shown by complete cleavage of all of the ovalbumin. We conservatively estimate that an SI of 12 or lower would have resulted in detectable covalent complex. To place a better limit on SI, inhibition was also assayed in solution at an ovalbumin:proteinase ratio of 124:1. Residual PPE activity was assayed after a 15 min incubation either with the α_1-PI control or with the recombinant ovalbumins. α_1-PI reacted with PPE with the expected SI of 1.0. None of the ovalbumins gave any detectable inhibition. Incubations were also carried out for 5 and 60 min, to check that the absence of observable inhibition was not due either to instability of an ovalbumin−PPE complex or to incompleteness of the reaction, respectively. In neither case was

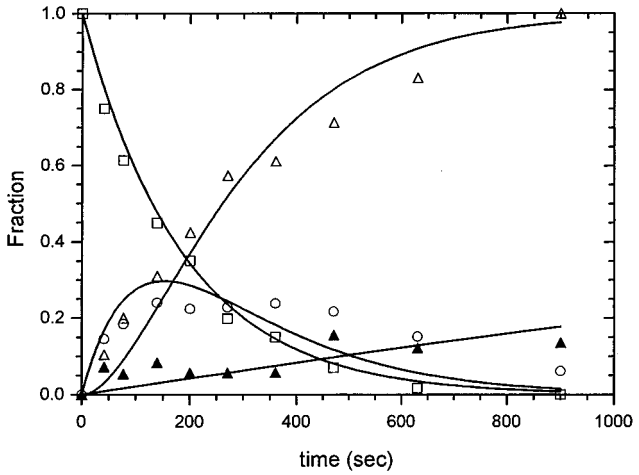


FIGURE 5: Extent of insertion of the reactive center loop probed by the susceptibility of the P14,P12−P10 variant to proteolysis at P8−P7 by subtilisin Carlsberg. Normalized band intensities from SDS−PAGE analysis of reactions of intact or P1−P1′-precleaved P14,P12−P10 variant ovalbumin with subtilisin Carlsberg at an ovalbumin:proteinase ratio of 1000:1 (for other conditions, see Materials and Methods): open symbols, reaction of the intact variant as starting material; filled triangles, reaction of the P1−P1′-precleaved variant; squares, uncleaved ovalbumin; circles, P1−P1′-cleaved ovalbumin (derived from uncleaved); and triangles, P8−P7-cleaved ovalbumin. The solid lines are fits to the data, as described. Comparison of the rate constants for formation of P8−P7-cleaved material starting with intact ovalbumin (open triangles) or strarting with P1−P1′-precleaved ovalbumin (filled triangles) gave a ratio of 36:1.

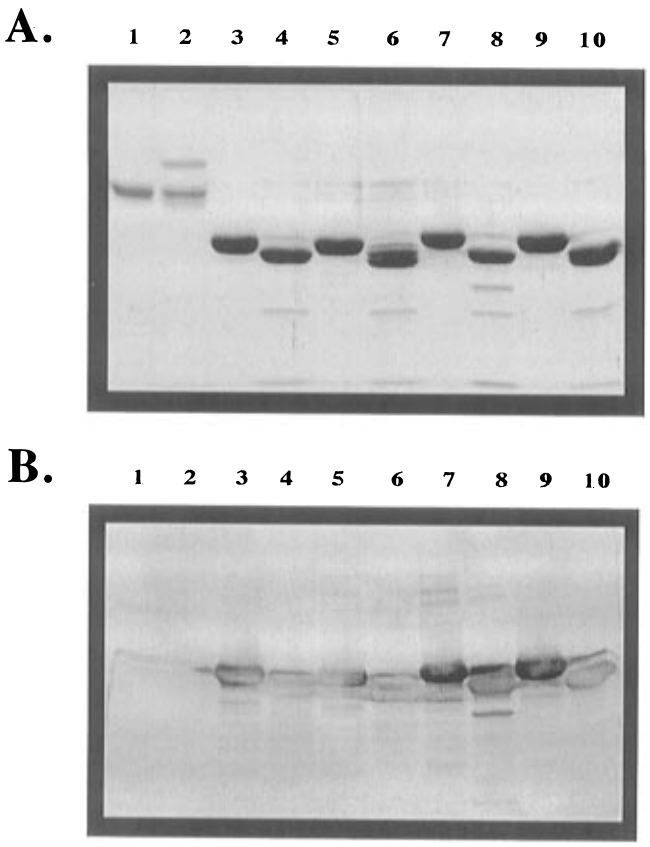


FIGURE 6: Failure of ovalbumin variants to form stable complex with PPE. Coomassie-stained SDS−PAGE (A) and anti-ovalbumin Western analysis (B) of ovalbumin−PPE reactions. An excess of ovalbumin was reacted with PPE to detect the presence of inhibitory complex formation. Positive control of α_1-PI and the α_1-PI−PPE complex (lanes 1 and 2, respectively) with the complex appearing as the upper band. Unreacted and reacted recombinant ovalbumins are in lanes 3 and 4 for recombinant wild-type ovalbumin, lanes 5 and 6 for the P12−P10 variant, lanes 7 and 8 for the P14 variant, and lanes 9 and 10 for the P14,P12−P10 combination variant. Ovalbumin was at 10 μg/lane, and PPE was at 1 μg/lane (ratio of 5.9:1). No higher molecular weight band corresponding to the ovalbumin−PPE complex was detectable upon reaction by either method.

inhibition observed. It is, however, possible that a normal serpin−proteinase complex forms between ovalbumin and PPE, but that it is so unstable that it is undetectable on the time scale of the measurements used. This represents a situation in which k_5 (Scheme 1) has been greatly increased for some reason compared with inhibitory serpin−proteinase pairs. Since the ratio of ovalbumin to PPE was 124:1 in each case, and we conservatively estimate that a 10% reduction in PPE activity would have been easily measurable, the SIs for inhibition of PPE by the ovalbumin variants must be >1240, even for the two variants for which spontaneous loop insertion is seen.

DISCUSSION

We have tested the hypothesis that ovalbumin can be converted into an inhibitor of PPE through mutation of hinge region residues, by examining the structural and proteinase inhibitory properties of three such ovalbumin variants. Replacement of the P14 arginine residue of ovalbumin by serine, either alone or in combination with replacement of VVG by AAA at P12−P10, results in a sufficient enhancement in the rate of loop insertion into β-sheet A that it occurs spontaneously following cleavage at the P1−P1′ bond by PPE. This contrasts with the need for extensive incubation at elevated temperatures to bring about an equivalent loop insertion in cleaved chicken ovalbumin (Shitamori & Nakamura, 1983). This increase in the rate of loop insertion alone was not, however, sufficient to convert ovalbumin into an inhibitor of PPE, even though PPE still recognized and cleaved these variants at the P1−P1′ bond.

There are two main possible explanations to account for the absence of inhibitory properties in these P14 variants, despite the enhanced rate of loop insertion. The first is that the rate of loop insertion is still not high enough, and the second is that the extent of loop insertion is not great enough. To understand the significance of each of these, it is necessary to consider the nature of the kinetic trapping mechanism whereby serpins inhibit proteinases. It has been proposed that, during the reaction of proteinase with a serpin, cleavage of the scissile bond at the stage of formation of the acyl enzyme intermediate (EI′ in Scheme 1) and release of the P1′−NH_3^+ leaving group permit insertion of the now unconstrained reactive center loop into β-sheet A (Wright & Scarsdale, 1995; Lawrence et al., 1995; Wilczynska et al., 1995; Gettins et al., 1996; Strömqvist et al., 1996). Since the proteinase is still covalently attached to the carboxyl of P1, such loop insertion would result in translocation of the proteinase from one end of β-sheet A to the distal end. The nature of the kinetic trap is proposed to be a disturbance of the catalytic site of the proteinase that greatly reduces the rate of deacylation (Lawrence et al., 1990). This could result from displacement of the acyl intermediate, alteration of the configuration of the catalytic triad or the oxyanion hole, or a combination of such changes, all as a consequence of an altered positioning of the proteinase relative to the attached reactive center loop that results from insertion of the reactive

center loop into β-sheet A. An alternative explanation is that access of solvent to the active site has been greatly reduced, thereby reducing the rate of hydrolysis of the acyl enzyme intermediate. The plausibility of such a translocation mechanism has been supported by recent fluorescence resonance energy transfer measurements, which are consistent with such large-scale proteinase movement (Stratikos & Gettins, 1997), and by NMR experiments that show a perturbation in the active site of chymotrypsin in complex with serpin (Plotnick et al., 1996). Successful proteinase inhibition would thus require rapid enough loop insertion for the structural perturbation of the proteinase catalytic apparatus, or access to it, to occur before deacylation can be completed and also that loop insertion occur far enough that such perturbation can occur.

Our findings on the magnitude of the increase in stability of the loop-inserted cleaved variants, as well as the continued accessibility of a secondary cleavage site in the loop-inserted form, indicate that full loop insertion does not occur upon cleavage of the P1−P1′ bond in ovalbumin. Thus, the increase in stability seen here for the P14 variant of ovalbumin is only 11 °C, with a further modest increase in the denaturation temperature of 5 °C upon replacement of VVG at P12−P10 in the combination P14,P12−P10 variant by AAA. The cleaved, loop-inserted, wild-type ovalbumin is also only ∼8 °C more stable than the noninserted form (Shitamori & Nakamura, 1983). These small increases in stability are in marked contrast to the cleaved inserted form of inhibitory serpins, in which residues from P15 to P3 insert into β-sheet A, and which are more stable than the native serpin by greater than 40 °C (Gettins & Harten, 1988; Hood et al., 1994). Even where an arginine was introduced at the P12 position of α_1-antichymotrypsin, such that the favorable energy of full loop insertion had been significantly reduced, the cleaved fully loop-inserted form had a midpoint for unfolding by guanidine hydrochloride that was still 3.4 M higher than that for the intact protein. The findings for the ovalbumin variants thus suggest that either full loop insertion occurs, but with a very large unfavorable energy for the sheet expansion necessary to allow the loop to insert, or only partial loop insertion occurs. The continued accessibility of the P8−P7 bond to cleavage by subtilisin Carlsberg in the major conformer of the loop-inserted state of the P14,P12−P10 variant, albeit at a 36-fold reduced rate, strongly suggests that it is only partial insertion that occurs, involving the six residues from P15 to P10, and that residues P9−P1 are not inserted. In the minor conformer of the P1−P1′-cleaved material, it is possible that the same *number* of residues are inserted but that the region of insertion is displaced by two residues to cover P13−P8 rather than P15−P10. This is expected to be thermodynamically possible, since the three side chains that would become buried in the protein interior upon loop insertion (P14, P12, and P10 vs P12, P10, and P8) are very similar, being SAA vs AAA, respectively. Such partial insertion is also consistent with the conclusions on the structure of the loop-inserted form of uncleaved chicken ovalbumin (*S*-ovalbumin), which indicated partial loop insertion only as far as P10 (Huntington et al., 1995). Whereas such a failure of the reactive center to completely insert may be a function of the primary structure of these reactive center residues, this is not considered likely, since an α_1-proteinase inhibitor variant in which the reactive center loop residues from P12 to P2 were replaced by those of ovalbumin gave a variant that could inhibit proteinase and a cleaved form that exhibited a >40 °C increase in stability (C. E. Chaillan-Huntington, P. G. W. Gettins, J. A. Huntington, and P. A. Patston, unpublished results). Instead, the failure to fully insert is likely to be due to a defect in the serpin conformational change mechanism outside of the reactive center.

In addition to the apparent failure of the reactive center loop to fully insert upon cleavage, the rate of such partial loop insertion also appears to be much lower than that in inhibitory serpins. When the P14,P12−P10 variant was incubated with subtilisin Carlsberg, the reactive center loop remained fully accessible for sufficiently long following cleavage at P1−P1′ for a relatively rapid secondary cleavage at P8−P7 to occur (Figure 5). Similarly, when the same variant was incubated with PPE at a low ovalbumin:elastase ratio, cleavage occurred at both P1−P1′ and subsequently at P10−P9 (Table 1), and the resulting P10−P9-cleaved species showed no evidence of loop insertion (Table 2). Only when a high ovalbumin:PPE ratio was used, such that cleavage at P10−P9 occurred slowly, was it possible to isolate a P1−P1′-cleaved species that then had time to spontaneously, but relatively slowly, insert into β-sheet A. We have estimated above that the cleaved loop inserts more than 26000-fold slower in the P14,P12−P10 ovalbumin variant than in cleaved plasminogen activator inhibitor 1. Using the reported k_{cat} for cleavage of ovalbumin at P1−P1′ by PPE of 10 s^{-1} (Wright, 1984) as a measure of k_3 (Scheme 1) and our current estimate of the upper limit of the rate of loop insertion of the P14,P12−P10 variant of 1.3×10^{-4} s^{-1} as a measure of k_4 (Scheme 1), we obtain a minimum expected SI of 77 000. This shows that, even if ovalbumin were capable of forming a stable complex through full loop insertion, the rate of such loop insertion would have to be increased a further 10000-fold to give the possibility of detecting complex.

In conclusion, it therefore seems that in wild-type ovalbumin both the rate and extent of loop insertion are inadequate for operation of the serpin proteinase inhibition mechanism. Although replacement of the P14 and P12−P10 residues with those that represent a consensus among inhibitory serpins greatly increases the rate of loop insertion, the increase is quantitatively not to the level found with inhibitory serpins. Perhaps more importantly, the extent of loop insertion that is possible with ovalbumin is far less than that for inhibitory serpins. It remains to be determined whether both inadequacies result from the same defect in the ovalbumin structure or from separate causes. It is possible that both result from an activation energy that is too high for fully separating strands 3 and 5 of β-sheet A to permit complete insertion of the reactive center loop. This in turn may result from stabilizing interactions from the underlying B and C helices greater than those that occur in other serpins (Stein & Chothia, 1991). In this regard, it may be significant that ovalbumin is ∼20 °C more stable than most other serpins in the native state (Smith & Back, 1965). While changes in the hinge region are clearly changes in the right direction for introduction of inhibitory properties into ovalbumin, the conversion of ovalbumin into an effective proteinase inhibitor will require additional changes outside of the reactive center, most probably at the interface between β-sheet A and α-helices B and C.

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