S-ovalbumin, an ovalbumin conformer with properties analogous to those of loop-inserted serpins

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Abstract

Most serpins are inhibitors of serine proteinases and are thought to undergo a conformational change upon complex formation with proteinase that involves partial insertion of the reactive center loop into a β -sheet of the inhibitor. Ovalbumin, although a serpin, is not an inhibitor of serine proteinases. It has been proposed that this deficiency arises from the presence of a charged residue, arginine, at a critical point (P14) in the reactive center region, which prevents loop insertion into the β -sheet and thereby precludes inhibitory properties. To test whether loop insertion is prevented in ovalbumin we have examined the properties of two forms of ovalbumin: the native protein and S-ovalbumin, a form that forms spontaneously from native ovalbumin and has increased stability. Calorimetric measurements showed that S-ovalbumin was more stable than ovalbumin by about 3 kcal mol⁻¹. CD spectra, which indicated that S-ovalbumin had less α -helix than native ovalbumin, and ¹H NMR spectra, which indicated very similar overall structures, suggest limited conformational differences between the two forms. From comparison of the susceptibility of the reactive center region of each protein to proteolysis by porcine pancreatic elastase and by subtilisin Carlsberg, we concluded that the limited native-to-S conformational change specifically affected the reactive center region. These data are consistent with a structure for S-ovalbumin in which part of the reactive center loop has inserted into β -sheet A to give a more stable structure, analogously to other serpins. However, the rate of loop insertion appears to be very much lower than for inhibitory serpins. Thus, although loop insertion does not appear to be prevented by the presence of arginine at P14, it occurs at too slow a rate to compete effectively with the substrate pathway. This alone is sufficient to account for the absence of detectable inhibitory properties in ovalbumin.

Keywords: conformational change; loop insertion; ovalbumin; serpin; S-ovalbumin

Serpins are a large family of structurally related proteins, most of which are inhibitors of serine proteinases, and many of which are involved in regulation of important physiological processes such as blood coagulation, fibrinolysis, and inflammation. They are unusual in four major respects: (1) the native state of the protein is not the most stable state, but instead a kinetically trapped

metastable intermediate; (2) a major conformational change involving partial insertion of the reactive center loop into β -sheet A of the inhibitor appears to accompany, and be required for, formation of a stable complex with target proteinase; (3) they appear to be suicide substrate inhibitors that act by a branched pathway mechanism, one branch of which leads to completion of the substrate reaction and the other to stable complex formation with the proteinase; and (4) inhibitory serpins that react by the substrate pathway give cleaved products in which the reactive center loop is completely integrated into β -sheet A and which are markedly more stable than the native protein (for reviews, see Huber & Carrell, 1989; Gettins et al., 1993). Although ovalbumin is a member of the serpin family it differs from other serpins in that it appears neither to act as a proteinase inhibitor nor to be able to undergo the conformational rearrangement to a more stable form upon cleavage as a substrate (Gettins, 1989; Stein et al., 1989, 1991; Wright et al., 1990).

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Abbreviations: T_m , midpoint temperature for thermal denaturation; α_1 PI, α_1 -proteinase inhibitor; T345R, variant in which residue 345 has been changed by mutagenesis from threonine to arginine; DSC, differential scanning calorimetry; PPE, porcine pancreatic elastase; PAI 1, plasminogen activator inhibitor 1.

In an attempt to explain why ovalbumin has such different properties, it was noted that the residue at position P14⁵ (the side chain of which becomes buried in the hydrophobic interior of cleaved, loop-inserted forms of inhibitory serpins) is charged in ovalbumin and angiotensinogen (another noninhibitory serpin), but uncharged in all known inhibitory serpins (Wright et al., 1990). It was proposed that an arginine side chain at this position would be thermodynamically incompatible with insertion of the reactive center loop into β -sheet A and that ovalbumin would thus be unable to form a stable complex with proteinase and thereby act as an inhibitor. This hypothesis is based on the X-ray structures of native ovalbumin (Stein et al., 1991) and a reactive center cleaved form (plakalbumin) (Wright et al., 1990). These structures show that the reactive center loop in native ovalbumin is a two and a half turn α -helix. Upon proteolysis by subtilisin Carlsberg, there is loss of six or seven residues from the reactive center loop but no insertion of the remaining reactive center loop residues into β -sheet A. However, we recently showed in a study on a P14 T345R variant of the inhibitory serpin α_1 -proteinase inhibitor (α_1 PI) that the introduction of an arginine at P14 did not prevent the reactive center loop from inserting into β -sheet A of α_1 PI to give a more stable form and did not abolish the inhibitory properties of the serpin toward several serine proteinases, though it did reduce the efficiency of inhibition (Hood et al., 1994). The properties of an analogous P14 T333R variant of PAI 1 have also been recently reported and are similar to those of the α_1 PI variant, though inhibitory efficiency is reduced even more (Lawrence et al., 1994b). Given that the outcome of an encounter between a serpin and a proteinase is kinetically determined, it may be that the reactive center loop of ovalbumin can insert, but does so too slowly to result in significant flux along the inhibitory branch of the reaction pathway relative to the flux along the substrate branch of the pathway.

To test whether ovalbumin is prevented from undergoing insertion of the reactive center loop region into β -sheet A to give a more stable structure, we compared the properties of native ovalbumin with those of a distinct form, S-ovalbumin,⁶ which has been known for more than 30 years and which has greater stability than native ovalbumin. From consideration of the relative stabilities, the secondary and tertiary structures, the susceptibility to specific proteolysis in the reactive center loop, the ease of dephosphorylation of the phosphoserine at P9, and the relative rates of formation of S-ovalbumin from the native protein under various conditions, we concluded that S-ovalbumin is probably a more stable partially loop-inserted form of ovalbumin that is only very slowly formed from native ovalbumin under physiological conditions. The apparent lack of inhibitory properties for ovalbumin appears to be due to a rate of loop insertion that is too slow for the inhibitory pathway to compete with the substrate pathway rather than from a total inability of the loop to insert partially.

⁵ The nomenclature system of Schechter and Berger (1967), used to describe the subsites of interaction between a proteinase and its substrate, designates the residues on either side of the substrate scissile bond as P1 and P1'. Residues N-terminal to this are designated P2, P3, ..., P_n, and residues C-terminal are designated P2', P3', ..., P_n'.

⁶ The designation "S-ovalbumin" is a historical one and should not be confused with the S and R nomenclature used by Carrell and colleagues (Carrell & Owen, 1985) to designate strained and relaxed conformations, respectively, of serpins.

Results

Thermodynamic properties of native and S-ovalbumins

The initial recognition of the S form of ovalbumin was based upon alteration in the unfolding temperature T_m (Smith, 1964). Indeed, the change in denaturation temperature is taken as a diagnostic for conversion of native ovalbumin into the S form (Donovan & Mapes, 1976). To more fully characterize the differences in thermodynamic properties of native and S-ovalbumins we carried out DSC studies on each protein. From the complete traces of the unfolding of native and S-ovalbumins (Fig. 1), we estimated the difference in stability ($\Delta\Delta G(T)$) of the two conformers ($\Delta_N^D G^S(T) - \Delta_N^D G^N(T)$) as 3 kcal mol⁻¹ at room temperature (Table 1) (the superscripts S and N refer to S- and native

Fig. 1. Calorimetric scans of native and S-ovalbumins. Samples were prepared by dialysis against 20 mM sodium phosphate buffer, pH 7.0. Buffer was used in the reference cell in each case. Traces have been baseline-corrected by subtraction of a scan of buffer in both reference and sample cells. **A:** Native ovalbumin, 5 mg mL⁻¹. **B:** S-ovalbumin, 5 mg mL⁻¹. The shoulder in the native scan represents an estimated 8% contamination with an ovalbumin species that is an intermediate in the conversion of native into S ovalbumin. The presence of the intermediate component was taken into account by using computer-generated peak deconvoluted data in determination of peak height and area for the native ovalbumin component.

Table 1. Thermodynamic characterization of native and S-ovalbumins

Ovalbumin	T_m (K)	ΔH_{cal}^a (kcal mol ⁻¹)	$\Delta H_{v.H.}^b$ (kcal mol ⁻¹)	$\Delta H_{cal}/\Delta H_{v.H.}$	$\Delta\Delta G$ (351.1 K) ^c (kcal mol ⁻¹)	$\Delta\Delta G$ (298 K) ^d (kcal mol ⁻¹)
Native	351.1	196 ± 9	131 ± 3	1.50 ± 0.10		
S-ovalbumin	358.9	194 ± 6	136 ± 5	1.43 ± 0.09		
S-N	7.8				2.1	3

^a Calorimetric enthalpy of denaturation calculated from the area under the unfolding transition. Values are given as the average of three scans (S-ovalbumin) and five scans (native ovalbumin).

^b van't Hoff enthalpy calculated using Equation 1 and parameters obtained from the DSC traces.

^c Difference in Gibbs free energy between S- and native ovalbumins at the T_m for native ovalbumin (351.1 K). The positive value indicates that S-ovalbumin is more stable than native ovalbumin.

^d Difference in Gibbs free energy between S- and native ovalbumins at 298 K. The positive value indicates that S-ovalbumin is more stable than native ovalbumin at this temperature.

ovalbumins, respectively). The shoulder at the high temperature side of the native ovalbumin unfolding transition (Fig. 1A) represents an intermediate form of ovalbumin, which can be converted to the same S-ovalbumin species as native ovalbumin⁷ (Donovan & Mapes, 1976). Attempts to remove this form by chromatographic means failed. It should be noted that all commercial samples of ovalbumin that we examined contained either this intermediate form or some S-ovalbumin. The material that we used (Calbiochem) was judged to contain the least of these minor species.

Analysis of the DSC trace corresponding to the native species was from a computer deconvolution of the experimental scan. Estimation of $\Delta_N^D G(T)$ for each ovalbumin species, and thus of $\Delta\Delta G$, depends on subtraction of large numbers (Equation 2) and is thus subject to larger percent errors than the parameters used to calculate $\Delta_N^D G$. Because $\Delta_N^D G = 0$ at the transition temperature, $\Delta\Delta G$ at the denaturation temperature of native ovalbumin (T_m^N) is equal to $\Delta_N^D G^S(T_m^N)$. This can be calculated with lower error than $\Delta\Delta G$ at room temperature because only one of the two $\Delta H_{calorimetric}$ values is used in the calculation. The value of 2.1 kcal mol⁻¹ at this temperature is, however, similar to the estimate at room temperature. It is clear that, although S-ovalbumin is more stable than native ovalbumin, the difference in stability is not large. We also calculated the ratio $\Delta H_{calorimetric}:\Delta H_{van't Hoff}$ for each ovalbumin (Table 1). This parameter can be used to distinguish between a simple one-step, two-state unfolding process and a more complicated one involving separate unfolding of discrete domains (Privalov, 1982). The values of 1.50 ± 0.10 for native ovalbumin and 1.43 ± 0.09 for S-ovalbumin indicate that the unfolding process is similar for both proteins and does not occur as a single co-operative step, but may involve two domains unfolding with considerable independence from one another.

Influence of pH and guanidine hydrochloride on formation of S-ovalbumin

Although pH 10 is typically used for generation of S-ovalbumin in vitro, high pH is not a requirement for its formation. The ef-

fect of the high pH is to increase the rate of formation of S-ovalbumin. However, significant amounts are also formed at pH values close to physiological (Fig. 2). Low concentrations of guanidine hydrochloride also brought about a fourfold increase in the rate of formation of S-ovalbumin at constant pH (Fig. 3). Both of these results suggest that the native-to-S transformation is a conformational change rather than a pH-dependent chemical modification such as deamidation (also see below).

¹H NMR spectra of ovalbumin species

To determine whether the native-to-S transformation involved a gross change in the structure of ovalbumin, one-dimensional ¹H NMR spectra were recorded to serve as fingerprints of the tertiary structure. The sensitivity of the chemical shifts of res-

Fig. 2. pH dependence of the formation of S-ovalbumin from native ovalbumin upon incubation at 55 °C for 18 h monitored by differential scanning calorimetry. The pH values of the samples are, from bottom to top, 7.2, 7.8, 8.3, 8.9, and 9.4. For clarity, only the portion of the scan around the unfolding transition is shown.

⁷ Indeed, the intermediate form appears to be an obligate intermediate in the formation of S-ovalbumin from native ovalbumin as evidenced by the kinetic analysis of the time course of conversion of native to S forms, which proceeds through formation of the intermediate form. Evidence for this can clearly be seen in Figures 2 and 3.

Fig. 3. DSC traces of ovalbumin as a function of incubation time at 55 °C in the absence (left) and presence (right) of 0.5 M guanidine hydrochloride. Times of incubation are, from bottom trace to top, 0, 1, 2.25, 3.25, 4.25, and 5.25 h. Conversion from native to S ovalbumin occurs via an intermediate species that has intermediate unfolding temperature.

onances in particular regions of the spectrum to the precise tertiary structure makes it possible to use invariance of such marker resonances as a reliable indication of conservation of tertiary structure. Comparison of the spectra of native and S-ovalbumin showed only small changes in these regions (Fig. 4), indicating that the structural change involved relatively minor perturbation of the overall protein fold.

CD spectra of ovalbumin species

Although the CD spectra of native ovalbumin and S-ovalbumin in the far-UV region are similar (Fig. 5), there are reproducible differences in shape and intensity consistent with loss of α -helix upon transformation of ovalbumin into S-ovalbumin. Secondary structure prediction based upon deconvolution of these spec-

Fig. 4. Conservation of tertiary structure shown by similarity of 500 MHz ^1H NMR spectra of native (top) and S- (bottom) forms of ovalbumin.

Fig. 5. CD spectra of native and S-ovalbumins. Mean residue differential absorption coefficient ($\Delta\epsilon$) is plotted against wavelength. The mean residue molecular weight was calculated to be 110.9, based upon a molecular weight of 42,700 and 385 amino acids.

tra using the basis set described in the Materials and methods is in agreement with this conclusion (Table 2). This prediction is also in good agreement with the known secondary structure present in the X-ray structure (Table 2). The 2–5% loss of total α -helix (based on 100% corresponding to 29% of residues) corresponds to three to six residues.

Relative rates of proteolytic cleavage of ovalbumin and S-ovalbumin

To test whether the limited native-to-S conformational transition affected the reactive center loop of ovalbumin, we examined the susceptibility of the two forms of the protein to proteolysis by PPE and subtilisin Carlsberg. These proteinases were chosen because they react with ovalbumin only in the reactive center loop region and are thus good probes of changes affecting this region. Subtilisin cleaves at three sites, between residues P1 and P1', P6 and P7, and P7 and P8 (Ottesen & Wollenberger, 1956; Ottesen, 1958), whereas PPE cleaves only at P1-P1' (Wright, 1984). We found that S-ovalbumin was more susceptible than ovalbumin to cleavage by both proteinases, measured by appearance of

Table 2. Best-fit secondary structure compositions for CD spectra of native and S-ovalbumins and comparison with secondary structure of native ovalbumin derived from the X-ray structure (Stein et al., 1991)

	Percent secondary structure composition				
	α -Helix	Anti-parallel β -sheet	Parallel β -sheet	β -Turn	Other
Ovalbumin (CD)	29	24	4	15	28
S-ovalbumin (CD)	28	24	4	15	29
Ovalbumin (X-ray)	30.6 ^a	25.3	3.2	16.6	24.3

^a This includes two short 3_{10} -helices, F1 and I1, and the C2 α -helix, which contains only four residues.

cleaved protein and loss of intact protein on SDS gels, consistent with greater accessibility of the sites of cleavage to the proteinase active site. Rates of cleavage were estimated from a semi-log plot of the residual uncleaved protein against time.⁸ A 38-fold difference in rates of reaction was found for PPE (Fig. 6) and a 4-fold difference for subtilisin (data not shown).

To ensure that the sites of proteolytic cleavage remained only within the reactive center region, we also ran SDS polyacrylamide gels of the reaction products on a discontinuous 7.5–20% gel so that the migration positions of both 40-kDa (N-terminal) and 2.9-kDa (C-terminal) polypeptide species could be compared for each ovalbumin (not shown). For either PPE or subtilisin, native and S-ovalbumins gave products with indistinguishable mobilities, implying that cleavage of each conformer occurred within the reactive center region but not at additional sites outside of it.

Extent of loop insertion

As a probe of the extent of changes in the reactive center loop, we examined the susceptibility of the serine-phosphate at position P9 (residue 350⁹) of the reactive center loop to dephosphorylation by *Escherichia coli* alkaline phosphatase, by recording ³¹P NMR spectra of native and S-ovalbumins as a function of time after addition of the phosphatase. Previous studies have provided ³¹P NMR resonance assignments for the two phosphoserine residues in ovalbumin (Vogel & Bridger, 1982) and shown that phosphoserine 350 is much more reactive to *E. coli* alkaline phosphatase than is serine 68 (Vogel & Bridger, 1982; Gettins, 1989).

Within experimental error (estimated to correspond to rate differences of 20%) the rates of dephosphorylation of phosphoserine 350 in S-ovalbumin and native ovalbumin were indistinguishable at pH 8.75 (data not shown). The much slower rate of dephosphorylation of phosphoserine 68 was also unaffected by the conformational change. The chemical shifts of the two phosphoserine residues were indistinguishable between the two forms of ovalbumin.

Because different lengths of the reactive center loop should remain after cleavage of ovalbumin by the two proteinases: up to residue P8 in the subtilisin-cleaved ovalbumin and up to P1 in the elastase-cleaved protein, we also examined the ability of each cleaved form to undergo the change to an S form and the stability of such products. In both cases it was possible to generate an S form by incubation at pH 10 and 55 °C. In both cases, the conversion to an S form resulted in the same increase in stability of 8 °C (data not shown) despite the different lengths of the reactive center loop available for possible loop insertion.

Fig. 6. Differential cleavage of ovalbumins by PPE followed by SDS-PAGE on 10% polyacrylamide gels under reducing conditions and quantitation by scanning of the Coomassie-stained gel. Ovalbumin or S-ovalbumin were incubated at room temperature at pH 7.0 in 20 mM sodium phosphate buffer with 1:100 PPE:protein. At the indicated times, aliquots were mixed with PMSF to 0.5 mM and an equal volume of sample buffer (nonreducing). ■, Ovalbumin. ●, S-ovalbumin. Solid lines represent single exponential decays. The large residual for the S form probably arises from the presence of some intermediate form ovalbumin in the preparations.

Discussion

In this paper we set out to determine whether the reactive center loop of ovalbumin is prevented from undergoing the loop-to-sheet interconversion that occurs in other inhibitory serpins. In doing so we have focused on characterizing the formation and properties of S-ovalbumin, a form of ovalbumin that, from literature reports of increased stability compared with the progenitor native species (Smith & Back, 1965), fulfills the minimum requirement for a loop-inserted serpin conformer. We found that the reactive center loop of ovalbumin does not appear to be prevented from inserting into β -sheet A, despite the presence of arginine at position P14. The rate of such loop insertion, however, is very slow under physiological conditions, which is sufficient to account for the absence of inhibitory properties in ovalbumin. There is thus a strong parallel with the effect of a threonine-to-arginine change at position P14 on the properties of the inhibitory serpin α_1 PI. This mutation greatly slowed down, but did not prevent, loop insertion into β -sheet A, with the result that the efficiency of the inhibitory pathway was greatly reduced relative to the substrate pathway. In ovalbumin the situation is even more extreme, such that loop insertion is so slow as to make inhibition undetectable.

In the absence of an X-ray crystallographic structure of S-ovalbumin, proposals for its structure must be based on indirect evidence. In trying to determine whether or not S-ovalbumin represents a loop-inserted form of ovalbumin and therefore whether or not ovalbumin can undergo such reactive center loop insertion, we considered not only our present findings, but also results of older studies on S-ovalbumin because this form of ovalbumin has been well characterized, albeit not in the context of it being a serpin.

⁸ Because all preparations of native ovalbumin contained some intermediate form and generation of S-ovalbumin from native always results in some residual intermediate form, apparently in equilibrium with it, the cleavage reactions with proteinase represent the sum of the cleavages of the major species and of intermediate form ovalbumin. Although the intermediate form has not been well characterized, it appears to behave more like native ovalbumin than S-ovalbumin. Thus, with proteinase it is only slowly cleaved. The leveling off of the cleavage of S-ovalbumin at a value close to 20% most probably represents the fraction of intermediate form ovalbumin.

⁹ The amino acid numbering system is based on sequence alignment with α_1 -proteinase inhibitor (Huber & Carrell, 1989).

Extent and nature of difference between native and S-ovalbumins

The first question is whether S-ovalbumin is a conformational isomer of native ovalbumin or a chemically distinct species. Although the usual method of generating S-ovalbumin has been by incubation at pH 10 and 55 °C, thus raising the possibility that it is a deamidation or cleavage product, we have shown that low concentrations of guanidine hydrochloride can significantly increase the rate of formation at pH 10 and also that it can be formed at a slower, but nevertheless appreciable rate, at pH 8, again accelerated by guanidine hydrochloride. Our findings are therefore consistent with a conformational interconversion that is pH dependent, rather than with a base-catalyzed chemical modification. An earlier study specifically addressed the susceptibility of ovalbumin to deamidation and found less than 0.2 mol/mol of ammonia released after incubation at 37 °C for 6 h at an even higher pH (pH 11) (Johnson et al., 1989). Peptide mapping has also shown that the disulfide bond position and sulfhydryl reactivities are identical in native and S-ovalbumins (Smith & Back, 1965, 1968). Molecular weight determination has shown that S-ovalbumin is monomeric (Smith & Back, 1965) and indistinguishable from native ovalbumin. Immunologically the two proteins are also the same (Smith & Back, 1965). S-ovalbumin differs from native, however, in being more compact and more hydrophobic (Nakamura & Ishimaru, 1981). A more dramatic difference in properties between native and S-ovalbumins is the increase in denaturation temperature of 8 °C, corresponding to an estimated $\Delta\Delta G$ of 3 kcal mol⁻¹ at room temperature and 2.1 kcal mol⁻¹ at 78 °C (the denaturation temperature of native ovalbumin). This implies that native ovalbumin is a metastable species. This phenomenon is not common outside of the serpin family, but is a hallmark of those serpins known to be proteinase inhibitors. The available data are thus consistent with S-ovalbumin being a conformational isomer of native ovalbumin rather than a chemically modified derivative.

The next question is what the nature and extent of the conformational differences are. Spectroscopic studies reported here and elsewhere indicate that the conformational change is very limited and involves a small alteration in secondary structure without a major alteration in the overall folding of the protein. Thus, the CD data and analysis (Fig. 5; Table 2) indicate a small difference in α -helix between the two proteins. This is the same conclusion that was found for the effect of cleavage and loop insertion in both wild-type α_1 PI and its P14 arginine-substituted variant (Hood et al., 1994). A Raman study also concluded that the change from native to S-ovalbumin involved a 3–4% reduction in α -helix and a small increase in antiparallel β -sheet (Kint & Tomimatsu, 1979). Although both the CD and Raman spectra report on the aggregate properties of the protein, making it possible to have small differences in spectra resulting from large, yet compensatory, changes in secondary structure, the ¹H NMR spectra are not consistent with such underestimation of the conformational change because the paucity of changes in structurally sensitive regions of the NMR spectrum (Fig. 3) requires that only minor changes in overall tertiary structure, and therefore in secondary structure, occur between native and S-ovalbumins.

Where then does this limited structural difference occur? The relative rates of proteolysis of native and S-ovalbumins by PPE and subtilisin Carlsberg indicate that the structural difference

involves only the reactive center region. This conclusion is based on the highly specific cleavages of the two proteinases, which are restricted to this region of the molecule (Ottesen & Wollenberger, 1956; Wright, 1984) and the differences in rates of reaction between the native and S forms of the protein. S-ovalbumin reacted 38-fold more rapidly than native ovalbumin with PPE, suggesting greater accessibility of the reactive center loop to cleavage. This is readily understood in terms of unfolding of the reactive center loop α -helix as part of the transformation of ovalbumin into S-ovalbumin. Although the rate difference was less for subtilisin, there was nevertheless a reproducible increase in cleavage rate for the S form. The absence of new cleavage sites elsewhere in the protein, evidenced by the absence of new lower molecular weight species on SDS-PAGE, also supports localization of the structural change to the reactive center loop.

A test of this proposal of unfolding of the reactive center region α -helix is provided by a previous study on the titratability of carboxyl groups in native and S-ovalbumins (Nakamura et al., 1981). It was found that 2 of the 49 carboxyl groups present in ovalbumin did not titrate normally in native ovalbumin, but appeared upon denaturation. In contrast, all 49 carboxyl groups were titratable in S-ovalbumin. To be consistent with our conclusions this requires that the two unusual carboxyl groups *must* be located in the reactive center region, or in contact with it, and must be involved in stabilizing interactions, shielded from solvent in native ovalbumin. Furthermore, upon unfolding of the reactive center α -helix to give the S form, these interactions should be disrupted. Inspection of the X-ray structure of native ovalbumin reveals two carboxyls with just these properties. They are Glu 352 (P7) and Asp 356 (P3), which are reported to be involved in ion pair interactions with lysine 283 and H-bonding to backbone amide atoms (Stein et al., 1991). Upon unfolding of the reactive center loop helix these interactions would be expected to be broken.

Evidence for loop insertion

This leads to the critical question of whether this change in structure of the reactive center region that involves unfolding of the α -helix also results in its insertion into β -sheet A, analogously to other serpins. To address this, we contrast the behavior of S- and native ovalbumins with the behavior of other serpins for which the nature of the conformational differences between different forms is better understood and ask whether ovalbumin behaves in the same way or differently.

The first comparison is the stability change of ovalbumin upon conversion to the S conformer. The latent form of PAI 1, which is a loop-inserted form, is more stable than the native protein (Munch et al., 1991) and unfolds at a temperature 17 °C higher (Lawrence et al., 1994a). This compares with a T_m increase of 8 °C and a $\Delta\Delta G$ increase of 3 kcal mol⁻¹ for ovalbumin. This qualitative similarity supports the proposal that S-ovalbumin is also a partially loop-inserted form.

The effect of guanidine hydrochloride in promoting formation of the S conformer is also similar to the effect it has in other serpins in promoting formation of loop-inserted forms. Thus, it has been shown for α_1 PI, antithrombin, α_1 -antichymotrypsin, heparin cofactor II, and C1 inhibitor that addition of 0.9 M guanidine hydrochloride induced formation of more stable loop-inserted forms of the serpin (though these may involve insertion of the reactive center loop of one molecule into

the sheet of another to form multimers) (Carrell et al., 1991). Low concentrations of urea also enhance the rate at which sheet-inserted binary serpin-peptide complexes can be formed (Schulze et al., 1990). It is important to note, however, that the relative ease of formation of the guanidine hydrochloride-induced forms is very different for ovalbumin than for the other serpins mentioned. The much higher temperature and longer incubation time used for ovalbumin, though at a lower guanidine hydrochloride concentration, indicate a much higher activation energy barrier for insertion of the ovalbumin reactive center loop. However, such variation in ease of loop insertion is also seen for inhibitory serpins. Thus, the native conformer of PAI 1 has such a high tendency to undergo loop insertion that it must be stabilized *in vivo* by association with vitronectin (Wiman et al., 1988), whereas other serpins such as antithrombin and α_1 PI are quite stable under physiological conditions.

Extent of loop insertion

The indistinguishable reactivities of the P9 phosphoserine in the two proteins indicate that no structural change involving this residue occurred and thus that insertion does not proceed beyond P10 and may involve only four or five residues (P14–P10) of the reactive center loop. This conclusion is consistent with the extent of increase in β -sheet inferred from the Raman study (Kint & Tomimatsu, 1979). The lower reactivity of phosphoserine 68 toward alkaline phosphatase in intact ovalbumin (Vogel & Bridger, 1982), but not in an isolated peptide fragment, suggests that accessibility does affect the reactivity of surface phosphate groups toward alkaline phosphatase. Thus, the absence of a significant change in reactivity of phosphoserine-350 in S- versus native ovalbumin is more consistent with similar accessibilities than with insensitivity of the method. This is also supported by the identical ^{31}P chemical shifts of the phosphoserine residues in the two proteins because the chemical shift is considered to be sensitive to environment (Lerner & Kearns, 1980).

The increased proteolytic susceptibility of S- compared to native ovalbumin can be understood in terms of the change in structure of the portion of the reactive center loop that does not insert. The site of cleavage by PPE and one of the sites of cleavage by subtilisin is at the end of the reactive center loop α -helix (P1–P1'), whereas the other is within the α -helical region (Stein et al., 1991). Neither cleavage site has the extended chain "canonical" conformation that is appropriate for interaction with

the active site of the proteinases (Bode et al., 1986, 1987). Thus, the greater reactivity of S-ovalbumin is consistent with an alteration in the reactive center region that makes this region less α -helical and more extended. Partial insertion of the reactive center loop would require unfolding of the remainder of the helix to physically permit this insertion. This would give a more extended "canonical" conformation for the noninserted part of the reactive center region and thus account for the observed greater reactivity with subtilisin and elastase (Fig. 7).

Consequences for proteinase inhibition and serpin mechanism

What then are the consequences of such putative loop insertion in ovalbumin for the mechanism of action of serpins and the failure of ovalbumin to function as an inhibitor? In some inhibitory serpins, both wild type and variant, it has been found that the serpin can act as both substrate and inhibitor. The proportion of each depends upon the serpin, the proteinase, as well as environmental conditions (Olson, 1985; Schechter et al., 1989; Rubin et al., 1990; Patston et al., 1991; Cooperman et al., 1993; Hopkins et al., 1993; Hood et al., 1994; Lawrence et al., 1994b). This can be understood in terms of a mechanism that involves a branched pathway in which a common intermediate is formed that can either be stabilized by loop insertion to give a stable complex or proceed by a second branch of the pathway to give substrate-like cleavage of the reactive center loop (Rubin et al., 1990; Patston et al., 1991; Gettins et al., 1993). The relative fluxes along each branch may be directly affected by the rate of loop insertion in the presence of proteinase, which in turn could be strongly affected by the nature of the first residue to be inserted into the β -sheet (P14). In the case of ovalbumin, loop insertion, although thermodynamically favorable, is very slow, resulting in apparently exclusive reaction by the substrate branch of the mechanism. Such slowness of loop insertion in the presence of proteinase is supported by rate constants estimated for the rate of conversion of ovalbumin into S-ovalbumin in the absence of proteinase that correspond to an activation energy of about 12 kcal mol $^{-1}$ (Donovan & Mapes, 1976). In a recent study on a P14 variant of α_1 PI (T345R) the substitution of arginine for threonine resulted in a reduced efficiency as an inhibitor, which was consistent with raising of the activation energy for loop insertion and consequent lowering of the rate of the inhibitory pathway relative to the substrate pathway. The cleaved

Fig. 7. Schematic representation of the reactive center loop and A sheet regions of ovalbumin in the native (A) and S (B) conformations. In the native conformation, the reactive center loop is α -helical. This is known to be the case from an X-ray structure determination (Stein et al., 1991). We propose that in the S conformation the α -helix has unfolded and partially inserted between the central strands of the A sheet. In this conformation the sites of proteolytic cleavage are in extended conformation and much more accessible to proteinase.

product of the substrate reaction also appeared to be completely loop-inserted, thus showing that arginine at P14 had not prevented loop insertion. Analogous findings were also obtained for a P14 T→R variant of PAI 1, though here the effect of the mutation on the relative rates of substrate and inhibition reactions was altered even more than for the α_1 PI P14 variant (Lawrence et al., 1994b). Although arginine at P14 can thus result in an increase in activation energy for loop insertion, it is not always sufficient to slow the rate of loop insertion, and therefore of inhibition, to undetectable levels.

Conclusion

We have presented evidence that S-ovalbumin represents a more stable conformational isomer of native ovalbumin, which differs from the native protein by a small alteration in secondary structure involving only the reactive center loop region. The properties of the S form parallel those of loop-inserted forms of related serpins and are most readily interpreted as S-ovalbumin being a partially loop-inserted form of ovalbumin. This implies that arginine at P14 in ovalbumin is not sufficient to prevent loop insertion, which is the same conclusion found for a recombinant variant of an inhibitory serpin α_1 PI that contained arginine at P14. Ovalbumin thus behaves in qualitatively the same way as inhibitory serpins in apparently being able to undergo loop insertion. The functional differences arise for kinetic reasons. Thus, the major difference between ovalbumin and other serpins is the relative rate of formation of a loop-inserted form. This is very much slower than even for the T345R α_1 PI variant or the T333R PAI 1 variant and is sufficient to explain the absence of inhibitory properties of ovalbumin.

Materials and methods

Preparation of S-ovalbumin

Except for studies in which the effects of pH or guanidine hydrochloride on the rate of formation of S-ovalbumin were examined, S-ovalbumin was prepared according to modification of a previously published procedure by incubating native ovalbumin in 100 mM phosphate buffer at 55 °C for about 20 h at pH 10 (Donovan & Mapes, 1976), followed by dialysis into the desired buffer. The conversion from native to S-ovalbumin was confirmed by DSC, which showed a characteristic increase in the T_m . Because the conversion to S-ovalbumin produces a species that is more stable than native ovalbumin, S-ovalbumin showed no tendency to revert to native ovalbumin. Polyacrylamide gels run under nondenaturing conditions showed that S-ovalbumin does not contain dimers or higher order polymers such as have been found to occur in some serpins upon heating, as a result of loop-sheet polymerization between the reactive center loop of one molecule and β -sheet A of another molecule.

Differential scanning calorimetry

DSC was carried out using a Microcal MC-2 calorimeter. Protein samples, in 20 mM sodium phosphate buffer, pH 7.0, were degassed prior to study. Protein concentrations were in the range 1.5–5 mg mL⁻¹ and were determined spectrophotometrically using an extinction coefficient of 2.93×10^4 M⁻¹ cm⁻¹ for both native and S-ovalbumin (Batra et al., 1990). A scan rate of one

degree per minute was used for all measurements. Data analysis employed the DA-2 Data Analysis and Acquisition System of Microcal Inc. (Amherst, Massachusetts), which is based on standard procedures. The calculation of $\Delta H_{\text{van't Hoff}}$ was according to the relationship:

$$\Delta H_{\text{van't Hoff}} = 4\Delta C_p(T_m)RT_m^2/Q_d, \quad (1)$$

where $\Delta C_p(T_m)$ is the height, T_m is the midpoint, and Q_d is the area under the unfolding transition in cal g⁻¹. $\Delta H_{\text{calorimetric}}$ is equal to $Q_d \times M$, where M is the molecular weight. $\Delta_N^D G(T)$, the difference in Gibbs free energy between the native and denatured states at temperature T , was calculated using the relationship (Privalov & Gill, 1988):

$$\begin{aligned} \Delta_N^D G(T) = \Delta H_{\text{cal}} \frac{(T_m - T)}{T_m} - \int_T^{T_m} \Delta_N^D C_p dT \\ + T \int_T^{T_m} \frac{\Delta_N^D C_p}{T} dT, \end{aligned} \quad (2)$$

where $\Delta_N^D C_p$ is the difference in heat capacity between native and denatured states, which is estimated from curve fitting the unfolding transition and is assumed to be independent of temperature (Privalov & Gill, 1988).

CD spectroscopy

CD spectra were recorded at 299 K on a JASCO 720 spectropolarimeter. For the region 178–214 nm, a 0.1-mm cell, scan rate of 2 nm min⁻¹, and response time of 16 s were used. For the region 214–260 nm, a 1-mm cell, scan rate of 5 nm min⁻¹, and response time of 8 s were used. Spectra are the average of four scans. A minimum of three replicates was run for each protein species. The instrument was calibrated prior to each set of runs with a standard of 0.06% ammonium camphor sulfonate in a 1-cm cell. Filtered and degassed protein samples were in 20 mM sodium phosphate buffer, pH 7.0, and had concentrations in the range 1–1.5 mg mL⁻¹. Secondary structure analysis of the spectra was carried out using the variable selection program (Manavalan & Johnson, 1987), with a basis set of 18 of the proteins provided with the program.

³¹P NMR spectroscopy

³¹P NMR spectra were recorded on an IBM AF200 wide-bore spectrometer, operating at 81 MHz for phosphorus, using 10-mm sample tubes and 2-mL samples. Acquisition parameters were a sweep width of 5,000 Hz, pulse angle of 55°, 8,192 time domain points, and WALTZ low-power broad-band proton decoupling. The repetition rate was 0.56 s⁻¹. A line broadening of 2 Hz was applied to the free induction decays prior to Fourier transformation.

¹H NMR spectroscopy

¹H NMR spectra were recorded on a Bruker AMX500 spectrometer in 5-mm tubes using a sweep width of 7,500 Hz and pulse angle of 70°. Residual solvent resonance intensity was reduced by presaturation with a selective 0.5-s pulse. Samples were

in 20 mM phosphate buffer, pH 7.0, in D₂O containing 150 mM NaCl.

SDS-PAGE

SDS-PAGE was carried out according to the procedure of Laemmli (1970), using 10% polyacrylamide gels unless noted otherwise. Protein bands were made visible by staining with Coomassie brilliant blue and quantitated using an Apple Color One scanner.

Materials

Hen ovalbumin came from Calbiochem (San Diego, California); subtilisin Carlsberg, PPE, and *E. coli* alkaline phosphatase, grade III, were from Sigma Chemical Co. (St. Louis, Missouri).

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References

- Batra PP, Roebuck MA, Uetrecht D. 1990. Effect of lysine modification on the secondary structure of ovalbumin. *J Protein Chem* 9:37–43.
- Bode W, Papamokos E, Musil D. 1987. The high-resolution X-ray crystal structure of the complex formed between subtilisin Carlsberg and eglin c, an elastase inhibitor from the leech *Hirudo medicinalis*. Structural analysis, subtilisin structure and interface geometry. *Eur J Biochem* 166: 673–692.
- Bode W, Papamokos E, Musil D, Seemueller U, Fritz H. 1986. Refined 1.2 Å crystal structure of the complex formed between subtilisin Carlsberg and the inhibitor eglin c. Molecular structure of eglin and its detailed interaction with subtilisin. *EMBO J* 5:813–818.
- Carrell RW, Evans DL, Stein PE. 1991. Mobile reactive centre of serpins and the control of thrombosis. *Nature* 353:576–578.
- Carrell RW, Owen MC. 1985. Plakalbumin, α_1 -antitrypsin, antithrombin and the mechanism of inflammatory thrombosis. *Nature* 317:730–732.
- Cooperman BS, Stavridi E, Nickbarg E, Rescorla E, Schechter NM, Rubin H. 1993. Antichymotrypsin interaction with chymotrypsin – Partitioning of the complex. *J Biol Chem* 268:23616–23625.
- Donovan JW, Mapes CJ. 1976. A differential scanning calorimetric study of conversion of ovalbumin to S-ovalbumin in eggs. *J Sci Food Agric* 27:197–204.
- Gettins P. 1989. Absence of large-scale conformational change upon limited proteolysis of ovalbumin, the prototypic serpin. *J Biol Chem* 264: 3781–3785.
- Gettins P, Patston PA, Schapira M. 1993. The role of conformational change in serpin structure and function. *BioEssays* 15:461–467.
- Hood DB, Huntington JA, Gettins PGW. 1994. α_1 -Proteinase inhibitor variant T345R. Influence of P14 residue on substrate and inhibitory pathways. *Biochemistry* 33:8538–8547.
- Hopkins PCR, Carrell RW, Stone SR. 1993. Effects of mutations in the hinge region of serpins. *Biochemistry* 32:7650–7657.
- Huber R, Carrell RW. 1989. Implications of the three-dimensional structure of α_1 -antitrypsin for structure and function of serpins. *Biochemistry* 28:8951–8966.
- Johnson BA, Shirokawa JM, Aswad DW. 1989. Deamidation of calmodulin at neutral and alkaline pH: Quantitative relationships between ammonia loss and the susceptibility of calmodulin to modification by protein carboxyl methyltransferase. *Arch Biochem Biophys* 268:276–286.
- Kint S, Tomimatsu Y. 1979. A Raman difference spectroscopic investigation of ovalbumin and S-ovalbumin. *Biopolymers* 18:1073–1079.
- Laemmli UK. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Lawrence DA, Olson ST, Palaniappan S, Ginsburg D. 1994a. Engineering plasminogen activator inhibitor 1 mutants with increased functional stability. *Biochemistry* 33:3643–3648.
- Lawrence DA, Olson ST, Palaniappan S, Ginsburg D. 1994b. Serpin reactive center mobility is required for inhibitor function but not for enzyme recognition. *J Biol Chem* 269:27657–27662.
- Lerner DB, Kearns DR. 1980. Observation of large solvent effects on the ³¹P NMR chemical shifts of nucleosides. *J Am Chem Soc* 102:7611–7612.
- Manavalan P, Johnson WC Jr. 1987. Variable selection method improves the prediction of protein secondary structure from circular dichroism spectra. *Anal Biochem* 167:76–85.
- Munch M, Heegaard C, Jensen PH, Andreasen PA. 1991. Type-1 inhibitor of plasminogen activators. Distinction between latent, activated and reactive centre-cleaved forms with thermal stability and monoclonal antibodies. *FEBS Lett* 295:102–106.
- Nakamura R, Ishimaru M. 1981. Changes in the shape and surface hydrophobicity of ovalbumin during its transformation to S-ovalbumin. *Agric Biol Chem* 45:2775–2780.
- Nakamura R, Takemori Y, Shitamori S. 1981. Liberation of carboxyl groups on conversion of ovalbumin to S-ovalbumin. *Agric Biol Chem* 45:1653–1659.
- Olson ST. 1985. Heparin and ionic strength-dependent conversion of antithrombin III from an inhibitor to a substrate of α -thrombin. *J Biol Chem* 260:10153–10160.
- Ottesen M. 1958. The transformation of ovalbumin into plakalbumin. *C R Lab Carlsberg Sér Chim* 30:211–270.
- Ottesen M, Wollenberger A. 1956. Stepwise degradation of the peptides liberated in the transformation of ovalbumin to plakalbumin. *C R Lab Carlsberg Sér Chim* 28:463–475.
- Patston PA, Gettins P, Beechem J, Schapira M. 1991. Mechanism of serpin action: Evidence that C1 inhibitor functions as a suicide substrate. *Biochemistry* 30:8876–8882.
- Privalov PL. 1982. Stability of proteins. Proteins which do not present a single cooperative system. *Adv Protein Chem* 35:1–104.
- Privalov PL, Gill SJ. 1988. Protein structure and hydrophobic interaction. In: Anfinsen CB, Edsall JT, Richards FM, Eisenberg DS, eds. *Advances in protein chemistry*, vol 39. San Diego: Academic Press. pp 191–234.
- Rubin H, Wang ZM, Nickbarg EB, McLarney S, Naidoo N, Schoenberger OL, Johnson JL, Cooperman BS. 1990. Cloning, expression, purification, and biological activity of recombinant native and variant human α_1 -antichymotrypsin. *J Biol Chem* 265:1199–1207.
- Schechter I, Berger A. 1967. On the size of the active site of proteases. *Biochem Biophys Res Commun* 27:157–162.
- Schechter NM, Sprows JL, Schoenberger OL, Lazarus GS, Cooperman BS, Rubin H. 1989. Reaction of human skin chymotrypsin-like proteinase chymase with plasma proteinase inhibitors. *J Biol Chem* 264:21308–21315.
- Schulze AJ, Baumann U, Knof S, Jaeger E, Huber R, Laurell CB. 1990. Structural transition of α_1 -antitrypsin by a peptide sequentially similar to β -strand 54A. *Eur J Biochem* 194:51–56.
- Smith MB. 1964. Studies on ovalbumin. I. Denaturation by heat and the heterogeneity of ovalbumin. *Aust J Biol Sci* 17:261–270.
- Smith MB, Back JF. 1965. Studies on ovalbumin II. The formation and properties of S-ovalbumin, a more stable form of ovalbumin. *Aust J Biol Sci* 18:365–377.
- Smith MB, Back JF. 1968. Studies on ovalbumin IV. Trypsin digestion and the cystine peptides of ovalbumin and S-ovalbumin. *Aust J Biol Sci* 21:549–558.
- Stein PE, Leslie AGW, Finch JT, Carrell RW. 1991. Crystal structure of un-cleaved ovalbumin at 1.95 Å resolution. *J Mol Biol* 221:941–959.
- Stein PE, Tewkesbury DA, Carrell RW. 1989. Ovalbumin and angiotensinogen lack serpin S-R conformational change. *Biochem J* 262:103–107.
- Vogel HJ, Bridger WA. 1982. Phosphorus-31 nuclear magnetic resonance studies of the two phosphoserine residues of hen egg white ovalbumin. *Biochemistry* 21:5825–5831.
- Wiman B, Almquist Å, Sigurdardottir O, Lindahl T. 1988. Plasminogen activator inhibitor 1 (PAI1) is bound to vitronectin in plasma. *FEBS Lett* 242:125–128.
- Wright HT. 1984. Ovalbumin is an elastase substrate. *J Biol Chem* 259: 14335–14336.
- Wright HT, Qian HX, Huber R. 1990. Crystal structure of plakalbumin, a proteolytically nicked form of ovalbumin. Its relationship to the structure of cleaved α -1-proteinase inhibitor. *J Mol Biol* 213:513–528.