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Structure and properties of ovalbumin

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Abstract

Ovalbumin is a protein of unknown function found in large quantities in avian egg-white. Surprisingly, ovalbumin belongs to the serpin family although it lacks any protease inhibitory activity. We review here what is known about the amino acid sequence, post-translational modifications and tertiary structure of ovalbumin. The properties of ovalbumin are discussed in relation to their possible functional significance. These include reasons for failure of ovalbumin to undergo a typical serpin conformational change involving the reactive centre loop, which explains why ovalbumin is not a protease inhibitor, and also the natural conversion of ovalbumin to the more stable ''*S*'' form.

Keywords: Food allergy; Ovalbumin

1. Introduction

Ovalbumin is the major protein in avian egg-white and was one of the first proteins to be isolated in a pure form [1]. Its ready availability in large quantities has led to its wide-spread use as a standard preparation in studies of the structure and properties of proteins, and in experimental models of allergy. In addition the synthesis of ovalbumin by hen oviduct and its regulation by steroid hormones has provided a model system in studies of protein synthesis and secretion. New interest in the structure and function of ovalbumin was stimulated by the unexpected finding that this protein belongs to the serpin superfamily [2]. The serpins [3] are a family of more than 300 homologous proteins with diverse functions found in animals, plants, insects and viruses, but not in prokaryotes [4,5]. They include the major serine protease inhibitors of human plasma that control enzymes of the coagulation, fibrinolytic, complement and kinin cascades, as well as proteins without any known inhibitory properties such as hormone binding globulins, angiotensinogen and ovalbumin.

2. The serpins

The functional activity of serpins as protease inhibitors is dependent on their unique ability to undergo a dramatic conformational change (illustrated in Fig. 1) on interaction with an attacking protease. The native serpin (Fig. 1a) has a highly flexible peptide loop (yellow), known as the reactive centre loop, which holds the reactive centre and mimics a good proteolytic substrate. The reactive centre peptide bond that is attacked by the protease is denoted P1–P1′ using the nomenclature of Schechter and Berger [6] where P1 is N-terminal and P1′ is

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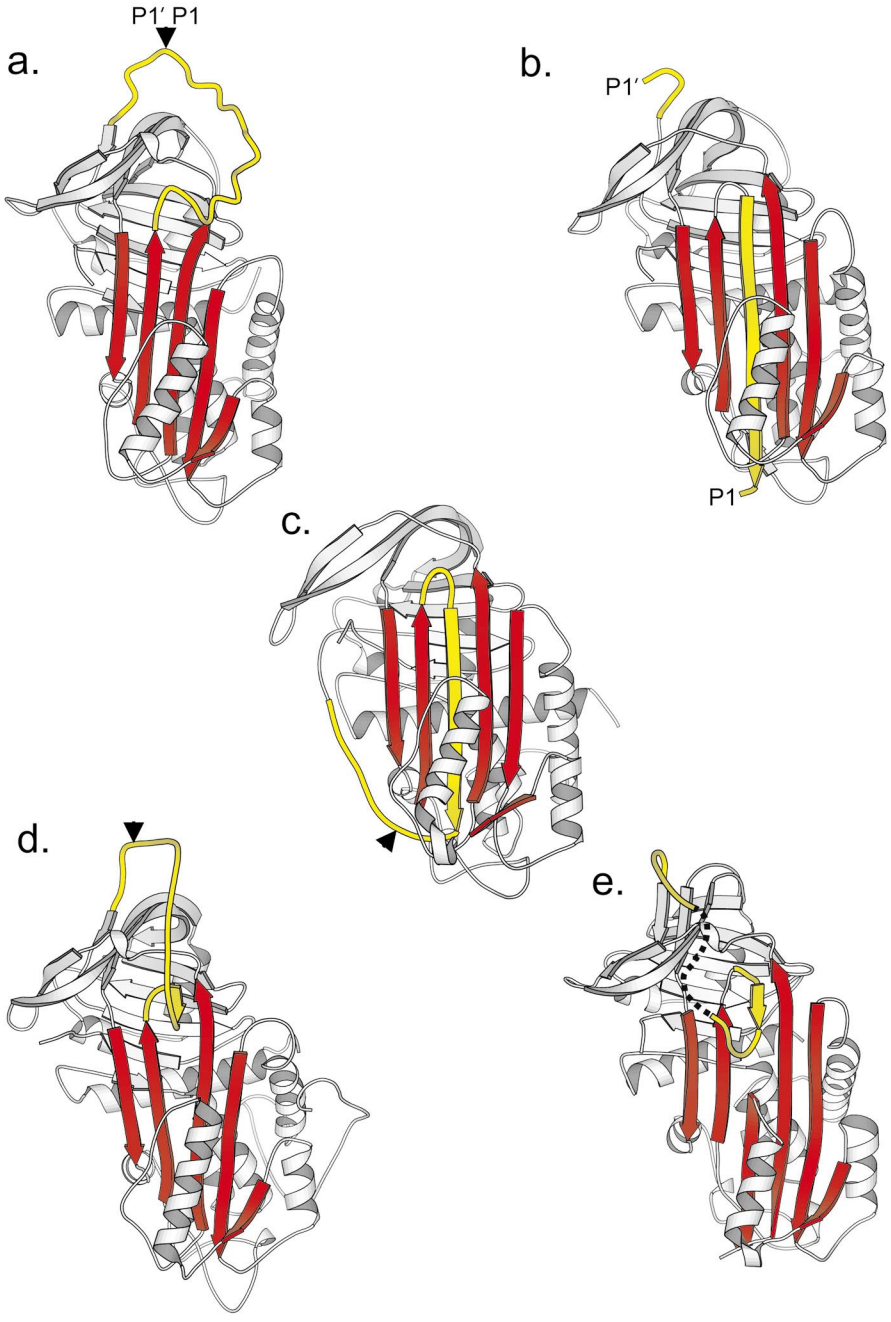

Fig. 1. The crystal structures of native α_1-antitrypsin, a, reactive centre cleaved α_1-antitrypsin, b, latent antithrombin, c, native antithrombin, d, and the delta variant of α_1-antichymotrypsin, e, illustrate the conformational mobility of the serpin superfamily. Inhibitory serpins react with proteases via an exposed, flexible reactive centre loop (yellow) which mimics a good substrate and leads to cleavage of the reactive centre bond, P1–P1′, indicated by an arrow. Cleavage results in subsequent incorporation of the N-terminal segment of the reactive centre loop as the fourth strand of the main β-sheet A (red). If deacylation occurs before full incorporation of the reactive centre loop the serpin is a proteolytic substrate, but if loop insertion is rapid, the protease becomes trapped while still attached to P1 via an ester bond. Loop insertion in the absence of cleavage also occurs. Latent antithrombin, c, is fully loop inserted and completely inactive. Antithrombin and plasminogen activator inhibitor-1 have been shown to convert to this conformation naturally in vivo. The partial insertion of the native antithrombin reactive centre loop, d, accounts for its need for the cofactor heparin to achieve rates of protease inhibition typical of other serpins. Serpins are sensitive to mutations which alter their conformational state. A recent structure of a pathogenic variant of α_1-antichymotrypsin, e, reveals another partially loop-inserted conformation resulting in complete loss of activity.

C-terminal to the site of cleavage. Unlike most proteins, the native serpin fold is not the most thermodynamically favoured conformation but a metastable folding intermediate. Interaction with the target protease releases conformational constraints and results in incorporation of the reactive centre loop as the middle strand (strand 4A) in the central β-sheet A (red) (Fig. 1b). The loop inserted form is more than twice as stable as the native conformer. It has recently been shown that full insertion of the reactive centre loop in β-sheet A, as illustrated in Fig. 1b, is required for inactivation of the protease [7–9], and that the energy of the conformational change is used to destabilise and distort the protease while still attached to the serpin via an ester bond at P1 [10]. The ability of serpins to undergo such a conformational change allows for tight regulation of their activity and provides a mechanism for clearance of serpin–protease complexes. However the dependence of the inhibitory mechanism on this complex structural transition makes the serpins especially sensitive to mutations which cause loss of function either by hindering loop insertion or by causing loop insertion in the absence of proteolytic attack (premature loop insertion) [11,12]. Premature loop insertion may occur within a single molecule (intramolecular loop insertion) or between molecules (intermolecular loop insertion). Intermolecular loop insertion, resulting in serpin polymerisation, is associated with various disease states including liver disease [13] and dementia [14].

The complex control mechanisms seen in inhibitory serpins explain the evolutionary success of this family as the predominant protease inhibitors in higher organisms. But what about the serpins without inhibitory functions? Many non-inhibitory serpins have simple roles: the hormone binding globulins act as ligand carriers in the circulation [15,16]; the only known function of angiotensinogen lies in its amino terminus which is cleaved by renin to release a small peptide involved in the control of blood pressure [17]. The function of ovalbumin is still unknown. It is interesting to consider why such a large and complex molecule as a serpin has been utilised for such apparently simple tasks and it is tempting to speculate that the serpin framework may have regulatory functions yet to be discovered.

In serpins without inhibitory activity a putative reactive centre can usually be readily identified by sequence alignment with typical inhibitory serpins. In ovalbumin, this is Ala–Ser at residues 353–354 which is also the sole cleavage site for elastase as predicted by the sequence [18]. Intriguingly, some non-inhibitory serpins have apparently retained the mobility of the reactive centre loop typical of inhibitory members of the family. The hormone binding globulins, corticosteroid binding globulin and thyroxine binding globulin, both undergo the characteristic serpin conformational change following cleavage at their putative reactive centre by enzymes released from activated neutrophils [19]. In corticosteroid binding globulin, the conformational change is associated with a reduction in hormone binding affinity suggesting a possible physiological mechanism for release of cortisol at inflammatory sites [19]. Other non-inhibitory serpins, including angiotensinogen and ovalbumin, do not show evidence for a large conformational change following cleavage at their putative reactive centres [20,21] and appear to have lost the extreme mobility of their inhibitory ancestors.

3. Amino acid sequence

Ovalbumin is a glycoprotein with a relative molecular mass of 45 000. The amino acid sequence of hen egg-white ovalbumin comprising 386 amino acids was deduced from the mRNA sequence by McReynolds et al. [22] and is in agreement with sequences of the purified protein [23] and the cloned DNA [24]. The sequence includes six cysteines with a single disulfide bond between Cys74 and Cys121 [25]. The amino terminus of the protein is acetylated [26]. Ovalbumin does not have a classical N-terminal leader sequence, although it is a secretory protein. Instead, the hydrophobic sequence between residues 21 and 47 may act as an internal signal sequence involved in transmembrane location [27]. Two genetic polymorphisms of ovalbumin have been reported: a Glu→Gln substitution at residue 290 [28] and an Asn→Asp substitution at residue 312 [29]. The sequences of chicken, Japanese quail, and common turkey ovalbumins have been determined and are 90% identical. Major differences include: a truncation of three amino acids from P1–P2′ in quail

ovalbumin, which supports the theory that ovalbumin is not inhibitory; an extra glycosylation site at 372 in turkey ovalbumin; chicken ovalbumin has six Cys residues while quail and turkey ovalbumin lack Cys31. As more ovalbumin sequences become available it may be possible to determine which domains are required for function through sequence comparison.

4. Post-translational modifications

A single carbohydrate side chain is covalently linked to the amide nitrogen of Asn293 at a typical Asn–X–Thr sequence recognised by glycosyltransferases. A second potential recognition site, Asn–X–Ser at residues 317–319, is not glycosylated in the secreted form found in the egg white, but has been observed transiently in the oviduct [30]. The carbohydrate chain is heterogeneous but the different ovalbumin glycopeptides share a common core structure: mannose β(1–4) glc*N*Ac β(1–4) glc*N*Ac–Asn293 [31]. Ovalbumin has two potential phosphorylation sites at serines 69 and 345. Comparison of ovalbumin sequences in different avian species shows an invariant glutamic acid two residues C-terminal to each phosphoserine which may form part of a recognition site for a protein kinase. Heterogeneity in the electrophoretic behaviour of ovalbumin is largely due to different degrees of phosphorylation at these sites [23,32]. Three major fractions can be separated by ion-exchange chromatography with, respectively two, one and zero phosphate groups per ovalbumin molecule in an approximate ratio of 8:2:1 [33,34].

5. Tertiary structure

The first serpin structure to be solved was of human α_1-antitrypsin that had been proteolytically cleaved at its reactive centre peptide bond. It showed an unexpected separation of the new chain termini by 70 Å (Fig. 1b) [35]. The N-terminal portion of the cleaved reactive centre loop forms the fourth strand of the six stranded β-sheet A that runs parallel to the long axis of the molecule. It was predicted that in intact antitrypsin this strand would be withdrawn from sheet A to form an external peptide loop, and β-sheet A would have only five strands. The first crystallographic models for the native serpin structure came from structures of ovalbumin (Fig. 2). The structure was initially solved for a proteolytically modified form of ovalbumin called plakalbumin [33]. Plakalbumin is formed by the interaction of ovalbumin with subtilisin, which excises six amino acids from the reactive centre loop [18]. The crystal structure of plakalbumin was reported by Wright et al. [36] and was refined at a resolution of 2.8 Å. The cleaved ends are separated by 27 Å in this structure, but the conformational change seen in cleaved inhibitory serpins, in which the cleaved reactive centre loop is inserted in β-sheet A as a new strand, has not taken place (Fig. 2b). In plakalbumin, sheet A has only five strands as had been predicted for an intact inhibitory serpin, but the plakalbumin structure provided only a partial model for an intact serpin since the reactive centre loop had been cleaved [37]. The conformation of the intact reactive centre loop was still unknown.

The crystal structure of native hen ovalbumin [38,39] was the first structure of an uncleaved serpin, and surprisingly, the intact reactive centre loop was found to take the form of an exposed α-helix of three turns that protruded from the main body of the molecule on two peptide stalks (Fig. 2a). The overall structural similarity between ovalbumin and active inhibitors of the serpin family suggested that the ability to adopt a helical form might be common to all serpins, although it would be an unexpected feature of a protease inhibitor since a helical reactive centre would have to unfold to dock to a protease. The ovalbumin structure includes four crystallographically independent ovalbumin molecules and the position of the helical reactive centre loop relative to the protein core differs by 2–3 Å between molecules. Although this shift is probably due to the different environments of the helices in the crystal lattice, it suggested that the reactive centre loop is flexible in solution.

The structures of various uncleaved inhibitory serpins have now been determined and confirm the exceptional mobility of the serpin reactive centre loop. These include inactive conformations resulting from intramolecular loop insertion in plasminogen activator inhibitor-1 [40] and in antithrombin [41]

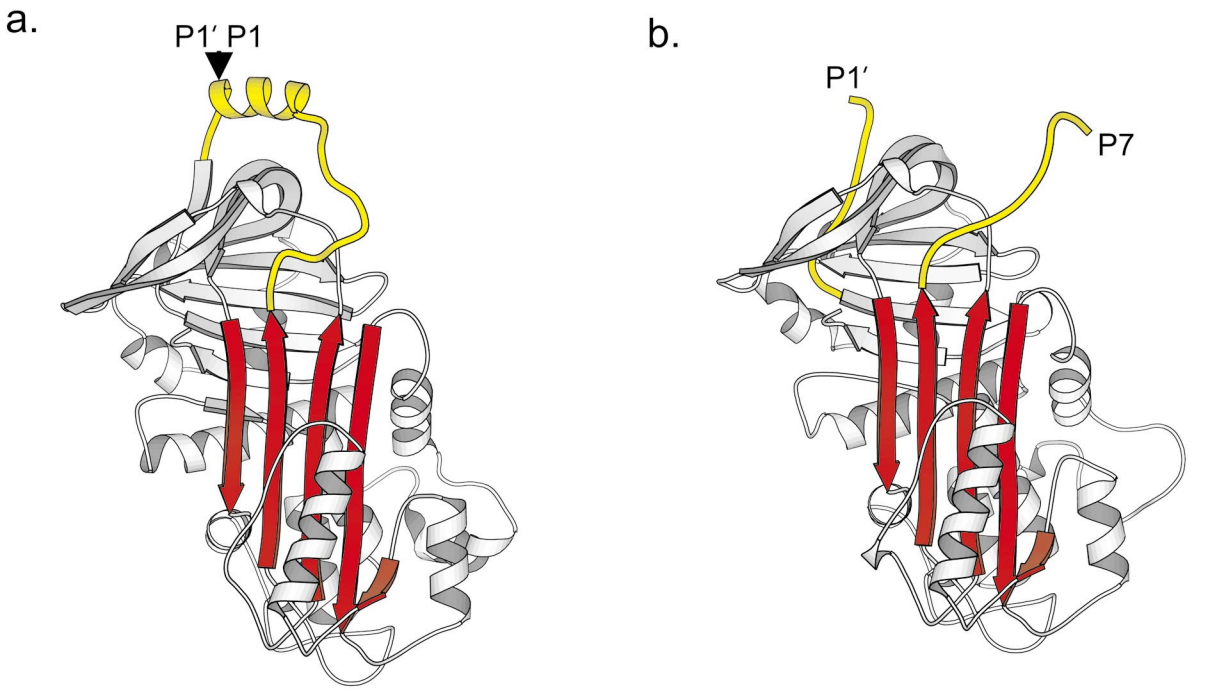

Fig. 2. The crystal structures of native, a, and subtilisin cleaved, b, ovalbumins. Native ovalbumin possesses a typical serpin fold, with a three-turn α-helical reactive centre loop (yellow). Unlike the inhibitory serpins, cleavage within the reactive centre loop does not result in its incorporation into β-sheet A (red). Subtilisin cleavage to form ''plakalbumin'' results in the excision of P1 to P6 and no loop insertion.

(Fig. 1c). Several serpins have now been crystallised in active conformations including a form of α_1-antichymotrypsin with a distorted helical reactive centre loop [42], an extended loop conformation in active plasminogen activator inhibitor-1 [71] and partially loop-inserted conformations of native antithrombin [41,43] (Fig. 1d) and a variant of α_1-antichymotrypsin [44] (Fig. 1e). In some of these crystal structures, the conformation of the reactive centre loop appears to be significantly influenced by packing contacts. However, the recent crystal structures of α_1-antitrypsin [45] and Manduca sexta serpin 1K [46] have shown that the serpin reactive centre loop can adopt a canonical conformation that would readily dock with a target protease. Structural studies of serpins in various conformations have shown how their unique flexibility is essential for function, and have formed the basis for a detailed understanding of the normal and abnormal function of this unusual family of proteins at a molecular level [11].

6. Ovalbumin is not a protease inhibitor

A comparison of the structures of native ovalbumin (Fig. 2a) and plakalbumin (Fig. 2b) shows that cleavage within the reactive centre loop of ovalbumin does not result in its incorporation into β-sheet A [20,21,36]. This failure of full loop insertion, which is an essential requirement for protease inhibition by serpins, explains why ovalbumin shows no inhibitory activity despite its sequence homology with functional inhibitors of the serpin family. But what is the molecular explanation for the failure of ovalbumin to undergo this conformational change?

In an active inhibitory serpin, it is generally accepted that insertion of the reactive centre loop into β-sheet A takes place sequentially, starting with P15, and in register with that observed in the structures of cleaved serpins. Such incorporation of a loop into a β-sheet results in alternating side chains being buried (Fig. 3). In typical inhibitory serpins

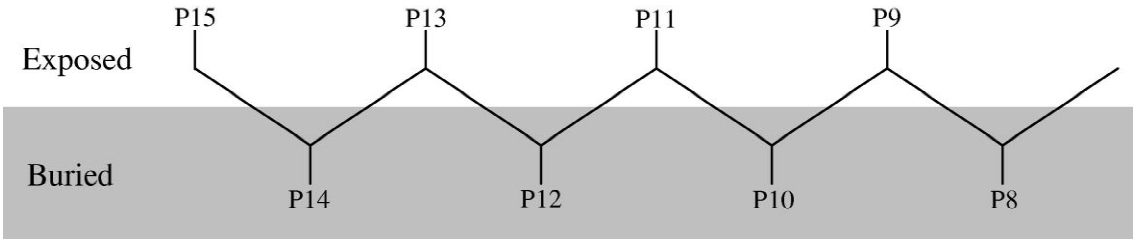

Fig. 3. Insertion of the serpin reactive centre loop into β-sheet A results in every other side chain being buried in the hydrophobic core of the serpin. The portion of the reactive centre loop most distant from the reactive centre bond (P15–P8) is called the hinge region and is crucial for efficient loop insertion. Each even numbered residue is buried and thus the sequence of this region is highly conserved as small amphipathic or hydrophobic amino acids.

the even numbered P residues of the reactive centre loop from P14 to P4 would have their side chains buried and are commonly small amphipathic or hydrophobic amino acids (Table 1). The first side chain to insert, P14, is thus critical for continued sequential incorporation of the reactive centre loop and is highly conserved as threonine among the inhibitory serpins. The arginine at the P14 position in ovalbumin led to the hypothesis that ovalbumin is thermodynamically incapable of loop insertion since burying the bulky charged side chain would be energetically too costly [36]. In ovalbumin, other residues in the so-called ''hinge region'' from P16 to P8 deviate from the conserved sequence (Table 1), notably P12 to P10 is VVG in ovalbumin compared with AAA in inhibitory serpins. The sequences in the hinge region of other non-inhibitory serpins are shown in Table 1, and in all cases there is a residue with an even P number which differs significantly from the conserved sequence and is often charged or bulky.

Work on recombinant variants of ovalbumin confirmed the hypothesis that bulky residues in the hinge region prevented loop insertion upon cleavage of the reactive centre loop [47]. Loop insertion is accompanied by a large increase in the thermal stability of serpins and is thought to be the driving force for the translocation and inactivation of proteases [10,48]. Whereas a cleaved inhibitory serpin is at least 60°C more stable than its native counterpart, cleaved ovalbumin is 1 to 2°C less stable than the native form [47,49]. An increase in stability on cleavage can be used as a marker for serpin reactive centre loop insertion. Variant ovalbumin with P14 arginine replaced by serine does undergo loop insertion on cleavage as indicated by an 11°C increase in thermal stability, and a further 5°C increase in stability is observed for the cleaved variant which includes the consensus P12 to P10 AAA [47]. Although these experiments demonstrate that the hinge region sequence of ovalbumin is responsible for its apparent lack of ability to spontaneously loop insert upon cleavage, it does not fully explain why ovalbumin is not inhibitory. An inverse mutation was made in the

Table 1
Comparison of the hinge region residues for non-inhibitory serpins with the consensus inhibitory sequence

	P16	P15	P14	P13	P12	P11	P10	P9	P8	Ref.
Inhibitory consensus	Glu	Gly	Thr	Glu	Ala	Ala	Ala	Ala	Thr	[4]
% Identity	38	98	80	85	98	75	65	75	83	[4]
Non-inhibitory serpins										
Ovalbumin	Ala	Gly	Arg*	Glu	Val*	Val	Gly	Ser	Ala	[22]
Human angiotensinogen	Asp	Glu	Arg*	Glu	Pro*	Thr	Glu*	Ser	Thr	[68]
Maspin	Glu	Ile	Thr	Glu	Asp*	Gly	Gly	Asp	Ser	[69]
PEDF	Asp	Gly	Ala	Gly	Thr	Thr	Pro*	Ser	Pro*	[70]

Residues which have an asterisk are considered detrimental to loop insertion.

inhibitory serpin antitrypsin where the consensus P14 threonine was replaced by arginine [50]. This variant was still capable of loop insertion and showed an increase in thermal stability comparable to wild-type. Furthermore, it retained the ability to inhibit certain target proteases, although with significantly diminished efficacy. Another reason to suspect that other factors may contribute to the lack of inhibitory activity of ovalbumin is the relatively small increase in thermal stability of the cleaved ovalbumin variants. The increase in denaturation temperature from 11 to 16°C for the cleaved combined variant with the P14, P12–P10 mutations indicates that insertion involves at least P14 through P12. Proteolytic susceptibility of the remainder of the reactive centre loop after cleavage at P1–P1′ indicate that the P8 residue is accessible to proteases and therefore not incorporated into β-sheet A. Thus, although the hinge region residues of ovalbumin are apparently incompatible with incorporation into β-sheet A upon cleavage, this provides only part of the reason for the lack of inhibitory properties of ovalbumin, since even with consensus hinge region residues ovalbumin is not capable of the full reactive centre loop incorporation observed for cleaved inhibitory serpins and required for protease inhibition. Support for this hypothesis is provided by work on a chimera of ovalbumin which required 64% of the sequence of PAI-2 before any inhibitory properties were observed [51].

7. *S*-Ovalbumin

By the time eggs reach the supermarket shelf and ultimately the consumer, typically more than half of the ovalbumin has changed form. *S*-Ovalbumin was discovered in 1964 by following the change in the melting profile of ovalbumin with the age of eggs [52]. The mid-point of thermal denaturation (Tm) of ovalbumin shifted from 78 to 86°C, and the new form was named ''*S*-ovalbumin'' to denote its increased stability [53]. The appearance of *S*-ovalbumin coincides with the loss of the ''food value'' of eggs since eggs with high *S*-ovalbumin content have runny whites and do not congeal as effectively on cooking. Most of the work on *S*-ovalbumin has been motivated by the loss in food value of stored eggs and not in relation to the potential function of ovalbumin in eggs.

S-Ovalbumin is easily formed in vitro by a 20 h incubation at 55°C in 100 m*M* sodium phosphate, pH 10 [54]. The high pH and temperature increase the rate of conversion, the basis of which has been extensively studied. Biochemical studies comparing the properties of native and *S*-ovalbumin confirm that the increase in stability on conversion to the *S*-form results from a unimolecular conformational change and not a change in the chemical make-up of ovalbumin [53,55,56]. The *S*-form differs from native ovalbumin only in its greater stability, compactness and hydrophobicity [57]. The conformational conversion requires the disulfide bond between cysteines 74 and 121 [58]. The chemically denatured states of native and *S*-ovalbumin also differ so that renaturation of *S*-ovalbumin does not lead to the native conformer [59]. *S*-Ovalbumin has also been formed from recombinant ovalbumin produced in *Escherichia coli* providing unequivocal evidence that the conversion is not dependent on post-translational modifications [60].

Only recently has the conversion of ovalbumin to the *S*-form been addressed in light of its membership in the serpin superfamily [61]. Most inhibitory serpins are capable of intramolecular reactive centre loop insertion in the absence of cleavage to form a ''locked'' or ''latent'' conformation [62]. Structures of such conformations have recently been determined by X-ray crystallography, including latent plasminogen activator inhibitor-1 and antithrombin (Fig. 1c), native antithrombin (Fig. 1d), and the ''delta'' conformer of α_1-antichymotrypsin (Fig. 1e). Each of these loop-inserted forms are more stable than the native (non-loop inserted) five-stranded β-sheet A forms. Recent work based on biochemical and thermal stability studies, has provided evidence that the conformational change in *S*-ovalbumin is also an intramolecular insertion of the reactive centre loop [61]. Studies using circular dichroism and Fourier transform infrared spectroscopies concluded that the limited conformational change in *S*-ovalbumin involves a small 2–5% loss of α-helix content and a concomitant increase in anti-parallel β-sheet [61,63]. This is what would be expected if the α-helical reactive centre loop of ovalbumin were to unravel to allow for limited incorporation into β-sheet A.

Consistent with an unravelled helix are the observations that the reactive centre loop of *S*-ovalbumin shows increased proteolytic susceptibility and loss of contacts with the main body of the molecule involving glutamates at positions P13 and P7 [61,64].

A working model for the conformation of *S*-ovalbumin was proposed to be a locked conformation with partial loop insertion from P15 to P10 [61]. This limited extent of loop insertion is necessary to explain the unaltered rate of dephosphorylation of the phosphoserine at P9 in *S*-ovalbumin. The model includes insertion of the P14 arginine to preserve the register of reactive centre loop insertion observed for the cleaved inhibitory serpins. However, the recent crystal structure of the cleaved P14 arginine variant of α_1-antichymotrypsin shows that the P14 arginine side-chain is not buried, but in-register insertion occurs C-terminal to P14 [65]. Out-of-register β-sheet A incorporation of serpin reactive centre loops and related peptides has been demonstrated structurally [66]. This allows for the postulation of out-of-register incorporation of the reactive centre loop of ovalbumin as the basis of conversion to *S*-ovalbumin to avoid insertion of the P14 arginine side-chain. The model is limited by the constraints that only P15 to P10 can be involved and that no charged side-chains may be buried. The improvement in stability observed for the cleaved P14, P12–P10 variant compared to the P14 variant of ovalbumin allows for the minimum number of residues which can be accommodated as strand 4 in β-sheet A to be set at six (P15–P12). The structure of *S*-ovalbumin is thus likely to be partially loop inserted in a manner analogous to other members of the serpin superfamily (Fig. 1d and e), and may involve out-of-register loop insertion to avoid burying the P14 arginine side chain.

8. Conclusion

Although ovalbumin comprises 60–65% of the total protein in egg white, its function remains unknown. Ovalbumin shows no protease inhibitory activity despite sequence identity of about 30% with antitrypsin and other functional inhibitors of the serpin family. The Ala–Ser bond at its putative reactive centre suggests specificity for elastase, but ovalbumin acts as a substrate not as an inhibitor of this enzyme. We believe that the structure of *S*-ovalbumin may be a central clue to determining the function of ovalbumin in eggs. The preservation of the serpin fold and its metastability is presumably not accidental and the natural conversion of ovalbumin to the more stable *S*-form is likely to be functionally relevant. The fraction of native ovalbumin may range from 20 to 80% in commercial preparations, and from 20 to 40% in fresh preparations from old eggs [53]. Perhaps the activity of ovalbumin has been overlooked due to inadvertent contamination with the *S*-form. It is possible that the activity of ovalbumin is lost on its conversion to *S*-ovalbumin, and thus the slow natural conversion provides a timing mechanism by which the needs of a chick embryo at different developmental stages can be met by the same protein. An alternative possibility is that the active form is the more stable *S*-form. This has recently been suggested by the finding of rapid conversion and subsequent migration to the chick embryo of a more stable form of ovalbumin found in fertilised eggs [67]. This more stable form has properties indistinguishable from that of *S*-ovalbumin formed in vitro, with the exception of an apparent loss of a phosphate group. It is thus possible that ovalbumin plays a role in chick embryo development which is mediated by the natural metastability of ovalbumin and affected through binding to an *S*-ovalbumin receptor in a phosphorylation dependent manner.

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