Chicken Ovomucoid: Determination of Its Amino Acid Sequence, Determination of the Trypsin Reactive Site, and Preparation of All Three of Its Domains†

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ABSTRACT: The complete amino acid sequence of chicken ovomucoid (OMCHI) is presented. OMCHI consists of three tandem domains, each homologous to pancreatic secretory trypsin inhibitor (Kazal) and each with an actual or putative reactive site for inhibition of serine proteinases. The major reactive site for bovine β-trypsin is the Arg^{89}–Ala peptide bond in the second domain. The equilibrium constant for hydrolysis of this peptide bond, K^0_{hyd}, is 1.85. The first and third domains of OMCHI are relatively ineffective inhibitors of several serine proteinases against which they were tested. OMCHI is a mixture of two forms: the major form with all of the amino acid residues and a minor form with Val^{134}-Ser^{135} deleted. This polymorphism is present in all chicken eggs and is the result of ambiguous excision at the 5′ end of the F intron. Procedures are given for preparation of modified chicken ovomucoid, OMCHI* (in which the Arg^{89}–Ala bond is hydrolyzed), of the first domain, OMCHI1 (residues 1–68), of the second domain, OMCHI2 (residues 65–130), and of the third domain, OMCHI3 (residues 131–186). In the case of the third domain, both the Asn^{175} glycosylated form, OMCHI3(+), and the carbohydrate-free form, OMCHI3(−), were obtained. These isolated native domains are useful in many studies of ovomucoid behavior.

Trypsin inhibitory activity of chicken egg white was known at the beginning of this century (Delezenne & Pozerski, 1903). The clear association of this activity with chicken ovomucoid (OMCHI)[1] came after Lineweaver and Murray (1947) developed a simple procedure for purification of ovomucoid. Since then, chicken ovomucoid has attracted considerable attention both as an inhibitor and as an object for physicochemical studies.

Probably the most remarkable work on avian ovomucoids is the paper of Rhodes et al. (1960), who isolated ovomucoids from egg whites of several species of birds and studied their inhibitory properties. They found that ovomucoids from closely related species often have strikingly different inhibitory specificities. This is given a molecular explanation in the the following paper (Laskowski et al., 1986). Furthermore, they found that some ovomucoids are, like the chicken ovomucoid molecule, single-headed, i.e., combine with only one molecule of a serine proteinase; others are, like the turkey ovomucoid molecule, double-headed and combine simultaneously with two enzyme molecules, and yet others, like the duck ovomucoid molecule, are triple-headed and combine with three molecules of enzyme at once.[2] Their seminal finding receives a molecular explanation in the three-domain structure of avian ovomucoids reported here.

Our interest in ovomucoids stemmed from two sources. We had used chicken ovomucoid along with soybean trypsin inhibitor (Kunitz) in our early studies of protein inhibitors of serine proteinase (Finkenstadt & Laskowski, 1965; Ozawa & Laskowski, 1966), but we knew little about its chemical or three-dimensional structure. The other motive was the work of Rhodes et al. (1960), which suggested that ovomucoids were an excellent system for the study of the relationship between sequence and reactivity of proteinase inhibitors.

In this paper, we present the amino acid sequence of chicken ovomucoid. We show that chicken ovomucoid consists of three tandem, homologous (Kazal) domains and that its trypsin reactive site peptide bond is the Arg^{89}–Ala bond in the second domain. We describe the equilibrium involved in the hydrolysis of this bond. The sequencing, or more properly the proof of the correctness of the previously surmised sequence, is obtained by making cleavages exclusively on the native protein, without prior reduction of the disulfide bridges. We also provide methods for preparing the first domain, OMCHI1 (residues 1–68), the second domain, OMCHI2 (residues 65–130), and the third domain (residues 131–186) with, OMCHI3(+), or without OMCHI3(−), a carbohydrate moiety on Asn^{175}. Finally, we deal here with the Val^{134}-Ser^{135} deletion that occurs in the minority of the OMCHI molecules and is almost certainly due to ambiguous excision of the F intron of the ovo-

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[1] Abbreviations: OMCHI, chicken ovomucoid; OMCHI*, chicken ovomucoid, modified (with the reactive site peptide bond Arg^{89}–Ala hydrolyzed); OMCHI1, chicken ovomucoid first domain; OMCHI2, chicken ovomucoid second domain; OMCHI3(−), unglycosylated chicken ovomucoid third domain; OMCHI3(+), glycosylated chicken ovomucoid third domain; PSBOV, bovine pancreatic secretory trypsin inhibitor (Kazal); CM, carboxymethyl; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid, sodium salt; CNBr, cyanogen bromide; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; Spase V8, *Staphylococcus aureus* proteinase V8.

[2] This classification, even though it served the early development of the field very well, is a major approximation. First, the authors used an arbitrary set of only two serine proteinases—trypsin and chymotrypsin. When the number of enzymes is increased to six by the addition of subtilisin, pancreatic elastase, and *Streptomyces griseus* proteinases A and B, the number of heads on some ovomucoids increases. It might be argued that when the set of serine proteinases became very large, all ovomucoids would become triple headed. An equally vexing problem in counting heads is that it requires us to replace a continuously variable function of each domain, the equilibrium constant for association with some proteinase, by a dichotomy: inhibits or does not inhibit. Such descriptions served us very well in the initial stages but have now become obsolete. Now we prefer to talk about K_a values for each domain and not to make the extreme yes–no distinction.

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mucoid gene (Stein et al., 1980).

Materials and Methods

Purification of Ovomucoid. A procedure developed in our laboratory that utilizes the classic method of Lineweaver and Murray (1947) as a first step was used. Chicken (*Gallus gallus*) egg white was placed in a Waring blender. Two volumes of freshly prepared, cold precipitating solution (1 volume of 0.5 M trichloroacetic acid and 2 volumes of acetone) were added, and the mixture was homogenized for 3–5 min. The resulting suspension was gently mixed overnight at 4 °C in a rotary shaker and then centrifuged at 3000*g* for 25 min. The ovomucoid was precipitated from the supernatant by adding 2–2.5 volumes of cold acetone (with vigorous stirring). The suspension was kept overnight at 4 °C. The precipitate was recovered by centrifugation and decantation and then dissolved in deionized water and freeze-dried. The yield of the crude ovomucoid was 8 g/L of egg white (35 eggs). In order to remove contaminating ovoinhibitor, 7.5 g of the preparation was subjected to size-exclusion chromatography (not shown) on a Bio-Gel P-10 (200–400-mesh) column (10 × 90 cm) in 5% (v/v) formic acid.

The major (retarded) peak was collected and lyophilized (yield 5 g). Two grams of this preparation was dissolved and dialyzed against 0.02 M sodium citrate buffer and then introduced to a CM-Sepharose CL-6B column (2.5 × 35 cm) equilibrated in the same buffer. The column was washed with the buffer and developed with a linear sodium chloride gradient (0–0.5 M) in the citrate buffer. The main peak was isolated, dialyzed against distilled water, and freeze-dried (yield 1 g).

Reduction and carboxymethylation were used extensively in this work as all the proteins and peptide fragments were reduced and carboxymethylated prior to sequencing. Treatment of entire ovomucoid is given as an example. Chicken ovomucoid was dissolved (final concentration of 1%) in 0.5 M Tris-HCl buffer (pH 8.5) containing 0.05 M dithiothreitol, 6 M guanidine hydrochloride, and 5 mM EDTA. The solution was flushed with nitrogen and incubated at 40 °C for 2 h. Sodium iodoacetate (2 mol/1 mol of dithiothreitol) was then added, and the solution was incubated in the dark for 15 min at 21 °C. The incubation mixture was exhaustively dialyzed against distilled water and freeze-dried.

Amino acid analyses were carried out after hydrolysis in 6 N HCl at 110 °C on a Durrum D-500 analyzer. For more details, see the following paper (Laskowski et al., 1986). All analyses agreed with expectations based on Figure 1.

Sequencing. The equipment currently used in our laboratory and the procedures used for sequencing connecting peptide fragment extended third domains, i.e., OMCHI3 (residues 131–186), are described in the following paper (Laskowski et al., 1986). The differences between the work done specifically on third domains (this includes the 131–186 runs in this paper) and the other runs done here are as follows: (1) Unlike in the third domain work, we did not always get the desired length of run, i.e., 70 residues on entire ovomucoid on the first attempt. In order to get the long runs referred to in Figure 1, two or three attempts were generally made. (2) The amount of material used ranged up to 5 mg/run. (3) Polybrene was used only in those runs (OMCHI1, OMCHI2, OMCHI3) where completion of the run was expected. (4) Many runs involved double coupling.

Determination of Fraction of Modified Chicken Ovomucoid in Mixture with Its Virgin Form. The method we used was based on an early observation of Kowalski and Laskowski (1972) that maleylation inactivates modified chicken ovomucoid (OMCHI*) by blocking the NH_2 terminus of P'_1 Ala^{90}, but does not influence virgin ovomucoid (OMCHI) whose P_1 Arg^{89} is not derivatized.

For maleylation, the method of Butler et al. (1969) was employed. A sample containing OMCHI and OMCHI* (final ovomucoid concentration 4×10^{-5} M) was incubated with maleic anhydride (1×10^{-2} M) in 0.2 M sodium borate (pH 9.0) for 10 min at 21 °C. Bovine β-trypsin[3] was then added (to a final concentration of 8×10^{-5} M), and the mixture was incubated for 10 min more to allow the enzyme–inhibitor complex formation. The concentration of free trypsin was then determining with a burst substrate (*p*-nitrophenyl p'-guanidinobenzoate) according to Chase and Shaw (1967). Trypsin inhibition by the OMCHI and OMCHI* mixture was determined in the same way except that excess maleic anhydride was allowed to decompose for 10 min prior to the addition of the inhibitor sample. Control determination of trypsin activity was performed in the same manner as the determination of trypsin inhibition except the inhibitor sample was omitted.

Results

Amino Acid Sequence of Mature Ovomucoid. After several years of work on conventional approaches to the sequencing of chicken ovomucoid (primarily reduction and carboxylation of OMCHI followed by exhaustive hydrolysis by trypsin and chymotrypsin, separation of peptides by high-voltage paper electrophoresis, isolation and sequencing, and conventional overlapping), we came to a sequence almost identical with that given in Figure 1. We then set out to prove that this sequence is correct by the strategy outlined in the legend to Figure 1. Only four highly limited cleavages were employed, and all were carried out on native, not on denatured, protein.

Confirmation That the COOH-Terminal Residue of OMCHI and of OMCHI3(−) Is Cys^{186}. Exhaustive tryptic hydrolysis of reduced and carboxymethylated samples of OMCHI and of OMCHI3(−) was followed by high-voltage paper electrophoresis. In each case, the most rapidly moving negative component could be identified as (carboxymethyl)cysteine, either by mixing of the digest with authentic material or by cutting out the spot and running the extract (without hydrolysis) in an amino acid analyzer. This finding is fully consistent with the ...Lys^{185}-Cys^{186} COOH-terminal sequence. This experiment should be relatively general for most avian ovomucoids as the ...Lys^{185}-Cys^{186} sequence is present in most (92 of the 106) third domains reported in the following paper (Laskowski et al., 1986).

Deletion Polymorphism Involving Val^{134}-*Ser*^{135}. The sequencing of chicken ovomucoid third domain revealed a puzzling phenomenon. The same phenomenon was evident on sequencing of OMCHI3(−) and OMCHI3(+) [these are the 131–186 fragments and differ only by Asn^{175} being free (−) or glycosylated (+)] and in the sequencing of the reduced carboxylated COOH-terminal fragment of OMCHI* (residues 90–186). The sequencing proceeds normally up to Val^{134}, but at Ser^{135}, both Ser and Asp are evident. At Val^{136} we see both Val and CM-Cys. From here on, a preview of *two* residues ahead continues throughout the sequence although the intensity of the minor sequence drops after it reaches Pro^{142} where, because Glu^{140} is expected, we program a short cleavage. In most runs, the intensity of the preview was about 20% and of the main sequence about 80%. From this result, one can

[3] Isolated from a commercial trypsin preparation (Worthington) by a chromatographic procedure of Schroeder and Shaw (1968) as modified by Luthy et al. (1973).

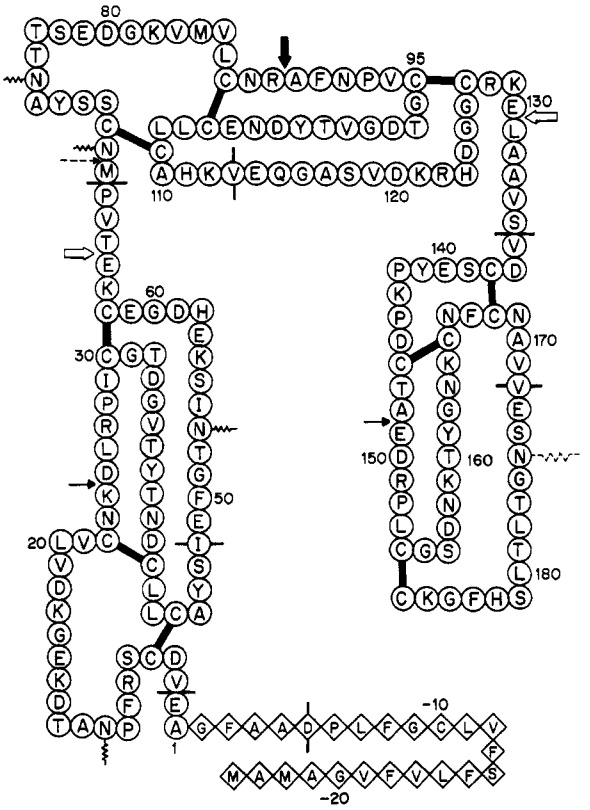

FIGURE 1: Amino acid sequence of preovomucoid. The signal peptide sequence residues (−24 to −1) shown in diamonds were determined by Thibideau et al. 1978). The mature protein sequence is given in circles. The strong black bars between Cys residues indicate disulfide bridges (for their assignment, see text). The slender black bars are the positions of introns in the preovomucoid gene as determined by Stein et al. (1980). (When these black lines go through residues, they indicate introns within codons.) The sawtoothed solid line indicates (almost) complete glycosylation sites; the sawtoothed broken line indicates the partial glycosylation site in the third domain. The strong black arrow indicates the trypsin reactive site of the second domain and the slender black arrows the reactive sites of the first and third domains. The broken arrow indicates the CNBr cleavage between the first and second domains. The white arrows indicate the sites of domain–domain cleavage by Spase V8. Additional cleavages by Spase V8 occur at Glu^{15}-Gly, Glu^{49}-Phe, and Glu^{57}-His. These are not indicated. The protein sequence is in complete accord with cDNA sequence (Catterall et al., 1980). The strategy for proving the protein sequence was (1) a run from 1 to 70 on the entire reduced and carboxymethylated ovomucoid, (2) isolation and complete sequencing of OMCHI1 residues 1–68, (3) isolation and complete sequencing of OMCHI2 residues 65–130, thus providing a five-residue overlap and determining residues 1–130, (4) preparation of modified ovomucoid OMCHI* (Arg^{89}-Ala hydrolyzed) and the reduced and carboxymethylated peptide 90–186 and sequencing of it from 90 to 165 (in this run, some identifications were marginal), and (5) isolation of OMCHI3 residues 131–186 and sequencing of reduced and carboxymethylated OMCHI3 to the end (there is a long overlap with the previous sequence, thus completing the entire sequence), and (6) providing an additional proof that Cys is the COOH-terminal residue of OMCHI and of OMCHI3.

deduce that 20% of the OMCHI3(−) molecules (and by inference of OMCHI molecules) are missing either the Val^{134}-Ser^{135} or Ser^{135}-Val^{136} dipeptide. The conclusion was recently strengthened when Lin et al. (1985) separated OMCHI3(−) into two fractions by HPLC and showed that the minor fraction has the Val^{134}-Ser^{135} (or Ser^{135}-Val^{136}) sequence completely deleted.

On our first encounter with the deletion polymorphism in OMCHI, we had suspected that it was analogous to the Gly^{162}/Ser^{162} polymorphism in Japanese quail ovomucoid (Bogard et al., 1980) where there are two allelic genes at a single locus—one for the Gly^{162} and the other for the Ser^{162} forms. As a consequence, there are three kinds of Japanese quail eggs—homozygous Gly^{162}, homozygous Ser^{162}, and heterozygous, in which the Gly^{162} and Ser^{162} forms of ovomucoid occur in equal amounts. We have, therefore, separately isolated OMCHI from three different chicken eggs, bought from different farms (two white eggs, one brown egg), and subjected these samples to identical Spase V8 digestions. All three isolated OMCHI3(−) domains were sequenced for 10 cycles. All showed 20 ± 2% of the short (deleted) form. The result is totally inconsistent with the polymorphism being a consequence of two allelic genes. Instead, it can be interpreted (although the statistics are too small) that all chicken eggs contain both forms of ovomucoid in roughly the same ratio.

Our preliminary results about the dipeptide deletion were communicated to the O'Malley group prior to their announcement of the gene and mRNA sequences of chicken ovomucoid (Stein et al., 1980; Catterall et al., 1980). These authors found that the codons for the Val^{134}-Ser^{135} sequence in the long form are followed by the F intron in the ovomucoid gene. More importantly, they found that the hexanucleotide sequence GTGATG (coding for Val^{134}-Ser^{135}) is exactly repeated at the exon/intron junction, thus allowing for the possibility of ambiguous excision at the 5′ end of the F introl. Their proposed scheme is illustrated in Figure 2. In further support of their proposal, Stein et al. (1980) demonstrated that there are two forms of chicken ovomucoid mRNA, the minor form containing a deletion at or near the 5′ splice site of the F intron. The Stein et al. (1980) proposal is entirely consistent with all of the data reported above as well as with the data on third domain sequences of many phasianoid birds reported in the following paper (Laskowski et al., 1986).

Characterization of Arg^{89}–Ala Reactive Site. Finkenstadt and Laskowski (1965) and Ozawa and Laskowski (1966) showed that a single Arg–Ala bond serves as the reactive site of chicken ovomucoid, but they could not locate its position in the molecule. In order to do this, we incubated OMCHI with 2 mol % of bovine β-trypsin at pH 3.0 for 24 h. The product was shown to be 93 ± 2% OMCHI* and 7 ± 2% OMCHI by the maleylation assay. This material was reduced and carboxymethylated, and the three fractions [intact molecules (1–186), fragment 1–89, and fragment 90–186] were separated on a 1.5 × 23 cm DEAE-Sephadex A-25 column at pH 8.8 with a 0.1–0.6 M NaCl gradient. pH 8.8 was maintained by 0.025 M Tris–0.025 M glycine buffer. The amino acid analysis of the two fragments as well as their extensive sequencing (see legend to Figure 1) showed conclusively that the split peptide bond in OMCHI* is Arg^{89}–Ala. OMCHI* was bound to an insoluble trypsin–Sepharose column at neutral pH. Elution by a sudden pH drop to pH 2 yielded only OMCHI (100 ± 2%) and no OMCHI*, thus proving that the trypsin complexes of OMCHI and of OMCHI* are the same substance.

Equilibrium between Virgin and Modified Inhibitor (K_{hyd}). OMCHI or OMCHI* was incubated with 2 mol % bovine β-trypsin at various pH values, aliquots were withdrawn, and α, the fraction of modified inhibitor, was determined at each point by the maleylation assay (see Materials and Methods). This gave at each pH value a set of curves such as in Figure 3A. As is seen, these reach a common asymptote arising from the achievement of virgin to modified inhibitor equilibrium. From such data at each pH, the value of the reactive site peptide bond hydrolysis equilibrium constant was calculated with the equation

$$K_{hyd} = [\text{OMCHI*}]/[\text{OMCHI}] = \alpha_{eq}/(1 - \alpha_{eq}) \quad (1)$$

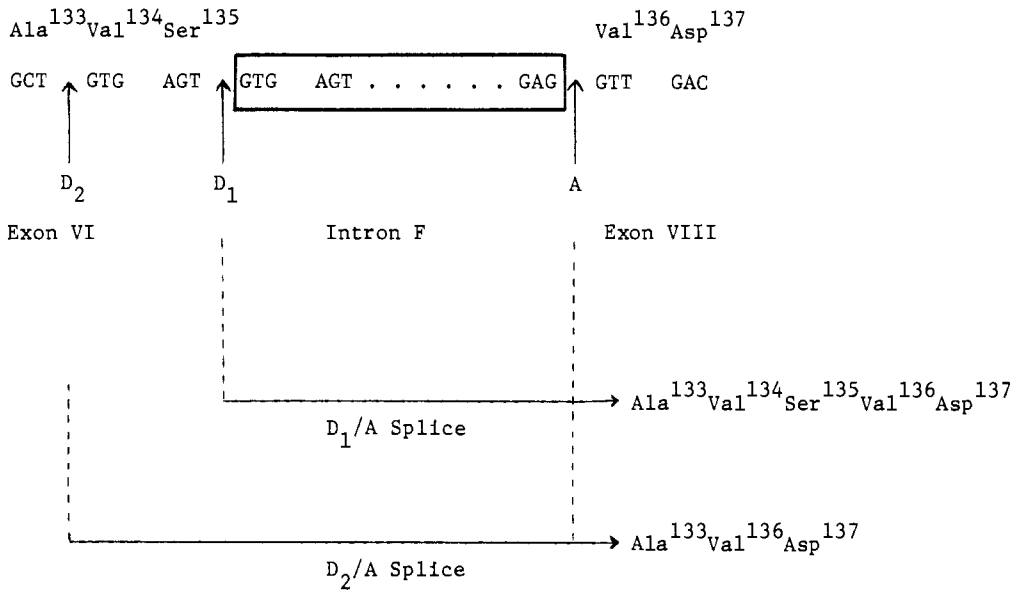

FIGURE 2: Probable mechanism for deletion polymorphism observed in this work. Adopted from Stein et al. (1980) essentially without modification. The nucleotide sequence of the chicken ovomucoid gene is shown with only the extreme 5′ and 3′ ends of the intron listed (within the box). The amino acids coded for by the exon sequences are listed on the top line. D_1 represents the predicted donor site and A the acceptor site. D_2 is a potential alternate donor site, whose usage in the splicing reaction would yield the polypeptide sequence Ala[133]-Val[136]-Asp[137] instead of the major sequence Ala[133]-Val[134]-Ser[135]-Val[136]-Asp[137] (Stein et al., 1980).

where α_{eq} is the fraction of OMCHI* at equilibrium. The values at different pHs are given in Figure 3B. It is seen there that the data can be fitted very well to the Dobry, Fruton, and Sturtevant (Dobry et al., 1952) equation for the simplest possible pH dependence on K_{hyd}:

$$K_{hyd} = K^0_{hyd}(1 + [H^+]/K_1 + K_2/[H^+]) \qquad (2)$$

with the parameters given in the legend to Figure 3B. In the simplest possible interpretation, pK_1 is the pK of the carboxyl group of Arg[89] (P_1 residue) and pK_2 is the pK of the alkylammonium group of Ala[90] in OMCHI*. The numerical values of K_{hyd}, pK_1, and pK_2 obtained here are quite interesting. However, we defer their detailed analysis to the publication of a large set of K_{hyd} values for Kazal inhibitors based primarily on measurements (Ardelt & Laskowski, 1984) on 45 of the 106 sequences of ovomucoid third domains reported in the following paper (Laskowski et al., 1986).

Isolation of Ovomucoid First Domain. Enzymatic hydrolysis of the connecting peptide between the first and second domain has not in our hands provided a useful method of preparation of the intact first domain of chicken or of any other avian ovomucoid because of extensive enzymatic nicking of the first domains. Therefore, we took recourse in CNBr cleavage at Met[68], a method developed independently by Beeley (1976a) and in our laboratory. Figure 4 shows the separation of the intact fragment 1–68 (68 is a Hse residue), whose amino acid analysis and sequence are in complete accord with the NH_2-terminal sequence of the entire OMCHI. The third peak in Figure 4A consists of domains 2 and 3, but it is internally nicked by the CNBr cleavage at the Met[84]–Val bond.

It should be pointed out that this procedure for obtaining the first domain is not general for ovomucoids from all avian species since the presence of Met[68] is not general in all species. Even having Met[68] present is not sufficient, since some ovomucoids with Met[68] also have one or more Met residues within the disulfide cluster of the first domain and, thus, have their first domains nicked by CNBr treatment. A more general procedure for first domain preparations is clearly needed, but thus far we have not been able to develop it.

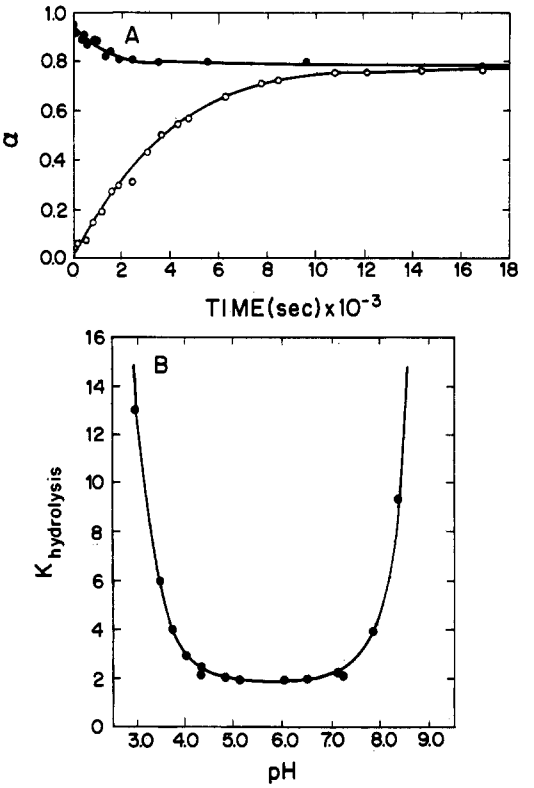

FIGURE 3: Hydrolysis and resynthesis of the Arg[89]–Ala peptide bond in entire chicken ovomucoid. (A) Chicken ovomucoid, OMCHI (○), or its modified form, OMCHI* (●) (final concentrations of 4×10^{-4} M), were incubated at pH 3.75 in 0.05 M $CaCl_2$ and 0.50 M NaCl at 20 °C for times indicated on the x axis with 2 mol % of bovine β-trypsin. A fraction of OMCHI* (α) was then determined in each incubation mixture. The value of α at the equilibrium (α_{eq}) was used in the eq 1 to get $K_{hyd} = 3.8 \pm 0.5$ at pH 3.75. (B) Experiment described in panel A was repeated except that the pH of incubation was varied. The K_{hyd} values found are given as a function of pH. The curve is the result of nonlinear least-squares fitting on the points to eq 2. This yields $K^0_{hyd} = 1.85$, $pK_1 = 3.82$, and $pK_2 = 7.77$.

Enzymatic Hydrolysis of Chicken Ovomucoid by Spase V8. The general objective of enzymatic digestions employed in this work is the hydrolysis of peptide bonds in the connecting

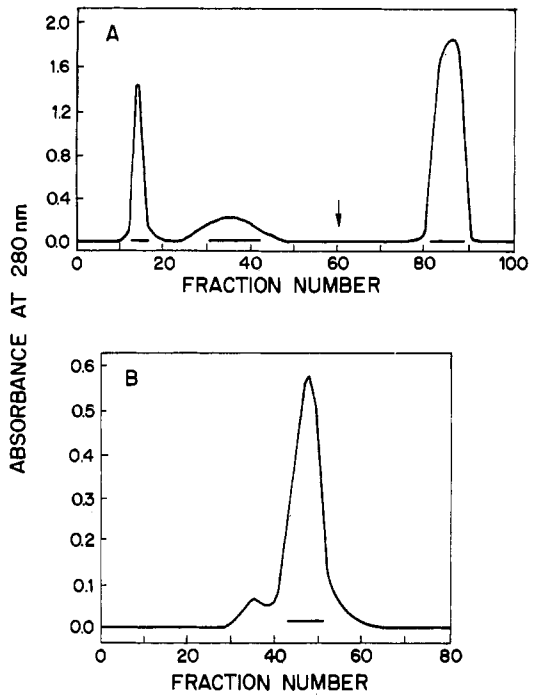

FIGURE 4: Cleavage of chicken ovomucoid by cyanogen bromide and isolation of first domain. (A) A total of 0.9 g of purified OMCHI was treated with 0.2 g of CNBr in 70% formic acid under nitrogen, at room temperature for 12 h. The resultant mixture was dialyzed against water and freeze-dried. The sample was redissolved in 0.02 M sodium citrate (pH 4.5) and applied to a CM-Sepharose column (2.6 × 34 cm) equilibrated with the same buffer. The column was developed with the equilibrating buffer and then (↓) with 0.5 M NaCl in the buffer. Fractions of 7.5 mL were collected at a flow rate of 15 mL/h. Pooled fractions are indicated by solid bars. The first peak is crude first domain (147 mg), the second entire ovomucoid (98 mg), and the third (379 mg) second domain and third domain nicked at Met^{84}-Val. (B) A total of 70 mg of the first peak of panel A was chromatographed (15 mL/h, 3-mL fractions) on a Bio-Gel P-10 (200–400-mesh) column (2.5 × 58 cm) in 5% formic acid. The pooled fractions are indicated by a solid bar.

peptides (residues 63–69 and residues 128–137) while leaving all other peptide bonds intact. This is quite difficult to accomplish, and in various attempts, we have encountered two types of failure: (a) failure to cut the connecting peptide and (b) nicking of the domains. We have succeeded in cutting the peptide bonds in the second connecting peptide with three different enzymes: pepsin (Kato et al., 1976), thermolysin (Laskowski et al., 1986), and Spase V8. Of these, Spase V8, while most expensive and most capricious, has the advantage of splitting only the Glu^{130}–Leu bond in this region. The other two enzymes split several bonds, and the isolated third domains have jagged ends. The most important advantage of Spase V8 is that it is also effective in cutting of the Glu^{64}-Thr bond in the first connecting peptide. Unfortunately, the Spase V8 digestion destroys the first domain by extensive internal nicking. The success of Spase V8 digestion is shown in Figure 5. This yields material for isolation of the second domain and of both the carbohydrate-containing and the carbohydrate-free third domains. If only the third domain is desired, the Spase V8 digestion should be shorter as the second domain appears in the digest more slowly than the third. As the Spase digestion is not highly reproducible, it is very helpful to monitor the digestion by analytical chromatography prior to applying the whole sample on a preparative column.

Isolation of Second Domain. The second peak on Figure 5, which corresponds to the second domain and to some other digestion products, is repurified by repeating the P-10 gel filtration under the same conditions as before. This material

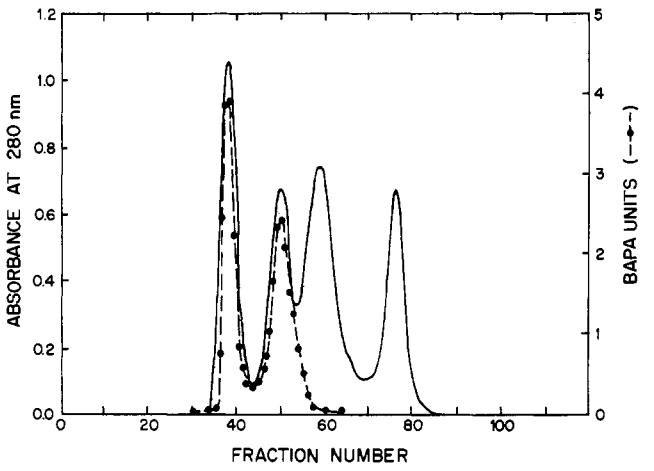

FIGURE 5: Gel-exclusion chromatography of Spase V8 digest of chicken ovomucoid. A total of 450 mg of purified OMCHI was dissolved in 10 mL of 0.05 M Tris-HCl buffer at pH 8.6. A total of 5 mg of Spase V8 (Miles) was added, and the mixture was incubated at room temperature for 44 h. A total of 3.5 mL of this digest was applied to Bio-Rad P-10 (200–400-mesh) column (2.6 × 98 cm). This column was equilibrated and developed with 5% formic acid at a flow rate of 25 mL/h. Three-milliliter fractions were collected. The solid curve represents absorbance at 280 nm and the points and dashed curve inhibitory activity against trypsin measured in arbitrary benzoyl-arginine-p-nitroanilide (BAPA) units. The peaks represent a rather complex mixture, but the predominant components are, from left and right, OMCHI (entire ovomucoid), OMCHI2, OMCHI3(+), and OMCHI3(−). Note that the gel-exclusion separation of OMCHI2 and of OMCHI3(+) is probably better for chicken than for many other avian ovomucoids since chicken second domain is exceptional in having two glycosylation sites. Most avian ovomucoids have Asp^{69} and thus are not glycosylated at this position.

is then subjected to DEAE-Sepharose CL-6B chromatography (Figure 6A) to yield purified second domain. It is a strong trypsin inhibitor. Its amino acid analysis and sequence agree exactly with that expected for residues 65–130.

Isolation of Carbohydrate-Free Third Domain, OMCHI3-(−). In most of our studies this is regarded as a more desirable material than OMCHI3(+) because it is easier to characterize by analytical ion-exchange chromatography (Ardelt & Laskowski, 1982) or by HPLC (Lin et al., 1985).

The fourth peak of Figure 5 is rechromatographed on Bio-Gel P-10 and then purified on DEAE-Sepharose. The results are given in Figure 6B. The trailing peaks after the main peak contain OMCHI3(−) in which one or more Glu–X peptide bonds have been hydrolyzed. The amino acid analysis and sequence of the main peak material correspond to residues 131–186 with the exception that in the majority of preparations the Val^{134}-Ser^{135} deletion is seen at about the 20% level.

Isolation of OMCHI3(+). This is obtained by rechromatography of the third peak of Figure 5 by methods described above. It differs from OMCHI3(−) on gel-exclusion HPLC where it elutes much before OMCHI3(−), in amino acid analysis where its hydrolysates are brown and show glucosamine, and in sequencing because there is no detectable signal at residue 175 (glycosylated Asn). However, the missing Asx is evident in amino acid analysis.

DISCUSSION

Comparison of Sequence (Figure 1) with Other Data. The NH_2-terminal residue of chicken ovomucoid was correctly determined by Fraenkel-Conrat and Porter (1952) as Ala. Numerous investigators attempted to determine the COOH-terminal residue in the 1950s and 1960s but, presumably because it is Cys, met with little success. Murthy et al. (1973) published the sequence of the first 40 residues from the NH_2

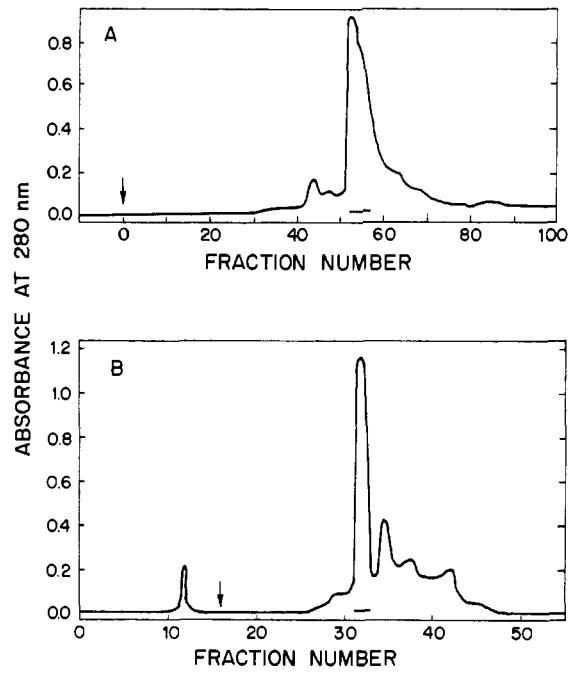

FIGURE 6: Purification of chicken ovomucoid second domain (A) and of third domain, unglycosylated (B), by anion-exchange chromatography. (A) Crude second domain (second peak in Figure 5) was rechromatographed on a Bio-Gel P-10 column under the conditions given in Figure 5, and 350 mg of the resulting preparation was applied to a DEAE-Sepharose CL-6B column (2.5 × 35 cm) in 0.05 M Tris-HCl buffer, pH 8.7. The column was washed with 1 L of the equilibrating buffer and then (↓) developed (20 mL/h, 7.5-mL fractions) with a linear sodium chloride gradient (0–0.5 M, total volume 1.4 L) in the buffer. The solid bar indicates the fractions pooled. (B) Crude third domain, unglycosylated (fourth peak in Figure 5), was charged on a DEAE-Sepharose CL-6B column (1.6 × 25 cm) in 0.05 M Tris-HCl buffer, pH 9.1. The column was developed (15 mL/h, 7.5-mL fractions) with the equilibrating buffer and then (↓) with a linear sodium chloride gradient (0–0.5 M, total volume 700 mL) in the buffer. The solid bar indicates the fractions saved.

terminus. They were generally correct, but a few residues were unidentified, and a few were identified incorrectly.

A good deal of work relevant to the chicken ovomucoid sequence was done by Beeley. In his first paper (Beeley, 1976a), he summarized his work on the location of the two Met residues in chicken ovomucoid. Not only did he locate Met^{68} and Met^{84} approximately correctly, but he was also able by CNBr cleavage at Met^{68} to prepare the first domain by the same method as we are employing here. In his second paper (Beeley, 1976b), he describes the isolation of the five glycopeptides of chicken ovomucoid. He provides sequences for the first four of them (residues 8–17, 45–56, 64–70, and 71–82). All of them are in complete accord with our sequence. The fifth glycopeptide was not sequenced because of low yield, but its amino acid composition corresponds to residues 169–185, and the low yield is consistent with partial glycosylation at the Asn^{175} site.

Thibideau et al. (1978), incidental to their work on the signal peptide of preovomucoid (Figure 1), sequenced the first 20 residues of the mature protein in complete accord with the data presented here. In the same year, we published an entire chicken ovomucoid sequence (Kato et al., 1978). It was labeled preliminary and it differs from the present results at Asn^{38} → Thr, Glu^{40} → Asp, and Thr^{84} → Met (the correct residue is given last). The last of these errors was typographical, raising the great fear [see also the following paper (Laskowski et al., 1986)] that, in large and careful sequence data sets, typographical errors may become major. In 1979 and early 1980, the sequence was totally reexamined, and the current correct sequence was sent to the O'Malley laboratory. Catterall et al. (1980) independently determined the entire sequence of chicken ovomucoid cDNA. To quote them, "The two sequences agree exactly lending credence to the accuracy of each."

Glycosylation [See Beeley (1976b)]. The chicken ovomucoid sequence has six Asn-X-Thr/Ser sequences. Of these the Asn^{158}-Lys-Thr sequence is not glycosylated; it is also not glycosylated in many other avian ovomucoid third domains where it occurs (Laskowski et al., 1986). The glycosylation sites at Asn^{10}-Ala-Thr and at Asn^{77}-Thr-Thr are at homologous positions in the first and second domains (see Figures 1 and 7). There is no corresponding sequence in the third domain. These sequences are very strongly, but not absolutely, conserved within the large set of avian ovomucoid sequences we have [Kato et al. (1978) and Kato, Kohr, and Laskowski (unpublished results)]. Both of these sites are probably glycosylated in a very large majority, but not all, of the molecules. There is an additional glycosylation site in the first domain, Asn^{53}-Ile-Ser. It is strongly conserved in other avian first domains, but it has no counterpart in the second domain or the third domain (the third domain glycosylation site misses

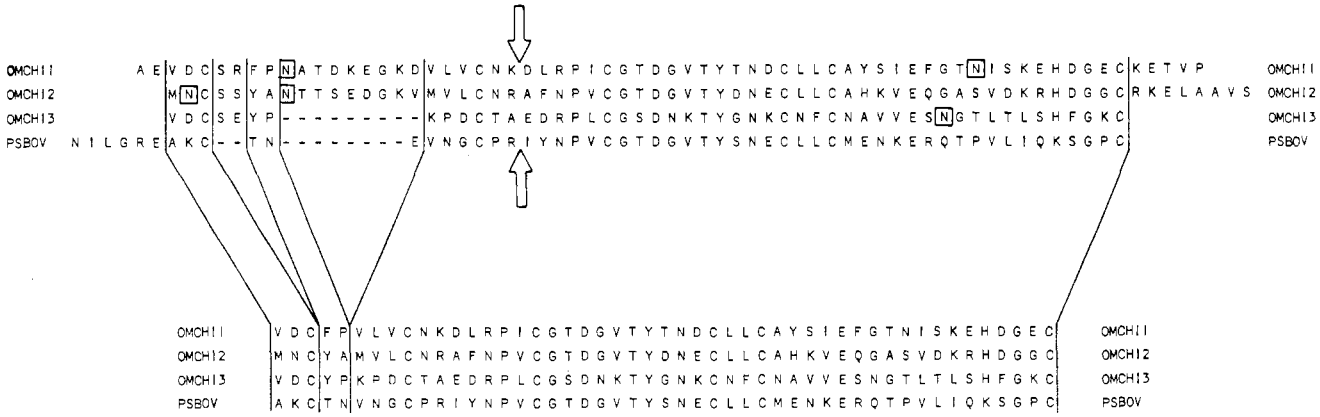

FIGURE 7: (Top) Alignment of the three domains of chicken ovomucoid with each other and with bovine pancreatic secretory trypsin inhibitor (Kazal) (PSBOV) whose sequence was determined by Greene and Bartelt (1969). This particular inhibitor is chosen here to represent the family as its isolation by Kazal et al. (1948) gave the family its name. The arrows mark the reactive sites. Residues in boxes are glycosylation sites. Two types of arbitrary decisions were involved in making the alignment. The first—deciding where domains start and end—is extensively discussed in the text. The second—the placement of the single deletion in OMCHI3 and of two deletions in PSBOV—is somewhat arbitrary, but it is based not just on the sequences shown but on our file of 250 Kazal family sequences. (Bottom) An indication of which residues were eliminated prior to obtaining the difference matrix (Table I).

alignment by only two positions) (see Figure 7). The additional glycosylation site in the second domain, Asn^{69}-Cys-Ser, is unusual. We have seen it thus far only in chicken (*Gallus gallus*) and Ceylon junglefowl (*Gallus lafayettii*). Other avian second domains have Asp^{69}-Cys-Ser homologous to Asp^{4}-Cys-Ser in the first domain and Asp^{137}-Cys-Ser in the third domains. Finally, there is Asn^{175}-Gly-Thr. This is a very strongly conserved sequence in avian ovomucoid third domains. In chicken it is partially glycosylated. Since the OMCHI3(–) sequence shows Asn^{175} and not Asp^{175}, glycosylation never took place; had carbohydrate been put on and later removed, we would expect Asp^{175}. While we do not deal with the nature of the CHO side chains, numerous papers on the composition and structure of the carbohydrate side chains of chicken ovomucoid are in the literature [e.g., Yamashita et al. (1984) and Parente et al. (1984)].

Disulfide Bridges. The ability to obtain the three intact domains by enzymatic hydrolysis or by cyanogen bromide cleavage shows only that the nine disulfide bridges occur in three intradomain clusters of three each. The pairing within the domains is assigned by homology to the known pairing in pancreatic secretory inhibitors. The known three-dimensional structures of OMTKY3 (three residues differ from OMCHI3) (Read et al., 1983), OMSVP3 (three residues differ) (Bode et al., 1985), and OMJPQ3G (seven residues differ) and OMJPQ3S (eight residues differ) (Papamokos et al., 1982) confirm the assignment. All other chemical data on specifically hydrolyzed domains are consistent with the proposed assignment.

Recognition of Domains. All of the results in this paper show that the chicken ovomucoid molecule is composed of three tandem homologous domains. The word domain has a variety of meanings in protein chemistry. In this case, it represents virtually separate, native protein molecules. This view is supported by the various properties of the domains. These are discussed under Structure, Inhibitory Activity, and Homology.

(*A*) *Structure.* Not only is it possible to divide the chicken ovomucoid molecule into three intact domains, but each of the domains is a native protein. This is shown most dramatically by the crystallization and determination of three-dimensional structure at high resolution of several ovomucoid third domains and of their enzyme–inhibitor complexes. In spite of several attempts, neither the first, second, nor third domain with carbohydrate has been crystallized as yet. This is probably only a reflection of the relatively greater difficulty of crystallization of glycoproteins and not a comment on their nonnative state.

Ogino et al. (1982) did extensive nuclear magnetic resonance studies on the third domains isolated by the methods described here. They found that all of them behaved as rigid, native, globular proteins and not as random structures or denatured polypeptides.

Waheed et al. (1977) and Baig and Sallahudin (1978) reported that urea and guanidine hydrochloride denaturation of chicken ovomucoid are multistate, not two state, and interpreted the results in terms of separate denaturation of domains. There is a difficulty in their work: they find only two transitions rather than the expected three. This is because the denaturation of the most stable third domain does not occur at the highest denaturant concentrations reached in the Sallahudin work (Otlewski and Laskowski, unpublished results).

The earlier announcement (Kato et al., 1976, 1978) that first and third domains can be prepared as separate entities led a number of workers to examine the denaturation behavior of the separated domains and to compare it to entire chicken ovomucoid (Matsuda et al., 1981a,b, 1982; Privalov, 1982; Griko & Privalov, 1984). From these studies it can be concluded that each domain undergoes a major denaturation transition and that the denaturation of entire ovomucoid can be described as the sum of independent (or almost independent) transitions of all three domains.

Donovan (1967) showed that entire chicken ovomucoid undergoes a low-pH transition that strongly affects the UV absorbance and fluorescence of several of its tyrosyl residues. March (1980), working with the set of chicken ovomucoid domains described in this paper, found that each of them undergoes a similar transition and that the entire ovomucoid transition of Donovan (1967) is a sum of these three intradomain transitions. Thus, we emerge with a picture of ovomucoid being composed of three tandem domains, each of which is a native globular protein.

(*B*) *Inhibitory Activity.* Avian ovomucoids are serine proteinase inhibitors. A strong three-domain model suggests that all three domains should be separately inhibitory.

However, chicken ovomucoid was reported to be single headed for bovine β-trypsin by Rhodes et al. (1960). We agree. In this paper, we report that the single reactive site is at Arg^{89}-Ala of the second domain and that the entire ovomucoid and the isolated second domain are essentially equally inhibitory. Indeed, the K_a values for the entire protein and for the second domain are about equal and $\simeq 1 \times 10^{10}$ M^{-1}. In contrast, neither of the isolated first or third domains is effective as an inhibitor of bovine β-trypsin.

The failure of the third domain to inhibit bovine β-trypsin is easy to rationalize in view of its Ala^{148}-Glu reactive site (see Figure 1). On the other hand, the failure of the first domain to serve as a bovine trypsin inhibitor either in intact ovomucoid or as an isolated domain is surprising in view of its Lys^{24}-Asp reactive site. It is also quite surprising that we did not detect facile cleavage of this Lys^{24}–Asp bond by bovine trypsin, at least under the conditions where we have tested it. However, the first domain is not "dead". Nagata and Yoshida (1984) showed that it inhibits a trypsin-like enzyme from *Streptomyces erythraeus* at the Lys^{24}-Asp reactive site but that the K_a for this inhibition is only 3×10^5 M^{-1}. Oe. Schoenberger (unpublished results) showed that both the first domain and the entire chicken ovomucoid are single-headed inhibitors for endoproteinase Lys-C from *Lysobacter enzymogens* with a K_a of $\simeq 1 \times 10^8$ M^{-1}. The second domain with the Arg^{89}-Ala reactive site is not an effective inhibitor of endoproteinase Lys-C. This might be expected as the enzyme is specific for P_1 Lys but not Arg.

Entire chicken ovomucoid is not known to be an efficient inhibitor of any serine proteinase with chymotrypsin–elastase-type specificity. Were such inhibition to occur, it would be anticipated at the Ala^{148}-Glu reactive site of the third domain. Isolated chicken third domain was extensively studied by Park (1985) as an inhibitor of bovine α-chymotrypsin, porcine elastase, subtilisin Carlsberg, and *Streptomyces griseus* proteinases A and B. All of them are inhibited, but the K_a values are low, ranging from 1.9×10^3 M^{-1} for chymotrypsin to 3×10^6 M^{-1} for *S. griseus* proteinase B.

Thus, each of the three isolated domains in chicken ovomucoid is active as an inhibitor. However, while the second domain is a strong inhibitor of bovine β-trypsin, the K_a values for the first and for the third domain are low. Whether the low values are a reflection of the fact that only inappropriate proteinases were tried and a proper one would be strongly inhibited or whether the third domain of chicken ovomucoid

Table I: Difference Matrix Showing in the Lower Left the Amino Acid Differences and in the Upper Right the Minimal Base Changes Required To Convert One of the Sequences into the Other[a]

	OMCHI1	OMCHI2	OMCHI3	PSBOV
OMCHI1	0	31	46	44
OMCHI2	24	0	49	41
OMCHI3	30	32	0	55
PSBOV	28	25	35	0

[a] Only those regions of the sequence where all proteins have actual residues are compared. The "reduced" 49-residue sequences are given in the lower part of Figure 7.

is inherently a poor inhibitor is difficult to decide.

(*C*) *Homology*. The alignment in Figure 7 leaves little doubt that the three domains of chicken ovomucoid are homologous to one another and also to other inhibitors in the Kazal family (see also Table I). The Kazal family has many members. They include the single-domain pancreatic secretory trypsin inhibitors and acrosin inhibitors from seminal plasma, the two-domain dog submandibular inhibitor, the three-domain avian ovomucoids, the four-domain alligator ovomucoid, and the seven-domain avian ovoinhibitors. The sequences of these inhibitors and their evolution are discussed in more detail in Laskowski et al. (1980). Strikingly, the homology is not limited to the sequence but also to intron placement (see Figure 1). There is an intron at the start of each domain: between residues 2 and 3 for the first, between residues 67 and 68 for the second, and between residues 135 and 136 for the third domains, respectively. In the last case, because of ambiguous intron excision for the minor fraction of the molecules (with Val^{134}-Ser deleted), the boundary is between residues 133 and 136. Furthermore, within each domain there is an intron splitting an internal codon and again in a homologous position in each case. These are at residues 48, 123, and 172 (Stein et al., 1980). Both the homology in sequence and the homology of intron placement clearly point to the construction of the ovomucoid gene by two consecutive gene elongations by duplication.

The striking conservation of introns solves for us an otherwise pesky problem. From a protein chemist's view, Figure 1 certainly makes OMCHI look as if it is composed of three domains (the disulfide bridge cluster), a leader peptide (residues 1–4), and two connecting peptides [63–69 and 128–137 (in the minor form 134–135 is deleted)]. How should we allocate these peptides between domains in alignments such as Figure 7 or in talking about domains? After consultation, Stein et al. (1980) and we decided to define domains as products of two consecutive exons starting with the third exon of preovomucoid. By this definition they are as follows: the first domain, residues 3–67; the second domain, residues 68–135; the third domain, residues 136–186. This leaves the first two NH_2-terminal residues of mature ovomucoid unassigned; they are encoded by the second of the signal peptide exons.

While this definition is very convenient for alignments such as Figure 7 and for storing the sequences in computer files, it causes a certain problem. In laboratory slang we call the 1–68 fragment, whose isolation by CNBr cleavage we describe, OMCHI1 (the first domain), yet by our definition it is really Ala^{1}-Glu^{2}-OMCHI1-Hse^{68}. Similarly, the Spase V8 generated OMCHI2 is the 65–130 fragment, yet by the definition above, it is Thr^{65}-Val^{66}-Pro^{67}-des-(Leu^{131}-Ala^{132}-Ala^{133}-Val^{134}-Ser^{135})-OMCHI2. The enzymatically isolated third domains are either Leu^{131}-Ala^{132}-Ala^{133}-Val^{134}-Ser^{135}-OMCHI3 if they were produced by Spase V8 hydrolysis or (predominantly) Val^{134}-Ser^{135}-OMCHI3 if they were produced by thermolysin or pepsin hydrolysis. Such subtleties matter little for those who use the domains for inhibitory activity studies, where the pieces of connecting peptide appear to have no effect on activity, but a great deal to those who study spectroscopic (especially NMR) or chromatographic behavior. In such studies all the residues must be accounted for, and the nomenclature must necessarily be complex.

Returning to Table I, we note that the first and second domains are far more homologous to one another than either of them is to the third. The third also differs from the first two by the large deletion (Figure 7). The special kinship of the first two domains becomes even clearer when the nucleotide difference matrix is considered [Table III of Catterall et al. (1980)]. On the basis of similar close kinship of domains 1 and 2 seen in our preliminary sequence of Japanese quail ovomucoid, we postulated (Kato et al., 1976) that the two gene duplication events giving rise to the three-domain avian ovomucoid gene were widely different in time. First, a two-domain ancestor composed of the present second and third domains was formed. Over its long existence, its two domains diverged considerably from one another. More recently (but before the last common ancestor of modern birds), the NH_2-terminal domain reduplicated to give rise to the modern three-domain ovomucoid. This view gained additional support from the discovery of many other multidomain Kazal inhibitors. In all of these, the COOH-terminal domains are exceptionally strongly homologous to one another; e.g., the seventh (COOH-terminal) domain of ovoinhibitor is more strongly homologous to ovomucoid third domains than to any of its own domains. This confirms the long existance of a two-domain ancestor which then reduplicated in the NH_2-terminal direction (Laskowski et al., 1980).

Acknowledgments

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Registry No. Ovomucoid (chicken protein moiety reduced), 76296-67-8; β-trypsin, 9002-07-7.

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