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A Ser^{162}/Gly^{162} Polymorphism in Japanese Quail Ovomucoid*

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Japanese quail ovomucoid exists in two polymorphic forms. One has serine, the other glycine at position 162. The tryptic peptide corresponding to positions 160 to 164 was purified from ovomucoids isolated from egg whites of eggs laid by 11 different hens and subjected to amino acid analysis. The quantitative distribution of serine and glycine in this pentapeptide is consistent with the interpretation that the ovomucoid gene exists in two codominant allelic forms at one locus. Even though the gene product is apparently expressed only in the female, these results indicate that the ovomucoid structural gene is transmitted as a simple Mendelian character which is neither sex-linked nor shows dominance.

Intact third domains (positions 131 to 186) isolated from the two allelic forms of ovomucoid interact with bovine β-trypsin in a similar but not identical manner; the complex with the glycine form dissociates more rapidly. Evidence is presented which suggests that glycine is the ancestral residue at position 162; yet, the serine form is the more frequent phenotype.

Ovomucoids are glycoproteins present in avian egg white; they are strong inhibitors of serine proteinases. Extensive studies on inhibitory specificity (Rhodes *et al.*, 1960; Feeney, 1971), primary structure (Kato *et al.*, 1976; 1978), and mechanism of inhibition (Finkenstadt & Laskowski, 1965; Ozawa & Laskowski, 1966; Schrode, 1974) led to the following conclusions.

1. Ovomucoids inhibit serine proteinases according to the standard mechanism which has been reviewed (Laskowski & Kato, 1980).

2. Ovomucoids consist of three tandem domains. Each domain is homologous to single domain pancreatic secretory trypsin inhibitors (Kazal). Each domain contains an actual or putative reactive site, which serves as an especially specific substrate for the cognate enzyme.

3. The sequences of ovomucoids from various birds are rather similar, but the amino acid residue contributing the carbonyl to the reactive site peptide bond is an exception. There are frequent changes in this position even among closely related species. These changes account for dramatic changes in specificity between ovomucoids from closely related species.

These observations make ovomucoids especially interesting for those concerned with protein evolution and therefore raise interest in its mode of inheritance. Such interest is heightened further by recent observations made by molecular biologists. A signal peptide was detected and sequenced in chicken ovomucoid (Thibodeau *et al.*, 1978) and the chicken ovomucoid gene was shown to contain at least seven introns (Catterall *et al.*, 1979).

During the primary structure determination of Japanese quail ovomucoid (Kato *et al.*, 1976; 1978), it was noted by one of us that position 162 was probably polymorphic for the amino acid residues serine and glycine. This afforded an experimentally simple opportunity to examine whether the ovomucoid structural gene is transmitted as a sex-linked or simple Mendelian character or if it is transmitted in some more complex manner by analyzing the distribution of serine and glycine in position 162 in ovomucoids isolated from individual hens.

EXPERIMENTAL PROCEDURES

Materials—Bovine trypsin (TRL37A870) and TPCK trypsin[1] (37C775) were purchased from Worthington Biochemical Corp. Subtilisin Carlsberg (48C-0164), soybean trypsin inhibitor (Kunitz) (93C-8140), N^{α}-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride, dithioerythritol, and iodoacetic acid were all obtained through Sigma. Vega Biochemical was the supplier of N^{α}-benzoyl-L-valyl-glycyl-L-arginine-*p*-nitroanilide hydrochloride. Proteinase from *Staphylococcus aureus* V8 was a generous gift from Gabriel Drapeau. Sepharose 4B and PD-10 Sephadex G-25 med columns were products of Pharmacia Fine Chemicals, while Bio-Gel P-10 (200 to 400 mesh) was obtained from Bio-Rad. Ultrapure guanidine hydrochloride was purchased from Schwarz/Mann. Constant boiling hydrochloric acid and ninhydrin were Pierce Sequanal grade. Reagent grade pyridine was further purified by distillation from ninhydrin (Hill & Delaney, 1967) and stored at 4°C. Trace amounts of water in reagent grade dimethyl sulfoxide and dimethylformamide were removed by the addition of molecular sieve beads (Matheson, Coleman and Bell, grade 512 type 4A, 4 to 8 mesh) which had been activated at 260°C for 15 h. The fluorescent burst titrant 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson *et al.*, 1973) was synthesized by Peter Fankhauser of this laboratory by a method similar to that given by Chase and Shaw (1967) for *p*-nitrophenyl-*p'*-guanidinobenzoate, while the *p*-nitrophenyl-*p'*-guanidinobenzoate itself was obtained from Nutritional Biochemicals. All other chemicals were reagent grade or the best commercially available.

General Methods—The molarity of trypsin active sites was determined using the spectrophotometric burst titrant *p*-nitrophenyl-*p'*-guanidinobenzoate (Chase & Shaw, 1967). The procedure of Fairbanks *et al.* (1971) with slight modification was employed for sodium dodecyl sulfate polyacrylamide gel electrophoresis. Soybean trypsin inhibitor (Kunitz) was coupled to Sepharose by standard procedures (March *et al.*, 1974). All water was distilled, charcoal-filtered, and deionized with a final specific resistance greater than 10^6 ohms. Spectrophotometric determinations were obtained in a Cary 118 spectrophotometer. Dialysis was accomplished in Spectrum's Spectrophor 2 tubing prepared by the manufacturer's instructions. Molecular weights for bovine trypsin, Japanese quail ovomucoid, and Japanese quail ovomucoid carbohydrate-free third domain of 24,000, 28,000, and 6,000, respectively, were used. All experiments were performed at 21 ± 1°C, unless otherwise indicated.

Eggs—Sets of 9 or 10 freshly laid Japanese quail (*Coturnix cotur-*

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[1] The abbreviation used is: TPCK trypsin, trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

nix japonica) eggs collected from each of 11 different hens were purchased from Marsh Farms, Garden Grove, Calif. The egg whites were carefully separated from the yolks and stored individually at −20°C. Additionally, a lot of 100 randomly laid Japanese quail eggs were obtained from the same source. Eighteen of these were treated in a similar manner to the nonrandom eggs, while the other 82 egg whites were pooled before freezing.

Purification of Ovomucoid—The purification procedure is a scaled down version of a method developed in our laboratory[2] which utilizes a modification of the precipitation purification of Lineweaver and Murray (1947) as a first step.

The thawed egg white from an individual egg (average volume 5.5 ml) was slowly diluted with 2 volumes of freshly prepared precipitation solution (1 volume of 0.5 M trichloroacetic acid and 2 volumes of acetone) and stirred overnight (4 to 12 h) at 4°C. After centrifugation at 3000 × *g* for 30 min, the supernatant was diluted with 2 volumes of cold acetone. The precipitate was collected by centrifugation at 3000 × *g* for 20 min and dissolved in a small volume of water. The solution was extensively dialyzed against water and lyophilized. This product is termed crude ovomucoid.

The lyophilized material was dissolved in 5% (v/v) formic acid, centrifuged to remove insoluble matter, and then chromatographed on a Bio-Gel P-10 column equilibrated with 5% (v/v) formic acid. The protein content of each fraction of the effluent was determined by measuring the absorbance at 280 nm (Fig. 1). Ovomucoid was located as those fractions which inhibited trypsin but not subtilisin (Feeney, 1971); the large molecular weight component which inhibits both trypsin and subtilisin was shown to be ovoinhibitor. The appropriate fractions were pooled, diluted with 2 volumes of water, and lyophilized.

One egg from each hen was processed in a similar manner. Sometimes the acetone precipitate was dissolved in 5% (v/v) formic acid and applied directly to the column without dialysis; this led to no appreciable change in the elution profile. The yield of ovomucoid was approximately 30 mg from each Japanese quail egg; variations in yield were consistent with the mass of the egg and the volume of the egg white. The entire pooled egg white sample was processed through the precipitation purification step; then, 30 mg of the lyophilized crude ovomucoid were treated as another sample.

Assay of Enzymatic Inhibitory Activities of Column Effluents—Trypsin assays were performed by the spectrophotometric method of Erlanger *et al.* (1961) with the following modifications for the assay of inhibitors. Small aliquots of enzyme or enzyme and inhibitor were added to 3.00 ml of 0.05 M Tris, 0.02 M calcium chloride, pH 8.2. After an incubation time of 5 ± 1 min, 30 μl of 0.1 M N^{α}-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride in dimethyl sulfoxide were added and the rate of increase in absorbance at 410 nm due to the release of *p*-nitroaniline was monitored.

Subtilisin inhibitor activity was monitored in a similar manner using 6 μl of the stock substrate 0.085 M N^{α}-benzoyl-L-valyl-glycyl-L-arginine-*p*-nitroanilide in dimethyl sulfoxide. In both cases, inhibitor activity is defined as the difference between the rate of *p*-nitroaniline production for an enzyme blank and an enzyme-inhibitor sample. The units are nanomoles of *p*-nitroaniline/min for a defined aliquot of inhibitor sample.

Preparation of Tryptic Peptide 160 to 164—Approximately 7 mg of ovomucoid from each hen were reduced in a manner similar to that reported by Konigsberg (1972). The reduction of the disulfide bonds was allowed to proceed in 1 ml of 6 M guanidine hydrochloride, 0.5 M Tris, 0.002 M EDTA, pH 8.2, at 50°C under nitrogen with a 25-fold molar excess of dithioerythritol to cystine. After 4 h, the temperature was lowered to 25°C and a 5% molar excess of iodoacetic acid over dithioerythritol was added and allowed to react in the dark for 15 min. The reaction was quenched by desalting on Sephadex G-25 med (Pharmacia PD-10 prepacked column) using 0.05 M ammonium bicarbonate as the eluate. The samples were digested with TPCK trypsin (1% by weight) for 16 h at room temperature and then dried under a stream of nitrogen at 37°C.

Electrophoresis of each peptide mixture which had been applied as a 12-cm streak in the center of Whatman No. 1MM paper (200 × 400 mm) was carried out in pH 6.5, pyridine/acetic acid/water buffer (40: 2:450; v/v/v) at 5000 V for 20 to 25 min in a flatbed Camag HVE Cell (model 6100). The electrophoresis was monitored by dipping 1-cm strips from each end of the sample zone in ninhydrin (0.2% in absolute ethanol, w/v) and allowing the color to develop overnight at room temperature over citric acid crystals. The transverse strip containing the desired tryptic peptide was eluted by descending chromatography with water and dried under a stream of nitrogen at 37°C. All peptide mixtures were purified identically. The overall yield of the peptide from the starting 7 mg of lyophilized ovomucoid was greater than 50% in all cases as judged by the recovery of aspartic acid in the amino acid analysis.

[2] I. Kato, unpublished observation.

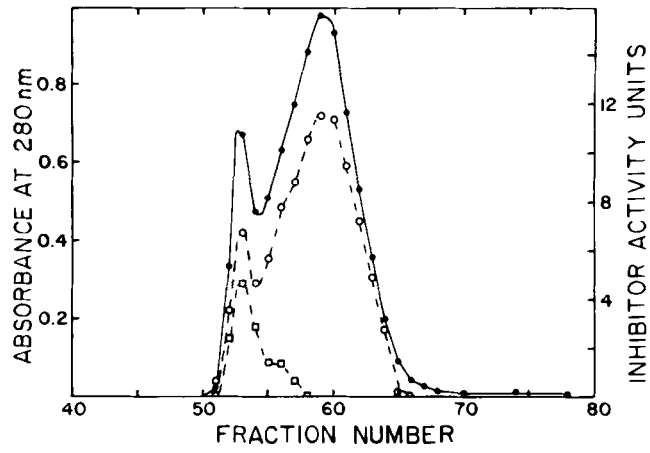

FIG. 1. **Gel filtration of crude Japanese quail ovomucoid on Bio-Gel P-10 (200 to 400 mesh).** The column (26 × 905 mm) was equilibrated and operated as follows: 5% (v/v) formic acid, 21°C, flow rate of 19.8 ml/h, 3.0-ml fraction volume, sample (from Hen L) 0.930 $A^{1\,mm}_{280\,nm}$ × 3.5 ml in 5% (v/v) formic acid. The effluent was monitored for $A^{10\,mm}_{280\,mm}$ (●——●), trypsin inhibitor activity (○- - -○) was expressed as units/7 μl of effluent, and subtilisin inhibitor activity (□- - -□) was expressed as units/0.8 μl of effluent. Fractions 57 to 62 were combined as ovomucoid.

Amino Acid Analysis—Protein and peptide samples were hydrolyzed in constant boiling hydrochloric acid at 110 ± 1°C for specified times *in vacuo*. Peptide samples eluted from paper were also made 0.1% (v/v) in 88% aqueous phenol before hydrolysis to improve tyrosine recoveries. The hydrolysates were dried at room temperature *in vacuo* over sodium hydroxide pellets. The amino acid analyses were performed on a Durrum D-500 amino acid analyzer according to the manufacturer's instructions. All analyzer chemicals and amino acid calibration standards were Pierce Sequanal grade.

Preparation of Bovine β-Trypsin—Bovine β-trypsin was prepared from commercial trypsin by a modification of a previously described procedure (Liepnieks & Light, 1974). Commercial bovine trypsin (200 mg) was applied to a column of soybean trypsin inhibitor (Kunitz)-Sepharose 4B (26 × 165 mm) in 0.05 M sodium acetate, 0.02 M calcium chloride, 0.5 M sodium chloride, pH 5.0. After eluting with several column volumes of the pH 5 buffer, a pH gradient was applied to the column. The pH gradient was formed with 900 ml of 0.1 M formate, 0.05 M calcium chloride, pH 4.2, and 900 ml of 0.1 M formate, 0.05 M calcium chloride, pH 2.9. The bovine α- and β-trypsins were identified by the pH of their elution positions (pH 3.8 and 3.5, respectively) and their purity was checked on sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of a reducing agent.

Preparation of Intact Ovomucoid Third Domain (Positions 131 to 186)—Intact third domain corresponding to positions 131 to 186 in Japanese quail ovomucoid was generated by limited proteolysis with staphylococcal proteinase (Kato *et al.*, 1978) and purified by the method of Kato *et al.* (1976). Digestion of the native Japanese quail ovomucoid (~25 mg) with staphylococcal proteinase (2% by weight) was allowed to proceed for 36 h at room temperature in 2.0 ml of 0.05 M Tris, 0.02 M calcium chloride, pH 8.2. The digestion mixture was applied directly to a Bio-Gel P-10 column which was operated and monitored in a manner similar to that utilized for the ovomucoid purification (Fig. 2). The various chromatographic components were identified by performing amino acid analysis on an aliquot from each peak fraction. The carbohydrate-free third domain fractions were pooled, diluted 1:1 with water, and lyophilized. The yield was ~4 mg/egg.

The Dissociation Rate Constant of Enzyme·Inhibitor Complex—A spectrofluorometric titration procedure (Laskowski *et al.*, 1974; Zahnley & Davis, 1970) was used to determine the rate constant for the dissociation of complexes of the two forms of ovomucoid third domains and bovine β-trypsin. Approximately 1 nmol of bovine β-trypsin and up to 3 nmol of the inhibitor variants were preincubated

for 6 ± 1 min in 3.00 ml of 0.1 M sodium veronal, 0.02 M calcium chloride, pH 8.3. The titration was initiated by the addition of 5 μl of 3.8 mM 4-methylumbelliferyl *p*-guanidinobenzoate in dimethylformamide and the fluorescence of the reaction as a function of time recorded. The assay conditions were: 0.34 μM bovine β-trypsin, 0.5 to 1.0 μM ovomucoid third domain, and 4.7 μM burst substrate. The fluorometric measurements were made using a Perkin Elmer MPF-2A fluorescence spectrometer with the following settings: excitation wavelength 360 nm, bandpass 6 nm; emission wavelength 450 nm, bandpass 10 nm. The only modification of the published procedure was that the first order rate constant was determined directly from a three parameter nonlinear least squares analysis of the $\Delta_{\text{fluorescence}}$ *versus* time data rather than a linear least squares analysis of the log $\Delta_{\text{fluorescence}}$ *versus* time data where $\Delta_{\text{fluorescence}}$ is the difference in arbitrary fluorescence units between the actual recording at time t and the reading at time t back extrapolated from infinite time.

RESULTS

Amino Acid Analysis of the Polymorphic Tryptic Peptide—High voltage electrophoresis was performed on a trypsin digest of several reduced and carboxymethylated Japanese quail ovomucoid samples. One sample was a standard prepared from a pool of egg whites obtained from 82 randomly laid eggs, while the other samples were ovomucoid purified from 11 individual quail eggs each known to be from a different hen. The electrophoretic maps of all the samples were identical. In addition to many partially resolved acidic fragments, two well resolved basic fragments were detected. These were identified as free lysine and the pentapeptide corresponding to positions 160 to 164 in the Japanese quail ovomucoid sequence. Two weakly detected fragments of similar mobility to the basic pentapeptide were also consistently seen. Table I summarizes the amino acid composition of the basic tryptic pentapeptide isolated from the various ovomucoid samples. The 11 individual hen samples were identical in composition with the following exception: 7 had 1 residue of serine but no glycine, 1 had a residue of glycine but no serine, and 3 had a half-residue each of serine and glycine. The pooled sample contained 0.83 residue of serine and 0.17 residue of glycine. As expected, a sample prepared from a second egg laid by hen A was identical with the first. The reason for the consistently high values for serine and glycine is not absolutely clear, but a contaminating peptide containing minimally arginine, serine, and glycine is suspected. Table I includes serine and glycine values which have been corrected by subtraction of the arginine value; this correction was always between 0.00 and 0.06 when applied. While leucine was also consistently detected at a level similar to that of arginine, trace amounts of other amino acids were only occasionally seen usually at a less than 0.04 residue/molecule level. Although this correction improves the data, it does not affect the interpretation.

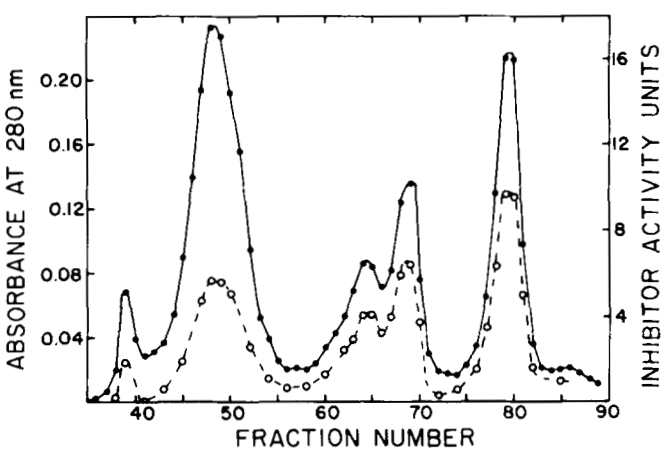

FIG. 2. **Gel filtration of the limited proteolytic digest of Japanese quail ovomucoid by staphylococcal proteinase at pH 8.2 on Bio-Gel P-10 (200 to 400 mesh).** The column (26 × 850 mm) was equilibrated and developed as follows: 5% (v/v) formic acid, 21°C, flow rate of 15.3 ml/h, 3.0-ml fraction volume, sample (from Hen L) 27.6 mg in 2.0 ml of digestion buffer. The effluent was monitored for $A_{280\,mm}^{10\,mm}$ (●——●), and trypsin inhibitor activity (○- - -○) was expressed as units/10 μl of effluent. Fractions 77 to 81 were combined as intact carbohydrate-free third domain ovomucoid.

These results clearly indicate that position 162 in ovomucoid isolated from individual Japanese quails is polymorphic. This position can exist as either serine or glycine or as an equimolar mixture of the two. These phenotypes can be symbolized as S, G, and SG. In view of these results, it is reasonable to suggest that the ovomucoid structural gene exists at one locus with two codominant alleles. On the basis of the 22 genes observed from the 11 diploid individuals, the

TABLE II

Ovomucoid phenotype frequencies in individual quail egg whites

The theoretical phenotype frequencies are based on the Hardy-Weinberg model applied to two alleles (s and g) at one locus. This predicts a $(p+q)^2 = p^2 + 2pq + q^2$ distribution where p is the allele frequency of s and q the allele frequency of g. p^2 represents the frequency of the *ss* genotype (S phenotype), $2pg$ the frequency of the *sg* genotype (SG phenotype), and q^2 the frequency of the *gg* genotype (G phenotype). See the text for the description of the phenotypes and the calculation of the allele frequencies.

Phenotype	Presumed genotype	Number of eggs		Frequency	
		Observed	Theoretical	Observed	Theoretical
S	*ss*	7	6.57	0.636	0.597
SG	*sg*	3	3.89	0.273	0.354
G	*gg*	1	0.57	0.091	0.052

TABLE I

Amino acid composition of ovomucoid peptide 160 to 164 from individual Japanese quail eggs

Amino acid[a]	Japanese quail egg											
	A	B	F	H	J[b]	K	L	N	F	T	U	Stnd[c]
	mol amino acid/mol peptide[d]											
Aspartic acid[d]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Threonine[e]	0.97	1.01	1.02	1.00	0.96	1.02	1.00	1.00	1.00	1.00	1.02	0.97
Serine[e,f]	1.00	1.03	0.48	1.07	0.94	0.02	1.04	1.06	0.52	0.55	1.04	0.87
Glycine[f]	0.02	0.02	0.51	0.01	0.02	0.97	0.02	0.03	0.52	0.48	0.04	0.18
Tyrosine	0.97	0.95	0.94	0.88	0.92	0.93	0.95	0.95	0.94	0.96	0.98	0.98
Lysine	1.03	1.01	0.99	1.00	1.00	0.99	1.01	1.02	1.01	1.00	1.00	1.00

[a] Standard amino acids not listed generally had values less than 0.04 mol/mol of aspartic acid.
[b] The 12-h hydrolysis values only.
[c] Sample prepared from a pool of egg whites isolated from 82 eggs of unknown maternal history.
[d] Determined by averaging 12- and 20-h hydrolysis values which had been calculated by assigning the aspartic acid frequency a value of unity.
[e] Determined by extrapolating 12- and 20-h hydrolysis values to zero time.
[f] Values corrected for presumed peptide contamination by subtraction of the arginine value (0.00 to 0.06).

allele frequency for s is 0.773 and for g is 0.227, where s represents the allele coding for serine and g the allele coding for glycine at position 162 in Japanese quail ovomucoid. Table II summarizes the observed phenotype frequencies and compares them to the theoretical phenotype frequencies calculated from the allele frequencies assuming Hardy-Weinberg equilibrium. Although the data set from which the allele frequencies were calculated is small, these frequencies are consistent with those seen in the pooled sample. Application of the χ^2 test yields a $P = 0.975$ ($n = 2$) for the Hardy-Weinberg model of two codominant alleles at one locus.

Complexes of the Polymorphic Forms of Ovomucoid Third Domains with Bovine β-Trypsin—Of three homologous tandem domains of Japanese quail ovomucoid, the second and third inhibit bovine β-trypsin (Kato *et al.*, 1976). In order to test the structure function consequences of the serine/glycine polymorphism at position 162, intact third domain was isolated from each allelic form of ovomucoid. The only difference between the two forms detected by amino acid analysis is the number of serine and glycine residues (Table III). The dissociation rate constant for a complex of Japanese quail ovomucoid third domain and bovine β-trypsin has previously been determined to be 1.13×10^{-3} s^{-1}.[3] Further, we pointed out that this number is both a very good indicator of the relative strength of inhibitors and easily quantitatively determined (Laskowski *et al.*, 1974). The first order rate constant for the dissociation of the complex between bovine β-trypsin and the two polymorphic forms of the ovomucoid third domains are given in Table IV. The two forms interact with bovine β-trypsin in a similar but not identical manner; clearly, the glycine^{162} form dissociates more rapidly and is thus judged to be a weaker inhibitor than the serine^{162} form.

TABLE III

Amino acid composition of nonglycosylated third domain isolated from hens with S and G phenotype ovomucoids

OMJPQ3 is an abbreviation for Japanese quail ovomucoid third domain (131 to 186). Two analyses were performed on each of three samples, 24-, 48-, and 72-h hydrolysates. The molar ratios for each analysis were calculated on the basis of 2 residues of glutamic acid, 4 of alanine, 3 of leucine, 5 of lysine, and 1 of arginine. Unless indicated otherwise, the composition listed is the average of these six analyses.

Amino acid	Ser^{162}-OMJPQ3	Gly^{162}-OMJPQ3	From sequence[a]
	mol/mol OMJPQ3		
Aspartic acid	9.01	9.01	9
Threonine[b]	3.12	3.01	3
Serine[b]	4.72	3.80	5
Glutamic acid	2.28	2.16	2
Proline	4.05	4.10	4
Glycine	3.03	4.00	3
Alanine	3.78	3.91	4
Cysteine[c]	5.22	5.32	6
Valine[d]	4.91	4.91	5
Methionine	0.06	0.02	0
Isoleucine	0.04	0.00	0
Leucine	2.97	2.98	3
Tyrosine	2.84	2.82	3
Phenylalanine	2.00	1.98	2
Histidine	0.99	0.95	1
Lysine	4.84	4.96	5
Arginine	1.07	1.02	1

[a] From Kato *et al.* (1978).
[b] Extrapolated to zero time of hydrolysis.
[c] Determined as half-cystine.
[d] Determined from the 72-h hydrolysis.

TABLE IV

Dissociation rate constants at pH 8.3 for the interaction of bovine β-trypsin and the polymorphic forms of ovomucoid third domain

OMJPQ3 is an abbreviation for Japanese quail ovomucoid third domain; carbohydrate-free forms of third domains were used.

Trial	Gly^{162}-OMJPQ3	Ser^{162}-OMJPQ3
	$10^4 \times k_D$	
	s^{-1}	
I	3.11	1.01
II	3.04	0.99
III	3.03	0.97
Average	3.06 (227 s)[a]	0.99 (700 s)[a]

[a] The value in parentheses is the dissociation half-life ($\ln 2/k_D$).

DISCUSSION

The Ovomucoid Structural Gene—Ovomucoid expression is apparently only a female characteristic. Although this is neither a necessary nor a sufficient condition for sex linkage of the ovomucoid gene, it warranted the consideration of such a possibility. In all birds, the female of the species is the heterogamete; that is, the female of the species carries an X and Y sex chromosome pair (White, 1973). A sex-linked locus would, therefore, lead to the existence of a single copy of the ovomucoid gene in an individual hen; in addition, the male would be totally devoid of the ovomucoid gene if the locus were on the Y chromosome. Two other structural gene alignments to consider were the normal autosomal locus and multiple loci as exemplified by human hemoglobin γ chains (Schroeder *et al.*, 1968). A survey of the distribution of a polymorphism in the gene product ovomucoid could differentiate between these three structural gene alignment possibilities. The distribution of three polymorphic forms of lysozyme, another egg white protein, from Peking duck (*Anas platyrhynchos*) has previously led Prager and Wilson (1971) to conclude that the lysozyme structural gene exists as one autosomal locus.

The survey of an ovomucoid polymorphism conducted here demonstrates the existence of three phenotypes S, G, and SG (presumed genotype *ss*, *gg*, and *sg*) at a frequency consistent with Hardy-Weinberg law prediction for multiple allelic forms at one locus. Had the ovomucoid gene existed at a sex-linked locus, only the S and the G phenotypes would be expected; clearly, the existence of the SG phenotype precludes this. If multiple loci for ovomucoid gene existed, the strict adherence to Hardy-Weinberg phenotype frequencies would not be expected. The distribution of the polymorphism at position 162 in ovomucoids isolated from individual Japanese quail eggs leads us to conclude that the ovomucoid structural gene exists as a non-sex-linked autosomal character. Thus, non-Mendelian modes of inheritance cannot account for the unusual evolutionary behavior of ovomucoid (Kato *et al.*, 1978). It should be pointed out, however, that these results do not dismiss the remote possibility of additional silent ovomucoid loci as is found for some primate hemoglobin α chains (Boyer *et al.*, 1971 and 1973).

Phylogenetic Considerations—Having demonstrated the existence of the polymorphism in Japanese quail ovomucoid and accepted the hypothesis of a single autosomal locus for the ovomucoid structural gene, it is of interest to ask which of the two amino acids was present at this position in the immediate ancestor. Table V summarizes the amino acids present at position 162 in several avian ovomucoids. Using the algorithm of minimum nucleotide replacement (Fitch, 1971; Fitch & Farris, 1974), the immediate ancestral residue was predicted for the polymorphic position on the basis of several reasonable tree topologies including that recently reported for avian lysozymes (Jollès *et al.*, 1979). This ancestral residue was found to be glycine (or more specifically codon GGY) for all trees tested. A more elaborate algorithm which reaches the

[3] K. A. Wilson, unpublished data.

TABLE V

Amino acid present at position 162 in phasianoid bird ovomucoids

Position 162 is defined in the Japanese quail ovomucoid numbering system.

Common name	Scientific name[a]	Amino acid[b]
Chicken	*Gallus gallus*	Glycine
Chukar partridge	*Alectoris chukar*	Glycine
Turkey	*Meleagris gallopavo*	Glycine
Bobwhite quail	*Colinus virginianus*	Valine
California quail	*Lophortyx californicus*	Alanine
Gambel's quail	*Lophortyx gambelii*	Alanine
Scaled quail	*Callipepla squamata*	Alanine
Ring-necked pheasant	*Phasianus colchicus*	Glycine
Silver pheasant	*Lophura nycthemera*	Glycine
Reeve's pheasant	*Syrmaticus reevesii*	Glycine
Golden pheasant	*Chrysolophus pictus*	Glycine
Lady Amherst's pheasant	*Chrysolophus amherstiae*	Glycine
Helmet guineafowl	*Numida meleagris*	Aspartic acid[c]
Mountain quail	*Oreortyx pictus*	Alanine[c]
Plain chachalaca[d]	*Ortalis vetula*	Aspartic acid
Duck[e]	*Anas platyrhynchos*	Glycine

[a] Scientific names are quoted from Gruson (1976).
[b] Residue reported by Kato *et al.* (1978).
[c] Residue determined by I. Kato, M. Empie, and W. Kohr (unpublished).
[d] A Cracid bird.
[e] An Anatid bird.

same conclusion was also developed (Bogard, 1980).

Although glycine is the presumed ancestor, serine is the major polymorphic form; thus, one concludes that serine is being fixed at position 162 in Japanese quail ovomucoid. The polymorphism was originally suspected in ovomucoid isolated from an unknown but very large number of Japanese quail egg whites obtained from Japan. It was definitely detected in ovomucoid isolated from a pool of 3000 egg whites which had been obtained 3 years earlier from the same American farm which supplied the eggs for the current experiment. Position 162 in ovomucoid isolated from this large lot of eggs was shown to be approximately 0.7 residue of serine and 0.3 residue of glycine. In view of these results, it is likely that the ovomucoid polymorphism at position 162 is relatively old within the Japanese quail population.

Structure-Function Relationships—Aside from being a storage protein the physiological function of ovomucoid is unknown. However, it seems highly likely that, whatever additional functions ovomucoid has, these include inhibition of some serine proteinases. The dramatic changes in inhibitory specificity of separated third domains obtained from various bird ovomucoids can be reasonably explained as a consequence of the hypervariability of the residue contributing the carbonyl function to their reactive site peptide bond (Kato *et al.*, 1978). In Japanese quail ovomucoid third domain, this residue is lysine^{148} (in entire Japanese quail ovomucoid numbering system) and as expected the Japanese quail ovomucoid third domain inhibits trypsin. Japanese quail ovomucoid is the only avian ovomucoid we examined (out of 25) whose third domain has a lysine residue contributing the carbonyl function to the reactive site peptide bond (none have an arginine) and the only one which inhibits trypsin.

In avian ovomucoid third domains, residues other than residue 148 are moderately strongly conserved. Residue 162 is a conspicuous exception (see Table V). It is the second most variable residue in the avian ovomucoid third domains we have examined. Most workers assume that positions in the polypeptide chain where maximal variability is observed are the least important to the maintenance of protein structure, protein function, and to organism survival. However, we have already shown (Kato *et al.*, 1978) that variations in amino acid residues at position 148, the hypervariable position, are associated with striking changes in inhibitory specificity and therefore may well affect the ovomucoid function. Therefore, it is difficult to decide *a priori* whether the variation in residue 162 shows that this position is especially unimportant or whether it too modulates inhibitory specificity.

The first order rate constants for the dissociation of complexes of bovine β-trypsin and the two allelic forms of carbohydrate-free third domain ovomucoids are nonidentical and, in fact, demonstrate the serine^{162} form to be an appreciably better inhibitor. It is interesting to speculate that the serine^{162} allelic form of ovomucoid is being fixed in the Japanese quail population because of its better inhibitory properties. If the speculation is correct, we have the very interesting observation of a slightly advantageous point mutation being fixed in a population. But even if the speculation is incorrect, the results indicate some functional importance of this position at least in the interaction with bovine β-trypsin. A three-dimensional structure determination of Japanese quail ovomucoid carbohydrate-free third domain[4] is currently in progress at Martinsried.

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