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Amino Acid Sequence of Pheasant Lysozyme. Evolutionary Change Affecting Processing of Prelysozyme†

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ABSTRACT: The amino acid sequence of ring-necked pheasant egg white lysozyme *c* was determined by automated Edman degradation of the reduced, alkylated tryptic peptides. Alignment of the tryptic peptides into a single chain containing 130 amino acids was done on the basis of automatic sequencing of the first 35 residues and by homology with chicken egg white lysozyme. Besides differing from the chicken enzyme by 9 amino acid substitutions, ring-necked lysozyme has an extra glycine at the amino terminus. Four other species of pheasant were shown to have lysozymes with a conventional amino terminus, beginning with lysine. The amino termini are now known for a total of 28 lysozymes *c*, and all except the ring-necked pheasant enzyme begin with lysine. To account for the extra residue in the ring-necked pheasant lysozyme, it is suggested that an evolutionary shift in the site of proteolytic cleavage of prelysozyme has occurred. Phylogenetic analysis of 10 avian and 3 mammalian lysozymes of known sequence placed the pheasant at a greater distance from the chicken than the turkey is from the chicken, in contrast to traditional taxonomic placement but consistent with evidence obtained from several other proteins. Immunological distances between ring-necked pheasant lysozyme and other bird lysozymes are consistent with the degree of sequence difference between ring-necked pheasant and other bird lysozymes and fit predictions based on the previously observed correlation between immunological cross-reactivity and sequence difference.

For several years we have been comparing the primary structures of lysozymes from various species. Amino acid sequences are already known for 12 lysozymes *c* (Jollès et al., 1976; White et al., 1977; Ibrahimi, 1977), and partial sequences are available for several others (Riblet, 1974; Morgan & Arnheim, 1974; Jollès et al., 1977; Ibrahimi, 1977). Knowledge of the positions at which sequence variation occurs has enhanced understanding of the structural requirements for lysozyme function. The sequence comparisons have also been valuable for studies of molecular evolution and of antigenic structure (Jollès et al., 1976; White et al., 1977, 1978).

While working out additional lysozyme sequences, which are necessary for in-depth studies of molecular evolution and antigenic structure, we discovered a novel structural feature, namely, an extra amino acid at the amino terminus of a bird

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lysozyme. This extra residue appears likely to result from an unusual mutation which affects the conversion of prelysozyme to lysozyme.

Prelysozyme was reported by Palmiter et al. (1977) to be a single polypeptide chain containing a leader sequence attached in peptide linkage to the amino terminus of lysozyme. Prelysozyme is thought to be a biosynthetic precursor of lysozyme. According to Blobel's signal hypothesis (Blobel & Dobberstein, 1975), the leader sequence acts as a signal that permits the nascent preprotein to pass through a biological membrane. After crossing the membrane, the leader is removed. Although little is known about the proteolytic machinery responsible for removing the leader peptide, the specificity of this machinery is highly conserved during the evolutionary process (Blobel & Dobberstein, 1975; Shields & Blobel, 1977; Lingappa et al., 1977; Habener et al., 1978). However, the position in the sequence at which the cleavage occurs can change during evolution. The first case of this sort was reported for preproinsulins (Shields & Blobel, 1977). Our studies apparently reveal a similar case for prelysozymes. The ring-necked pheasant lysozyme, whose primary sequence we report in this article, differs at the amino terminus from other lysozymes of known structure in a way that can be explained by an evolutionary shift in the site of processing of prelysozyme.

This pheasant lysozyme is also of interest from the standpoint of molecular taxonomy. The ring-necked lysozyme is more different from chicken lysozyme than we were led to expect by traditional ornithological classification (Wetmore, 1960). This finding with lysozyme is consistent with other molecular evidence (Prager & Wilson, 1971a,b, 1979; Nolan et al., 1975).

Materials and Methods

Eggs and Lysozymes. Freshly laid ring-necked pheasant (*Phasianus colchicus*) eggs were obtained from the State Game Farm, Vacaville, CA. Korean RNP (*Phasianus colchicus*) eggs were collected in Korea by Dr. S. Y. Yang, and 40 mL of egg white were shipped to us frozen. Eggs from nine additional phasianoid species—Lady Amherst pheasant (*Chrysolophus amherstiae*), golden pheasant (*Chrysolophus pictus*), silver pheasant (*Lophura nycthemera*), Reeve's pheasant (*Syrmaticus reevesi*), Hyderabadi francolin (*Francolinus pondicerianus*), scaled quail (*Callipepla squamata*), Benson quail (*Lophortyx douglasi*), mountain quail (*Oreortyx picta*), and Mearn's quail (*Cyrtonyx montezumae*)—were obtained from various suppliers in the United States (Ibrahimi, 1977).

All lysozymes were purified according to Prager & Wilson (1971a). A final purification step on Bio-Rex 70 was included only for part of the RNP material. Purification of the Korean pheasant lysozyme was carried out only through the Sephadex G-50 step, at which point we estimate the material to have been at least 95% pure. Two distinct lysozymes, designated A and B, were obtained from the Reeve's pheasant (Prager et al., 1978b), and each was rechromatographed on CM-Sephadex.

Enzymes and Reagents. In Paris, where the RNP[1] lysozyme sequence was determined, materials were obtained from the following sources: trypsin (EC 3.4.4.4) and carboxypeptidases A and B (EC 3.4.2.1 and 3.4.2.2) were purchased from Worthington. Sephadex G-25 fine was obtained from Pharmacia, and Dowex 1-X2 (200–400 mesh) was obtained from Bio-Rad. All other reagents were obtained from Merck or Prolabo, except mercaptoethanol (Koch-Light) and those used for the sequencer (S.D.S., Marseilles).

In Berkeley, where enzyme purification, peptide mapping, and manual sequencing of the N termini took place, materials were obtained from sources previously described (Prager & Wilson, 1971a; Prager et al., 1972; Ibrahimi, 1977) or were standard analytical grade reagents.

Antisera and Immunological Methods. The antiserum pools to RNP, chicken, bobwhite quail, Japanese quail, turkey, duck A, duck B, and chachalaca lysozymes were those described by Prager & Wilson (1971a) and Jollès et al. (1976). The antiserum pool to California quail lysozyme is described by Ibrahimi (1977). The immunological comparisons were done according to quantitative microcomplement fixation (Champion et al., 1974), and many of them have appeared elsewhere (Prager & Wilson, 1971a; Prager et al., 1974a; Jollès et al., 1976). The degree of antigenic difference is given in terms of immunological distance, which is equal to 100 times the log of the factor by which the antiserum concentration must be raised for a heterologous antigen to produce a complement fixation curve whose peak height is equal to that produced by the homologous antigen (the immunogen) (Prager & Wilson, 1971a; Champion et al., 1974).

Reduction, Alkylation, and Enzymatic Digestion. Reduction (mercaptoethanol) and alkylation (iodoacetamide) of 68 mg of RNP lysozyme were performed according to Jollès et al. (1972). Reduced, alkylated RNP lysozyme (40 mg) was digested with 1.2 mg of trypsin for 30 h at 37 °C and pH 8 (0.1 M ammonium bicarbonate). The trypsin was pretreated for 16 h at 37 °C with 0.0625 M HCl. Carboxypeptidase digestion was carried out at 37 °C for different time intervals in 0.1 M ammonium bicarbonate (Jollès & Jollès, 1969), and the digests were analyzed on an automatic amino acid analyzer.

Separation, Purification, and Analysis of the Tryptic Peptides. The tryptic peptides were chromatographed at 20 °C on a 140 × 1.2 cm column of Dowex 1-X2 (Jollès et al., 1972). The peptides contained in the different peaks were desalted on Sephadex G-25 (180 × 2 cm) with 30% acetic acid as the eluant. The peptides were further purified by preparative paper chromatography (Whatman No. 1) in 1-butanol–pyridine–acetic acid–water (15:10:3:12 v/v/v/v) or paper electrophoresis (Whatman No. 1; 50 V/cm; 45 min) at pH 6.5 in pyridine–acetic acid–water (100:3.5:900 v/v/v). The amino acid compositions of the peptides were established with a Technicon autoanalyzer after total hydrolysis (6 M HCl; 110 °C; for 18, 48, or 72 h; under vacuum).

Sequence Determination. The amino-terminal sequence of native or reduced and carboxymethylated RNP lysozyme was determined by automated Edman degradation in a Socosi sequencer, Model PS-100, by the 1 M quadrol buffer method. The structures of the tryptic peptides were also automatically determined, either by the dimethylallylamine buffer method or by the 0.1 M quadrol buffer procedure in the presence of polybrene (Klapper et al., 1978). The thiazolinones were converted into phenylthiohydantoin (PTH) amino acids, and the latter were characterized by thin-layer chromatography (chloroform–methanol, 90:10 v/v; pure chloroform), by gas-liquid chromatography (Beckman GC-45 chromatograph), or with a Technicon amino acid analyzer after regeneration of the free amino acid (6 M HCl in the presence of mercaptoethanol; 24 h; 150 °C; under vacuum).

Manual Determination of Amino Termini. Native lysozyme (1–2 mg) was subjected to manual Edman degradation

[1] Abbreviations used: RNP, ring-necked pheasant; lysozyme, lysozyme *c* (Prager et al., 1974b, 1978b); PTH, phenylthiohydantoin.

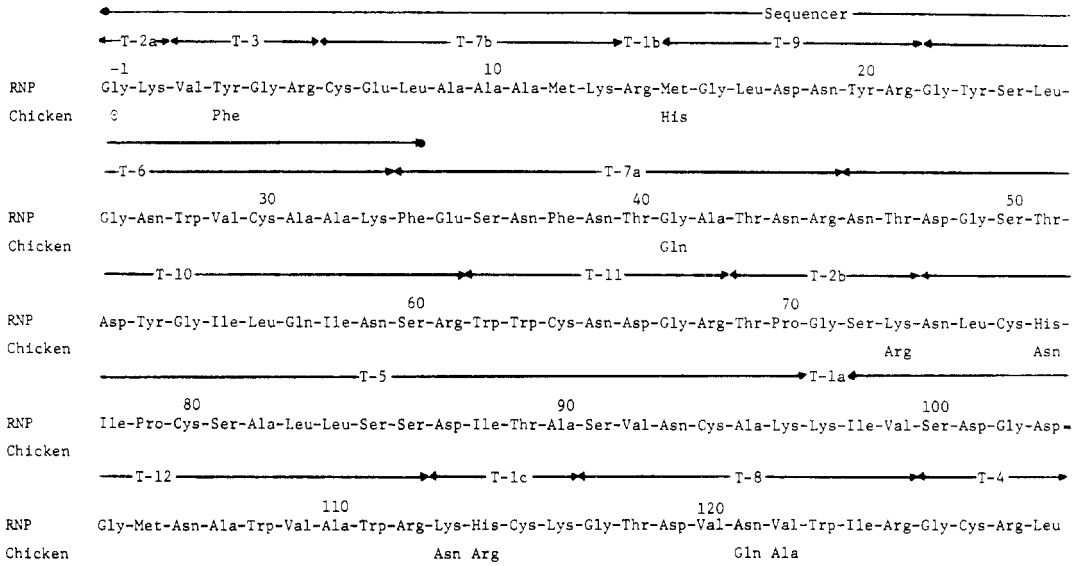

FIGURE 1: Primary structure of ring-necked pheasant lysozyme. The tryptic peptides which were isolated and studied are indicated, as are the first 35 residues determined directly on the reduced, alkylated lysozyme with the sequencer. The sequence of the chicken enzyme is given at those positions where the two lysozymes differ. To facilitate comparison of the RNP sequence with all other known lysozyme sequences, the numbering of the chicken enzyme is retained and the RNP N-terminal glycine designated as residue −1. (Θ) deletion. Additional information concerning the isolation and sequencing of individual peptides and the identification of each residue is available as supplementary material.

(Ibrahimi, 1977). The PTH amino acids were hydrolyzed to the free amino acids (5.7 M HCl; 140 °C; 15–20 h; under vacuum), which were then identified with a Beckman 121 automatic amino acid analyzer. The degradation was carried out for six to eight cycles, though the N terminus was of primary importance. The 10 lysozymes obtained from nine species of gallinaceous birds other than the RNP and the lysozyme from the Korean RNP were studied in this manner.

Peptide Mapping. A RNP lysozyme tryptic digest (3 mg or less) was mapped on paper essentially as described (Arnheim et al., 1969; Prager et al., 1972). Maps were stained with ninhydrin or fluorescamine, and the peptides were eluted sequentially with 20% pyridine, 50% acetic acid, and distilled water (Ibrahimi, 1977). Peptide compositions were determined after hydrolysis (6 M HCl; 110 °C, 24 h; under vacuum). Analysis of peptides obtained from maps was done to faciliate the subsequent large-scale sequence study by suggesting where nearly all the amino acid substitutions relative to chicken lysozyme occurred.

Results

Lysozyme Purification and Characterization. The purification, demonstration of purity, characterization, and amino acid composition of RNP lysozyme have already been reported (Arnheim et al., 1969; Prager & Wilson, 1971a). The lysozymes from the other nine species were pure according to starch gel electrophoresis at pH 5.3, 7.0, and 11.9 (Prager & Wilson, 1971a) and polyacrylamide gel electrophoresis at pH 8.9 (Prager et al., 1978b), though a small amount of lysozyme was seen distributed over two to four small peaks in addition to the main peak upon chromatography on Bio-Rex 70 (Prager & Wilson, 1971a) in the case of golden and silver pheasants and scaled and mountain quails.

N- and C-Terminal Sequences. The N-terminal sequence of native or reduced and alkylated RNP lysozyme (330 nmol) was established with the sequencer for 35 cycles (Figure 1) (see also paragraph at the end of this paper regarding supplementary material). The different tryptic peptides contained in this sequence were characterized again when the tryptic digest was studied. The N-terminal amino acid was glycine, with lysine being obtained in the second cycle. Until now, only lysine had been reported as the amino-terminal residue in lysozyme.

Table I: Amino-Terminal Sequences of 28 Lysozymes c^a

taxonomic group	position:	amino-terminal sequence −1	1	2	3
phasianoid birds					
ring-necked pheasant[b]		Gly	Lys	Val	Tyr
four other pheasants,[c] Japanese quail, turkey			Lys	Val	Tyr
chicken, partridge, francolin, guinea fowl, seven American quails			Lys	Val	Phe
anatid birds					
duck II, duck III, black swan			Lys	Val	Tyr
cracid birds					
chachalaca			Lys	Ile	Tyr
reptiles					
tortoise			Lys	Ile	Tyr
mammals					
human			Lys	Val	Phe
baboon			Lys	Ile	Phe
mouse			Lys	Val	Tyr
rat			Lys	Thr	Tyr

[a] The sources of 10 of the complete sequences are given in Jollès et al. (1976). The remaining three complete sequences are from White et al. (1977), Ibrahimi et al. (1979), and this work. The five previously reported partial sequence data are from: Gambel quail and chukar partridge, Ibrahimi (1977); black swan, Morgan & Arnheim (1974); tortoise, Jollès et al. (1977); and mouse, Riblet (1974). The remaining 10 amino-terminal sequences are reported as part of this work, with detailed results given in the supplementary material. [b] Two independent determinations, one on a sample from introduced game birds in California and the other from a wild Korean population. [c] Two different lysozymes were examined from the Reeve's pheasant.

The Korean RNP lysozyme also yielded the amino terminal sequence Gly-Lys, indicating that this novel structural feature was not solely a property of the *Phasianus colchicus* flock maintained in Vacaville, CA. All the other 10 lysozymes newly examined here began with lysine, followed by valine (Table I). No other sequence differences beyond those shown in Table I were seen among the first few amino terminal residues examined.

Leucine (0.9 residue) was the unique carboxy-terminal amino acid obtained from digestion of RNP lysozyme for 6 h at 37 °C with carboxypeptidase A. With carboxypeptidases A and B leucine (0.9 residue) and arginine (0.4 residue) were liberated. Thus Arg-Leu was established as the C-terminal sequence.

Tryptic Peptides. After chromatography of the tryptic hydrolysate on Dowex 1-X2, 12 peaks (T-1 to T-12) were isolated. From peak T-1 two free amino acids (T-1a, lysine; T-1b, arginine) and a peptide (T-1c) were isolated by paper chromatography and electrophoresis. Peaks T-2 and T-7 each contained two peptides (T-2a and T-2b; T-7a and T-7b), which were separated by paper chromatography.

Table II gives the composition of all the tryptic peptides, as well as their R_f, mobility (m) at pH 6.5, and yields. These 16 peptides add up to a total of 130 amino acids present in the molecule. Moreover, the amino acid composition of the whole molecule determined from the tryptic peptides and ultimately from the sequence agrees completely with the composition reported by Arnheim et al. (1969). The composition originally reported (Arnheim et al., 1969), furthermore, included the then-puzzling possibility that the RNP enzyme had one more amino acid than other bird lysozymes examined.

The structures of the tryptic peptides were established with the sequencer. Peptides T-2a, T-3, T-7b, T-1b, T-9, T-6, and the first residue of T-7a were contained in the N-terminal sequence determined directly on the reduced and alkylated undigested lysozyme.

Peptides T-1a, T-4, T-10, T-11, and T-12 had the same amino acid composition, R_f, and m values at pH 6.5 as the corresponding peptides of chicken lysozyme. Automated Edman degradation revealed no structural difference with the latter.

Peptides T-2b, T-5, and T-7a each had only one change in their amino acid composition when compared to the corresponding tryptic peptides of chicken lysozyme. Complete sequence determination revealed only one change per peptide (Figure 1). In the nine-residue peptide T-8, two compositional and two sequential differences occurred relative to the corresponding chicken lysozyme peptide.

The tetrapeptide T-1c (Lys-His-Cys-Lys) had no direct counterpart in the chicken lysozyme tryptic digest, but it had already been characterized in the guinea fowl (Jollès et al., 1972) and chachalaca (Jollès et al., 1976) lysozymes as replacing Asn-Arg-Cys-Lys in the chicken enzyme. Moreover, the sequence Cys-Lys occurs in only one place in the chicken.

Alignment of the Tryptic Peptides. All the tryptic peptides of RNP lysozyme were completely sequenced, and none contained more than two differences compared with the corresponding peptides of chicken lysozyme. Furthermore, the 35 amino acid long N terminus, characterized independently, contained six tryptic peptides and the start of a seventh, allowing for determination of the first 46 residues without recourse to homology. The four C-terminal residues could also be placed based on peptide T-4 and determination of Arg-Leu by carboxypeptidase digestion. The remaining eight tryptic peptides could be easily ordered by homology with the known chicken lysozyme sequence and, in one case, with the assistance of the known guinea fowl and chachalaca sequences.

Sequence Difficulties. The only real sequence difficulty involved was the use of homology to order eight of the tryptic peptides. With the exception of peptide T-1a (free lysine), these peptides range from 4 to 23 residues in length. Thus, in view of the great similarity of all bird lysozymes sequenced to date, a change in the order of two peptides other than T-1a is a virtual impossibility; furthermore, any such shift would generate an unprecedented dramatic change in the positions of half-cystines. The major weakness in the sequence, then, is placement of lysine (T-1a) at position 97. Free lysine could have arisen also from tryptic cleavage of T-1c (positions 113–116) and conceivably could be absent at position 97 or placed elsewhere. We consider these possibilities very unlikely since all lysozymes sequenced, including those examined using chymotryptic overlap peptides, have either lysine or arginine at position 97.

One further difficulty in the sequence is the assignment of aspartic acid to position 103. We indicate (Figure 1) that both chicken and RNP have aspartic acid at that position, and, indeed, peptide T-12 (Table II) has a mobility of −0.30 at pH 6.5, virtually identical with the mobility of −0.35 observed by Jollès et al. (1963) for the same peptide isolated from chicken lysozyme and tested under the same conditions. However, it has since been reported (Imoto et al., 1972; Phillips, 1974) that the chicken has asparagine at position 103. Moreover, the California quail (Ibrahimi, 1977; Ibrahimi et al., 1979) definitely has asparagine at that position, and reexamination (E. M. Prager, unpublished experiments) of the electrophoretic mobility at pH 6.4 of the homologous peptides from chicken and bobwhite quail lysozymes showed them to be neutral, not acidic, consistent with asparagine and not aspartic acid at position 103. Facile deamidation (Robinson & Rudd, 1974) could be the cause of reports of aspartic acid at position 103, and serine and aspartic acid at positions 100 and 101, respectively, would be expected to accelerate markedly the deamidation of an asparagine at position 103, particularly during desalting in an acidic medium (A. B. Robinson, personal communication). Thus, it is possible that the RNP lysozyme has an asparagine at position 103. Since several, if not all, bird lysozymes reported to have aspartic acid at position 103 may have asparagine there, we have for the number of sequence differences and phylogenetic analyses made no distinction between aspartic acid and asparagine at that position.

Immunological Cross-Reactivity. Table III presents the results of microcomplement fixation experiments comparing RNP lysozyme with seven gallinaceous and five duck lysozymes. The pheasant lysozyme is clearly distinguishable immunologically from every other lysozyme of known sequence. Furthermore, the relationship between the amino acid sequence difference and the immunological distance for reactions involving RNP lysozyme is consistent with that observed among other avian lysozymes of known sequence (Jollès et al., 1976; Ibrahimi et al., 1979).

Discussion

Sequence Aspects. RNP lysozyme is quite similar to the six other phasianoid lysozymes sequenced, differing from them by 10–14 amino acid substitutions (Table III). Save for the N-terminal glycine, the RNP sequence revealed no newly variable positions in the lysozyme molecule. However, the RNP sequence is uniquely different from all other lysozymes of known sequence in that it contains methionine at position 15 and glycine at position 41.

Only 50 of the 131 positions in the lysozyme molecule are invariant to date. The 50 invariant positions include the eight half-cystine residues involved in disulfide bonds together with nearly all the residues implicated in the catalytic activity of lysozyme (Osserman et al., 1974). Sequencing of additional bird lysozymes will probably not contribute many new variable

Table II: Properties of the Tryptic Peptides of Reduced, Alkylated Ring-Necked Pheasant Lysozyme: Amino Acid Composition, R_f, Mobility (m), and Yield

	tryptic peptide																
amino acid[a]	T-1a	T-1b	T-1c	T-2a	T-2b	T-3	T-4	T-5	T-6	T-7a	T-7b	T-8	T-9	T-10	T-11	T-12	total
Asp								2.65 (3)	1.00 (1)	2.80 (3)		2.05 (2)	2.15 (2)	3.56 (4)	2.15 (2)	3.20 (3)	20
Thr					0.92 (1)			1.00 (1)		1.50 (2)		0.79 (1)		1.45 (2)			7
Ser					0.98 (1)			3.30 (4)	0.88 (1)	0.82 (1)				1.75 (2)		1.00 (1)	10
Glu										0.84 (1)	1.00 (1)			0.72 (1)			3
Pro					0.92 (1)			1.00 (1)									2
Gly				1.06 (1)	0.95 (1)	1.06 (1)	1.10 (1)		1.84 (2)	0.98 (1)		1.02 (1)	1.10 (1)	1.90 (2)	1.30 (1)	2.02 (2)	14
Ala								2.64 (3)	1.73 (2)	1.00 (1)	2.40 (3)					1.60 (2)	11
Val						0.74 (1)		0.99 (1)	0.86 (1)			2.00 (2)				1.29 (2)	7
Cys/2[b]			0.75 (1)				0.69 (1)	1.76 (3)	0.74 (1)		0.90 (1)				0.72 (1)		8
Met											0.62 (1)		0.71 (1)			0.69 (1)	3
Ile								1.62 (2)				0.81 (1)		1.55 (2)		0.56 (1)	6
Leu							0.72 (1)	2.30 (3)	1.00 (1)		0.80 (1)		1.00 (1)	0.87 (1)			8
Tyr						0.86 (1)			0.50 (1)				0.61 (1)	0.50 (1)			4
Phe										1.63 (2)							2
Trp[c]									+ (1)			+ (1)			+ (2)[d]	+ (2)[d]	6
Lys	(1)		2.00 (2)	1.00 (1)	1.00 (1)			1.03 (1)	0.85 (1)		1.00 (1)						8
His			0.92 (1)					0.79 (1)									2
Arg		(1)				1.00 (1)	0.82 (1)			0.90 (1)		0.87 (1)	0.90 (1)	1.00 (1)	0.84 (1)	0.88 (1)	9
total	1	1	4	2	5	4	4	23	12	12	8	9	7	16	7	15	130
R_f[e]	0.19	0.22	0.08	0.14	0.20	0.47	0.40	0.60	0.55	0.30	0.65	0.19	0.65	0.60	0.47	0.54	
m[f]	+1.1	+1.0	+0.78	+0.86	+0.53	+0.48	0	−0.10	0	0	−0.24	0	0	−0.25	0	−0.30	
yield (%)	33	10	43	70	80	76	40	16	24	35	45	37	56	26	28	28	

[a] Values are based on hydrolysis for 18 h. The number of residues per peptide is given in parentheses to the nearest integer. Values obtained after 48 and 72 h of hydrolysis were taken into consideration in arriving at the integral values. [b] Determined as *S*-(carboxymethyl)cysteine. [c] Identified by the Ehrlich reaction. [d] Automated sequence studies demonstrated the presence of two Trp residues. [e] Solvent: 1-butanol–pyridine–acetic acid–water (15:10:3:12 v/v/v/v). [f] Solvent: pyridine–acetic acid–water (100:3.5:900 v/v/v), pH 6.5. $m = 0$ for Gly, +1 for Arg, and −1 for $CySO_3H$ (cysteic acid).

Table III: Immunological Distances of Bird Lysozymes Relative to RNP Lysozyme

lysozyme X	no. of amino acid substitutions[a] total	no. of amino acid substitutions[a] internal[c]	immunological distance[b] measured with anti-RNP lysozyme	immunological distance[b] measured with anti-lysozyme X
chicken	10	0	30	25
turkey	10	1	34	21
Japanese quail	12	0	63	68
guinea fowl	13	4	25	*d*
California quail	13	3	30	21
bobwhite quail	14	3	47	35
chachalaca	24	0	112	100
duck II	24	1	95	*d*
duck III	25	1	101	*d*
duck A	*e*		89	121
duck B	*e*		93	100
duck C	*e*		101	*d*

[a] The additional N-terminal glycine in the RNP has been counted as one amino acid difference and considered to be fully exposed. [b] Most of the immunological comparisons are taken from Prager & Wilson (1971a), Prager et al. (1974a), and Jollès et al. (1976). The percent standard deviation from perfect reciprocity (Champion et al., 1975) is 14.6% for the eight reciprocal comparisons in Table III and 18.2% for all 36 reciprocal lysozyme comparisons involving a total of nine antisera and antigens (E. M. Prager, unpublished experiments). [c] Based on the degree of exposure of amino acid side chains in chicken lysozyme according to Browne et al. (1969) and Lee & Richards (1971) [cf. Jollès et al. (1976)]. [d] Antisera unavailable. [e] Sequence unknown.

positions. To reveal new variable positions, one would need to examine additional nonavian lysozymes.

Six of the nine amino acid substitutions in RNP lsyozyme relative to chicken lysozyme occur in the N–C region and two more occur in the loop region. These regions have previously been discussed as being especially variable and also of immunological importance (Jollès et al., 1976). Positions 121 and 122 may be considered hypervariable, since five different amino acids are represented at each of these positions among the 10 bird lysozymes of known sequence. Further, only the RNP, guinea fowl, and chachalaca lysozymes have both lysine at position 113 and histidine at position 114, and among bird lysozymes only the RNP and chachalaca have histidine at position 77.

Origin of the Extra Glycine. Ring-necked pheasant lysozyme is notable for its extra glycine at the amino end. The amino termini of 28 lysozymes, from 26 different species, have been examined, and the RNP lysozyme is the only one to have this additional glycine residue (Table I). To explain this novel structural feature, we hypothesized that a prelysozyme molecule exists, and while this work was in progress a prelysozyme was demonstrated in the chicken (Palmiter et al., 1977). The extra glycine residue in RNP lysozyme corresponds to the glycine residue in the leader sequence of chicken prelysozyme. The chicken leader sequence consists of 18 residues which are thought to be removed during secretion by proteolytic cleavage of the glycyl–lysyl bond linking the leader sequence to lysozyme (Palmiter et al., 1977; Habener et al., 1978). We propose that this cleavage does not occur during the production of RNP lysozyme and that, instead, the presumptive RNP prelysozyme is cleaved on the amino side of the glycine residue.

An evolutionary shift in the site of processing has already been documented for another protein. In certain fishes, preproinsulin is cleaved at a different site from that in

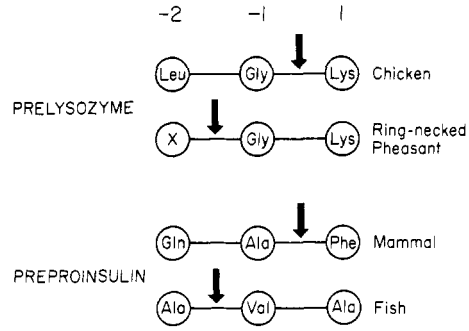

FIGURE 2: Evolutionary shifts in the site of preprotein processing. The sites of proteolytic cleavage in prelysozyme and preproinsulin are indicated by the arrows. The numbers represent amino acid residue positions in the region where the leader sequence meets the protein sequence. Position numbers are by homology with the known sequences of chicken prelysozyme and mammalian preproinsulin (Palmiter et al., 1977; Shields & Blobel, 1977; Habener et al., 1978). Negative and positive numbers represent respectively positions in the leader sequence and in the processed protein of the reference species. The ring-necked pheasant is thought to have diverged only about 30 million years ago from the lineage leading to other phasianoid birds (Prager & Wilson, 1979). Mammals and fish, in contrast, last shared a common ancestor 400 million years ago. (The particular preproinsulin sequences given in the figure are rat and angler fish.)

mammals (Shields & Blobel, 1977). As shown in Figure 2, the site of cleavage has moved by one amino acid in the preproinsulin sequence. We propose that a similar shift has taken place in the case of prelysozyme.

The two cases differ, however, in one notable respect. In the preproinsulin case, the shift in processing has been accompanied by substitutions at the original cleavage site. However, in the prelysozyme case the presumed shift has occurred without amino acid substitutions at the original site of cleavage; the glycyl–lysyl bond is present but is not cleaved in the pheasant (Figure 2). The implication is that mutations elsewhere in the pheasant genome have been responsible for the altered processing.

These mutations could have occurred elsewhere in the prelysozyme sequence, either in the leader or in the lysozyme itself. We know (see above) that RNP lysozyme differs from all other lysozymes of known sequence by having methionine at position 15 and glycine at position 41. Both of these positions are in the amino-terminal region, and, intriguingly, residue 41 occludes a significant area on residue 1 in the three-dimensional structure of chicken lysozyme [cf. the Ooi plot in Rupley et al. (1974)]. Residues 1 and 40 are hydrogen-bonded to one another (Phillips, 1966, 1974). In all other species there is a residue with a bulky side chain at position 41, but in the RNP there is the smallest possible residue, with no side chain, thereby providing space for the extra N-terminal residue in the three-dimensional structure of RNP lysozyme. In addition, an evolutionary substitution at position 15 has been associated with small conformational changes in lysozyme (Banyard et al., 1974). Our immunological results (Table III) with RNP lysozyme, in turn, make any large conformational change very unlikely.

How reasonable is it that processing could be affected by the nature of the residues at positions 15 and 41 in the lysozyme sequence? At the time of processing, the growing polypeptide chain of chicken prelysozyme is roughly 57 residues long, according to Palmiter et al. (1977), and this is the very time at which residues 40 and 41 are being added to the growing chain. However, one expects the first 57 residues to be embedded in the ribosome and the associated membrane. The experiments of Malkin & Rich (1967) and Blobel & Sabatini (1970) show that, at 0 °C, the innermost 30–39

residues of the growing chains of mammalian hemoglobin and immunoglobulins are intimately associated with the ribosome. One expects an additional 20 residues of the growing chain to be within the associated membrane. These considerations make it unlikely, but do not exclude the possibility, that the three-dimensional structure which juxtaposes residues 1, 40, and 41 has an opportunity to form before processing occurs, particularly if folding of the polypeptide can take place within the ribosome–membrane complex. Phillips (1966) has stated that, "The first 40 residues from the terminal amino end [of lysozyme] form a compact structure ... with a hydrophobic interior and a relatively hydrophilic surface that seems likely to have been folded in this way, or in a simply related way, before the molecule was fully synthesized". Thus, it is possible that in the RNP the small residue at position 41 together with any conformational change induced by the methionine at position 15 is responsible for the shift in the cleavage site. The possibility of an altered leader sequence in RNP lysozyme also seems reasonable of course and should be investigated.

Although the mutation responsible for the processing change most probably took place in the prelysozyme gene, one must also consider the possibility of a mutational change in the processing machinery. A change of the latter sort would be expected to result in abnormal processing of other preproteins in the RNP. Ovomucoid is of interest in this connection. The leader sequence of chicken preovomucoid shares several identities in the carboxy-terminal region with the leader sequence of chicken prelysozyme (Habener et al., 1978); most notably, glycine is the last residue in both leader sequences. It is significant that RNP ovomucoid has no glycine at the amino terminus (I. Kato and M. Laskowski, Jr., personal communication). Thus, there is no indication of altered processing of the presumptive RNP preovomucoid. The postulated shift in the site of processing in the RNP is confined so far to prelysozyme.

We suspect that many other cases of evolutionary shifts in the site of processing will be found. This suspicion is based on the many cases of "ragged ends"[2] seen when one aligns the sequences of related proteins so as to achieve maximum homology. Good candidates, in addition to proinsulin and lysozyme, include somatotropin (Seeburg et al., 1977; Dayhoff, 1976), ribonuclease (Beintema et al., 1977), lutropin (Dayhoff, 1976), fibrinopeptides (Dayhoff, 1972), and cytochrome *c* (Dayhoff, 1976). In most instances the evolutionary shifts are very small.

Phylogenetic Considerations. From the minimal mutation distances among all 13 animal lysozymes of known sequence, we have constructed by the Farris method (Farris, 1972) the tree shown in Figure 3. An ancestral sequence analysis gives a tree with the same branching order as shown in Figure 3. The tree indicates how the pheasant sequence may be related genealogically to the other lysozyme sequences. The tree in Figure 3 is consistent with immunological evidence obtained by comparing these lysozymes (Arnheim & Wilson, 1967; Arnheim et al., 1969; Prager & Wilson, 1971a,b) and contrasts markedly with that expected from the traditional ornithological classification, which is based on qualitative morphological evidence (Wetmore, 1960; Arnheim & Wilson, 1967; Arnheim et al., 1969). The traditional scheme would classify the chicken as the pheasant's closest relative. The lysozyme tree, however, places the pheasant furthest from the chicken among the phasianoid birds tested. A molecular tree could be constructed

[2] The term "ragged ends" refers to the existence of additional, or fewer, N-terminal residues in some species relative to the homologous protein in other species when these proteins are aligned so as to maximize homology.

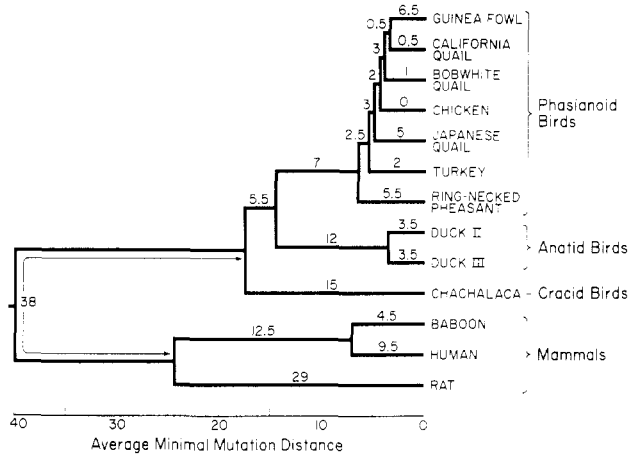

FIGURE 3: Phylogenetic tree of animal lysozymes constructed from minimal mutation distances according to the Farris method (Farris, 1972; Prager & Wilson, 1978). The *F* value (Prager & Wilson, 1976, 1978) is 10.9%. The total length of the tree is 171.5 evolutionary events.

in which RNP lysozyme is placed as the closest relative of chicken lysozyme, but this would require the fixation of six more point mutations than the most parsimonious scheme, shown in Figure 3.

Studies on nine other proteins, using the microcomplement fixation technique, are consistent with the suggestion that the lineage leading to the RNP diverged from that leading to the chicken at least as long ago as the lineage leading to the turkey (Nolan et al., 1975; Prager & Wilson, 1976). Recent quantitative morphological comparisons suggest also that the turkey should be placed within rather than outside the taxonomic group containing the chicken and RNP (Schnell & Wood, 1976).

Immunological Aspects. The sequence data are consistent qualitatively and quantitatively with published and hitherto unpublished data concerning the immunological distances between RNP lysozyme and other lysozymes (Prager & Wilson, 1971a,b; Table III). These results lend further support to the proposal that there is a correlation between the immunological distance and the degree of sequence difference (Champion et al., 1975; Jollès et al., 1976; Prager et al., 1978a). This correlation is not perfect, and it is of interest that some anomalies in the sequence–immunology relationship can be explained in terms of residue exposure. For example, the immunological relationship between RNP and California quail lysozymes and between RNP and guinea fowl lysozymes is slightly stronger than expected considering the degree of sequence difference (Table III). This could be explained if we consider that several of the positions at which these lysozymes differ are internal and unlikely to have an antigenic effect (Wilson & Prager, 1974; Jollès et al., 1976). Conversely, the relatively large immunological difference between RNP and Japanese quail lysozymes can be explained by noting that all of the substitutions by which these two lysozymes differ occur on the surface, and nine occur in regions previously suggested as being in or adjacent to antigenically important regions (Jollès et al., 1976).

The average immunological distance between RNP and chacalaca lysozymes is smaller than that between the chachalaca and any other bird lysozyme tested (Jollès et al., 1976). This was a puzzling finding because the RNP is no closer taxonomically to the chachalaca than are other phasianoid birds. The sequence data now help to explain this finding: RNP lysozyme is more similar to chachalaca lysozyme, by a minimum of three residues, than is any other bird lysozyme

examined. Furthermore, the RNP and chachalaca lysozymes, uniquely among bird lysozymes sequenced, are identical at positions 77, 113, and 114, two of which are thought to be parts of an antigenic determinant (Atassi & Lee, 1978). A complete presentation of our intraavian lysozyme immunological data and a discussion of those data in light of a recently proposed model for the complete antigenic structure of chicken lysozyme (Atassi & Lee, 1978) are being published separately (Ibrahimi et al., 1979).

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Supplementary Material Available

Detailed information concerning isolation, properties, and structural determination of tryptic peptides and application of the automatic sequencer for 35 cycles to whole RNP lysozyme; a discussion of the peptides obtained from maps; data and discussion on determination of the amino termini of the 10 additional lysozymes; and a summary of number of amino acid differences and minimal mutation distances among the 13 animal lysozymes of known sequence (13 pages). Ordering information is given on any current masthead page.

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Magnesium and Calcium Binding to Parvalbumins: Evidence for Differences between Parvalbumins and an Explanation of Their Relaxing Function†

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ABSTRACT: The calcium- and magnesium-binding properties of four parvalbumins, two from each genetic lineage α and β, have been studied by equilibrium dialysis, flow dialysis, and ultraviolet differential spectroscopy. In the absence of calcium, α-parvalbumins from rabbit pI = 5.55 and from frog pI = 4.88 and β-parvalbumin from frog pI = 4.50 bind two Mg^{2+} ions with high affinity (K_d = 16, 21, and 27 μM, respectively) at two equivalent and independent sites. Magnesium binding to the ion-free proteins induces conformational changes at the level of the hydrophobic core and of the AB loop, which can be monitored by UV differential spectroscopy. Absorbance differences increase linearly with the molar ratio up to 2 mol of Mg^{2+}/mol of parvalbumin. The affinity of parvalbumins for Ca^{2+} decreases in the presence of Mg^{2+} according to a simple competition for the same sites. In the absence of Mg^{2+}, two Ca^{2+} ions are bound at equivalent and independent sites with a calculated K_d = 6.6, 7.8, and 2.2 nM for rabbit, frog pI = 4.88, and frog pI = 4.50 parvalbumins, respectively. Substitution of Mg^{2+} by Ca^{2+} ions induces structural changes that are especially visible at the level of the hydrophobic core and of the AB loop, but the overall structure is similar in proteins that bind either Ca^{2+} or Mg^{2+}. Therefore, these three parvalbumins exhibit two independent and equivalent high-affinity Ca^{2+}–Mg^{2+} sites. In contrast, when studied by the above techniques, β-parvalbumin from hake exhibits two nonequivalent high-affinity Ca^{2+}–Mg^{2+} sites. Since no more than 1.5 Mg^{2+}- or Ca^{2+}-binding sites could be found, the protein may have lost part of its Ca^{2+}-binding capacity upon removal of divalent metals. Hake parvalbumin was shown to bind 2 Ca^{2+} ions at independent sites with K_{d_1} = 3–5 nM and $K_{d_2} \geq$ 17 nM, when conformational changes induced by addition of EGTA were followed by UV differential spectroscopy. Binding of EGTA to parvalbumin ($K_{0.5}$ = 35 mM) was also demonstrated by this technique. β-Parvalbumins appear to bind Ca^{2+} ions more strongly than α-parvalbumins. The Ca^{2+}–Mg^{2+} sites of parvalbumins exhibit either a lysyl residue (CD sites) or a glycyl residue (EF sites) between the Y and Z coordination sites. There is, therefore, no correlation between the nature of the residue in this position and the class of site. Parvalbumins bind 2 Mg^{2+} ions in resting muscle. The delay in calcium binding resulting from the dissociation of bound Mg^{2+} explains why Ca^{2+} can trigger contraction. Relaxation occurs when Ca^{2+} is displaced from the low-affinity sites of troponin C to the high-affinity Ca^{2+}–Mg^{2+} sites of parvalbumins. More generally, low-affinity Ca^{2+}-specific sites, which pick up Ca^{2+} with diffusion-limited kinetics, are "triggering sites", whereas high-affinity Ca^{2+}–Mg^{2+} sites are "relaxing sites".

Parvalbumins are low M_r (ca. 12000) acidic proteins present in abundance in the sarcoplasm of vertebrate fast skeletal muscles (Pechère et al., 1973; Blum et al., 1977; Pechère, 1977). They belong to two evolutionarily distinct lineages α and β, which were recognized in phylogenetic trees built by the maximum parsimony method (Goodman & Pechère, 1977) and confirmed by the absence of immunological cross-reactions between α and β proteins (Demaille et al., 1974). The knowledge of a number of primary structures (see Goodman & Pechère, 1977, for a review) and of the tertiary structure (Moews & Kretsinger, 1975a) of parvalbumins is in sharp contrast with the fact that their biological function is as yet poorly understood. There is some evidence, however (Pechère, 1977), that this function is related to their calcium-binding properties. By using the Chelex partition technique, parvalbumins were shown (Benzonana et al., 1972) to bind 2 mol of Ca^{2+}/mol with a $K_d = 2 \times 10^{-7}$ M in the presence of physiological levels of Mg^{2+}, i.e., 2 mM. They are mostly found in fast muscle and nervous tissue (Baron et al., 1975), which both possess Ca^{2+} sequestration devices such as sarcoplasmic reticulum, allowing fast triggering and release (Hasselbach, 1978; Blaustein et al., 1978).

It was thus recently proposed that parvalbumins play the role of a soluble relaxing factor, capable of removing Ca^{2+} from the myofibrillar troponin C before being themselves deionized by the sarcoplasmic reticulum Ca^{2+}–Mg^{2+}–ATPase (Pechère et al., 1977; Pechère, 1977). This hypothesis has received experimental support from kinetic experiments conducted on myofibrils (Pechère et al., 1977) as well as from the ability of sarcoplasmic reticulum vesicles to remove Ca^{2+} from parvalbumins (Blum et al., 1977; Gerday & Gillis, 1976).

Parvalbumins must therefore exist in resting muscle in the Ca^{2+}-free form. This raises the question as to how calcium ions can reach troponin C through the parvalbumin "barrier"

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