Studies on the Primary Structure of Chicken Erythrocyte Histone Fraction V

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Histone fraction V was prepared by selective acid extraction from chicken erythrocyte nuclei. Amino acid sequence studies on the tryptic and thermolytic peptides are reported. Only a limited amount of overlapping sequence data was obtained.

Histones are basic proteins that are associated with nuclear DNA. The function of these proteins is uncertain; they may be involved in genetic control or with the maintenance of chromosome structure (Hnilica, 1967; Stellwagen & Cole, 1969).

Ion-exchange chromatography of histone preparations on Amberlite CG-50 yields five major fractions. In general, corresponding histone fractions from different sources have essentially identical amino acid compositions, gel-electrophoretic mobilities and terminal amino acids (Satake, Rasmussen & Luck, 1960; Rasmussen, Murray & Luck, 1962; Stellwagen & Cole, 1969). Chromatographic analysis of the tryptic peptides of some of the histone fractions of calf thymus, liver and kidney, and guinea-pig testis indicated that corresponding fractions had similar primary structures (Crampton, Stein & Moore, 1957). The sequence analysis of histone fraction IV from calf thymus and pea buds demonstrated an almost identical amino acid sequence for this protein from the two organisms; the differences in sequence involve only two conservative amino acid changes (DeLange, Fambrough, Smith & Bonner, 1969b).

Histone fraction V, which occurs uniquely in nucleated erythrocytes (Stedman & Stedman, 1950; Neelin, 1964), is an outstanding example of specificity in histones. The amino acid compositions of this histone fraction isolated from fish, amphibian and avian erythrocytes show significant variation (Hnilica, 1967); histone fraction V from chicken and turkey erythrocytes also shows small differences in composition (P. J. Greenaway, unpublished work). Chicken erythrocyte histone fraction V was shown to be heterogeneous by ion-exchange chromatography on Amberlite CG-50. This heterogeneity was related to an ambiguity in sequence, involving arginine and

glutamine, at residue 15 and it was concluded that chicken erythrocyte histone fraction V is polymorphic (Greenaway & Murray, 1971). Histone fraction V may therefore show an evolutionary diversity that is not apparent in the other major histone fractions.

This paper describes structural studies on the tryptic and thermolytic peptides of chicken erythrocyte histone fraction V.

MATERIALS

Pooled chicken blood was generously given by Messrs J. Sainsbury Ltd., Bury St Edmunds, Norfolk, U.K., and D. B. Marshall Ltd., Newbridge, Midlothian, U.K. The blood was collected, immediately after the chickens were slaughtered, into polythene centrifuge bottles containing 10% (w/v) trisodium citrate (100 ml/l of blood) and was taken to the laboratory in melting ice. Trypsin, chymotrypsin and carboxypeptidase B were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Carboxypeptidase A was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Thermolysin was obtained from Daiwa Kasei K.K., Osaka, Japan. Polyamide layers were obtained from the Cheng-Chin Trading Co. Ltd., Taipei, Taiwan. All chemicals were, where possible, analytical grade.

METHODS

Preparation of histone fraction V. Histone fraction V was prepared from chicken erythrocyte nuclei by selective acid extraction (Murray, Vidali & Neelin, 1968). Extraction of saline-washed nucleoprotein at pH 1.8, after one prior extraction at pH 2.1, gave a preparation of histone fraction V that was slightly contaminated with histone fraction I. The contaminating histone fraction was removed by chromatography on Amberlite CG-50 (Neelin, Callahan, Lamb & Murray, 1964).

Amino acid analysis. Amino acid compositions were determined with a Beckman model 120C automatic amino acid analyser (Spackman, Stein & Moore, 1958). The samples were hydrolysed in 6M-HCl (0.5 ml) at 105°C for

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24 h in evacuated, sealed tubes. The hydrolysates were dried *in vacuo* and stored at 4°C until analysis.

Ionophoresis and paper chromatography. Apparatus similar to that described by Michl (1951) was used for ionophoresis. The buffer systems were pyridine-acetic acid-water (25:1:225, by vol.), pH 6.5 (Ryle, Sanger, Smith & Kitai, 1955), pyridine-acetic acid-water (1:10:89, by vol.), pH 3.5 (Ryle *et al.* 1955) and formic acid-acetic acid-water (1:4:45, by vol.), pH 2.1 (Ambler, 1963). Ionophoretic separations of peptides were carried out on Whatman 3MM paper (loading approx. 0.5–0.1 μ mol/cm) or on Whatman no. 1 paper (loading approx. 0.1–0.05 μ mol/cm).

Peptide mixtures were also separated by descending chromatography with the solvent mixture butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.) (Waley & Watson, 1953). Whatman 3MM paper was used with the same loading as for ionophoresis and the chromatograms were normally developed for 12–16 h at room temperature. Chromatography was never used as the final stage in the purification of peptides (Ambler, 1963).

Peptides were located after ionophoresis or chromatography by dipping the paper through 0.2% ninhydrin in acetone (Toennies & Kolb, 1951). The specific colour tests for peptides containing histidine (Dent, 1947) and tyrosine (Jepson & Smith, 1953) were also used. Peptides were eluted from paper with 0.1 M-NH₃.

Digestion of histone fraction V with trypsin or thermolysin. Trypsin was treated with diphenylcarbonyl chloride to inhibit chymotryptic activity (Erlanger & Cohen, 1963). The protein was dissolved in 0.1 M-ammonium acetate, pH 8.5 (10 mg/ml) and a solution of either trypsin (10 mg/ml) or thermolysin (1 mg/ml) was then added (enzyme/substrate ratio 1:80). The mixture was incubated at 37°C for 10–14 h and then freeze-dried.

Purification of peptides. Peptides produced by proteolytic digestion were initially fractionated on a column (93 cm $\times$ 3 cm diam.) of Sephadex G-25 with 5% (v/v) formic acid. Fractions containing peptides with similar elution volumes were pooled and freeze-dried. The small, basic tryptic peptides were further fractionated on a column (100 cm $\times$ 1.5 cm diam.) of SE-Sephadex A-25 with an increasing concentration- and pH-gradient of ammonium acetate. Peptides contained in fractions obtained from both chromatographic separations were subsequently purified by ionophoresis at pH 6.5, by paper chromatography and by ionophoresis at pH 3.5.

Determination of peptide sequences. The dansyl (DNS) chloride method was used to determine the *N*-terminus of peptides (Gray, 1967). DNS-amino acids were separated on polyamide layers (Woods & Wang, 1967) by using formic acid-water (3:200, v/v) for separation in the first dimension, toluene-acetic acid (9:1, v/v) in the second dimension and ethyl acetate-methanol-acetic acid (20:1:1, by vol.) for separation in the same direction, but after the second separation.

Sequential degradation of peptides from the *N*-terminus was carried out with phenyl isothiocyanate (Königsberg & Hill, 1962; Ambler, 1963); the new *N*-terminus exposed at each successive cycle was identified by dansylation. The *C*-terminal amino acid, released at the final cycle of degradation, was identified by ionophoresis at pH 2.1 in addition to dansylation.

Peptide *C*-terminal groups were determined by digestion

with carboxypeptidase A or carboxypeptidase B by the procedures described by Ambler (1967). The amino acids released were identified by ionophoresis at pH 2.1.

Peptide nomenclature. The first letter shows the digest from which the peptide was obtained: T, trypsin; H, thermolysin. The second letter shows the mobility of the peptide at pH 6.5: B, basic; N, neutral; A, acidic. The peptides in each series are then distinguished by Arabic numerals.

The ionophoretic mobility at pH 6.5 (*m*) and at pH 3.5 (*m'*) was measured relative to lysine for basic peptides (+ sign) and aspartic acid for acidic peptides (– sign) and was corrected for electro-endosmosis. The mobility of peptides at pH 6.5 was used to determine whether their side-chain carboxyl groups were free or amidated (Offord, 1966). The mobility of peptides in descending chromatography (*R_x*) was measured relative to Xylene Cyanol FF; the *R_F* of Xylene Cyanol FF is approx. 0.69.

Residues joined by hyphens are in sequence, residues in parentheses are in unknown order. The subscript $\rightarrow$ shows the amino acids identified by sequential degradation with phenylisothiocyanate and the subscript $\leftarrow$ shows the amino acids sequentially released by carboxypeptidase A or B.

RESULTS

Amino acid composition. The amino acid composition of chicken erythrocyte histone fraction V is shown in Table 1. The distinguishing features of this composition are the relatively high quantities of lysine, arginine, serine and alanine residues and the absence of cysteine and tryptophan residues. The minimal molecular weight, calculated from the amino acid composition, is

Table 1. *Amino acid composition of chicken erythrocyte histone fraction V*

Experimental details are given in the text.

Amino acid	Amount	
	Mol/100 mol of amino acids recovered	Residues/molecule of histone fraction V (nearest integer)
Lys	24.1	40
His	1.4	2
Arg	10.9	18
Asx	1.8	3
Thr	3.3	6
Ser	13.0	22
Glx	4.4	7
Pro	7.1	12
Gly	4.8	8
Ala	15.8	26
Val	3.9	7
Met	0.6	1
Ile	3.0	5
Leu	4.6	8
Tyr	1.7	3
Phe	0.6	1

Table 2. *Amino acid sequences of the tryptic peptides of histone fraction V*

Peptides enclosed in brackets have overlapping sequences. SerP, *O*-phosphoserine. Further details are given in the text.

Peptide	Yield (μ mol)	R_x	m	m'	Sequence
TA1	0.39	0.34	-0.40	+0.16	<u>SerP-Asp-Lys</u>
TN6	0.08	0.20	+0.06	+0.61	<u>Ser-Asp-Lys</u>
TB22	0.34	0.21	+0.47	+0.57	<u>Ser-Asp-Lys-Lys</u>
TN1	1.04	0.26	0.00	+0.69	<u>Ala-Glu-Lys</u>
TB13	0.24	0.21	+0.36	+0.84	<u>Ala-Glu-Lys-Lys</u>
TB28	0.23	0.33	+0.61	+0.65	<u>Ala-Glu-Lys-Lys-(Ser,Pro,Ala,Ser,Gly)-Arg</u>
TB7	0.29	0.32	+0.23	+0.76	<u>Ser-Pro-Ala-(Ser,Gly,Arg,Ala)-Lys</u>
TN2	3.11	0.55	+0.01	+0.27	<u>(Gln,Ile)-Glu-Ser-Lys</u>
TN3	1.06	0.43	+0.02	+0.31	<u>(Thr,Glu)-Lys</u>
TN4	0.17	0.61	+0.02	+0.36	<u>Gln-Val-Lys</u>
TN5	0.31	0.20	+0.02	+0.58	<u>Asp-Lys</u>
TB1	2.29	0.66	+0.11	+0.44	<u>Thr-Glu-Ser-Leu-Val-(Leu,Ser,Pro,Ala,Pro,Ala,Lys,Pro,Lys)</u>
TB4	0.10	0.79	+0.19	+0.83	<u>Ala-Ile-Arg</u>
TB5	0.34	0.58	+0.20	+0.33	<u>Glx-Asx-Leu-Ile-(Pro,Ala,Val,Leu)-Lys</u>
TB6	1.33	0.58	+0.20	+0.63	<u>Val-(His,Asx,Asx,Glx,Gly,Ala,Ile,Leu)-Lys</u>
TB8	0.27	0.58	+0.26	+0.58	<u>Thr-Val-Lys</u>
TB9	0.40	0.24	+0.27	+0.70	<u>Gly-Gly-Ser-Ser-Arg</u>
TB10	0.10	0.53	+0.31	+0.77	<u>Ala-Val-Arg</u>
TB11	0.29	0.64	+0.34	+0.55	<u>Gly-Val-Gly-Ala-(Ser,Ser,Gly,Phe)-Arg</u>
TB12	0.69	0.34	+0.35	+0.72	<u>Ala-Pro-Arg-(Ser,Ala)-Lys</u>
TB14	0.82	0.78	+0.42	+0.66	<u>Ile-Ser-Leu-Arg</u>
TB15	0.94	0.78	+0.43	+0.72	<u>Tyr-Ile-Lys</u>
TB51	0.23	0.22	+1.08	+1.18	<u>Lys-Pro-Lys</u>
TB24	0.54	0.37	+0.47	+0.82	<u>Ala-Ser-Arg-Arg</u>
TB16	0.34	0.20	+0.43	+1.06	<u>Lys-Pro-Lys-Ala-Ser-Arg</u>
TB17	0.50	0.78	+0.44	+0.72	<u>Ala-Ser-Lys-(Pro,Ser,Arg)-Arg</u>
TB18	0.78	0.43	+0.44	+0.74	<u>Ala-Thr-Ala-Arg</u>
TB20	0.20	0.26	+0.46	+0.69	<u>(Ser,Gln,Gly,Ala)-Lys</u>
TB21	0.81	1.05	+0.47	+0.53	<u>Leu-Leu-Ala-Ala-Gly-(Val,Leu)-Lys</u>
TB38	0.11	0.32	+0.70	+0.87	<u>Ala-Lys-(Arg,Ser,Ser,Thr,Pro)-Lys</u>
TB26	0.09	0.24	+0.51	+0.90	<u>Arg-Ser-(Ser,Thr,Pro)-Lys</u>
TB23	1.52	0.40	+0.47	+0.67	<u>Ser-Ser-Thr-Pro-Lys</u>

Table 2 *Cont.*

TB49	0.14	0.20	+1.05	+1.05	<u>Lys-Ser-(Pro,Ala)-Lys</u>
TB45	0.19	0.11	+0.99	+0.61	<u>Ser-Pro-Ala-(Lys,Arg)</u>
TB25	0.58	0.37	+0.49	+0.82	<u>Ser-Pro-Ala-Lys</u>
TB27	0.17	0.27	+0.52	+0.85	<u>Ser-Ala-Lys-(Ala,Pro)-Arg</u>
TB29	0.45	0.39	+0.61	+0.73	<u>Ala-Ser-Ala-Arg</u>
TB32	0.10	0.26	+0.61	+0.82	<u>Ala-Ser-Lys</u>
TB35	3.86	0.31	+0.64	+0.94	<u>Ala-Lys</u>
TB36	0.18	0.20	+0.64	+1.02	<u>Lys-Ser-Lys</u>
TB39	0.56	0.40	+0.76	+0.83	<u>Ala-Arg</u>
TB40	0.48	0.25	+0.81	+0.92	<u>Ser-Arg</u>
TB42	0.08	0.33	+0.86	+1.07	<u>Lys-Ala-Arg</u>
TB43	0.36	0.18	+0.88	+1.11	<u>Arg-Ala-Pro-Gly-Pro-(Lys,Lys,Arg,Ser)</u>
TB44		0.31	+0.92	+0.93	Free Arginine
TB46	0.39	0.11	+0.99	+0.68	<u>Lys-Ser-Pro-Lys</u>
TB47		0.21	+1.00	+1.00	Free Lysine
TB48	0.14	0.22	+1.02	+1.10	(Lys ₄ ,Pro,Ala)
TB50	0.19	0.16	+1.08	+1.00	(Lys ₅ ,Pro ₂ ,Ala)
TB52	2.19	0.23	+1.10	+1.26	Lys-Lys or Lys-Lys-Lys

Table 3. *Amino acid sequence of peptide TB2*

Peptide TB2 (0.2 μ mol) was dissolved in 0.1 M-ammonium acetate, pH 8.5 (0.5 ml). Chymotrypsin was added (enzyme/substrate ratio 1:50) and the solution incubated at 37°C for 3 h. The peptides were purified by ionophoresis at pH 3.5. Further details are given in the text.

Peptide	<i>m'</i>	<i>N</i> -Terminus	Composition (mol of amino acid/mol of peptide)										Sequence
			His	Arg	Thr	Ser	Glu	Pro	Ala	Met	Ile	Tyr	
TB2	+0.12	Ser	1.0	0.9	1.1	3.1	1.2	1.1	4.3	1.0	1.9	0.6	See Fig. 1.
TB2C1	+0.05	Ser				1.0	1.0			1.0			<u>Ser-Glu-Met</u>
TB2C2	+0.12	Ser	0.9	1.0	0.7	2.7	1.3	0.9	4.0	0.7	1.8	0.5	Undigested peptide
TB2C3	+0.31	Ala		1.0					1.7		1.0		<u>Ala-Ala-Ile-Arg</u>
TB2C4	+0.46	Ile		1.0					3.2		1.9		<u>Ile-Ala-Ala-Ala-Ile-Arg</u>
TB2C5	+0.53	Ser	0.8	0.2	0.8	2.7	1.2	1.0	1.0	0.8		0.6	<u>Ser-Ala-Ser-His-(Pro,Thr,Tyr,Ser,Glu,Met)</u>
TB2C6	+0.65	Ala		1.0					0.9		1.1		<u>Ala-Ile-Arg</u>
TB2C7	+0.72	Ser	1.0		0.9	2.1		1.0	0.9		0.7		<u>Ser-Ala-Ser-His-Pro-Thr-Tyr</u>

approx. 18400. Acetylation (Phillips, 1963), methylation (Murray, 1964) and phosphorylation (Ord & Stocken, 1966*a,b*) of certain amino acids has been

detected in histone fractions. Histone fraction V contains essentially no acetyl groups, trace amounts of ω -*N*-methylarginine (equivalent to approx.

Fig. 1. Amino acid sequence of tryptic peptide TB2. The sequences of the chymotryptic peptides were determined as shown in Table 3.

Table 4. *Amino acid sequences of the thermolytic peptides of histone fraction V*

Experimental details are given in the text.

Peptide	Yield (μmol)	R_x	m	m'	Sequence
HA1	0.17	0.28	-0.65	+0.30	<u>Ala-Asp</u>
HA2	2.33	0.60	-0.65	+0.07	<u>Tyr-Ser-Glu-Met</u>
HA3	0.54	0.28	-0.65	+0.09	<u>Thr-Glu-Ser</u>
HA4	0.10	0.43	-0.58	-0.12	<u>Gln-Asp-Leu</u>
HA5	0.20	0.21	-0.55	+0.46	<u>Val-Gly-Asn-His-Ala-Asp</u>
HN3	0.30	1.44	0.00	+0.32	<u>Ala-Ala</u>
HN4	0.05	0.38	+0.04	+0.37	<u>Ala-Gly</u>
HN5	0.16	0.40	+0.05	+0.16	<u>Val-Gly-Ala-Gly-Ser-Ser</u>
HB2	0.26	0.57	+0.35	+0.55	<u>Ile-(Lys,Gln)-Tyr</u>
HB3	0.23	0.50	+0.35	+0.57	<u>Val-(Lys,Leu)-Gln</u>
HB4	0.43	0.50	+0.35	+0.71	<u>Tyr-Lys</u>
HB5	0.37	0.68	+0.35	+0.72	<u>Phe-Arg</u>
HB7	0.35	0.20	+0.35	+0.91	<u>Lys-(Arg,Arg,Arg,Ser)-Ala-Ser</u>
HB9	0.13	0.45	+0.35	+0.98	<u>Ile-Arg-Arg</u>
HB10	0.22	0.11	+0.35	+1.06	<u>Ala-(Lys,Arg,Pro)-Ala-Lys</u>
HB11	0.57	0.44	+0.40	+0.75	<u>Val-Lys-Ala</u>
HB12	0.45	0.23	+0.42	+0.71	<u>Ala-Ser-Lys</u>
HB13	0.14	0.78	+0.42	+0.88	<u>Ile-Arg-Arg-Leu</u>
HB14	0.70	0.55	+0.49	+0.80	<u>Ile-Lys</u>
HB16	1.03	0.34	+0.53	+0.85	<u>Val-Arg-Ile-Ser-His-Lys</u>
HB17	0.31	0.15	+0.53	+1.18	<u>Ala-Arg-Lys-Lys</u>
HB18	0.18	0.27	+0.55	+0.99	<u>Val-Arg-Ala-Lys-Lys</u>
HB19	0.45	0.14	+0.56	+0.93	<u>Ala-Ser-Pro-Lys-Lys</u>
HB20	0.80	0.18	+0.63	+1.06	<u>Ala-Arg-Lys</u>
HB21	0.75	0.14	+0.67	+1.15	<u>Ala-Lys-Lys</u>
HB23	0.28	0.25	+0.70	+0.96	<u>Ser-Arg-Arg-Ser</u>

0.03 mol/mol of protein) but significant quantities of *O*-phosphoserine (equivalent to approx. 0.23 mol/mol of protein) (Greenaway, 1970).

Tryptic peptides of histone fraction V. The sequences of the peptides isolated from the tryptic digest of histone fraction V are summarized in Table 2. Peptide TB2 was further digested with chymotrypsin and its sequence determined as indicated in Table 3 and Fig. 1. Digestion of the protein by trypsin was incomplete. Some of the purified peptides contained more than one basic amino acid and overlapping sequences were obtained; these are grouped together by brackets in Table 2.

Thermolytic peptides of histone fraction V. The thermolytic peptides of histone fraction V were initially separated by gel filtration on Sephadex G-25 into three size fractions. The thermolytic peptides contained in the first two fractions were large, very basic, difficult to purify in high yield and were not characterized further. The sequences of the peptides purified from the remaining fraction are summarized in Table 4.

DISCUSSION

Some of the tryptic and thermolytic peptides isolated during this study fit into the known *N*-terminal sequence of the protein (Greenaway & Murray, 1971). Peptide TB2 extends this sequence by six amino acids. It is difficult to deduce other fragments of sequence from the information obtained. There are several obvious relationships between some tryptic and thermolytic peptides. For example, peptide HA4 is the *N*-terminus of peptide TB5; peptides HN5 and HB5 can be accommodated in the sequence of peptide TB11. For the majority of the results unambiguous overlapping sequence information is lacking.

The results obtained from this study indicate an irregular distribution of basic amino acids along the polypeptide chain. The isolation of free arginine, free lysine and a polylysine peptide indicates that there may be clustering of basic amino acids along the sequence. Clustering of basic amino acids may be a reason for the inefficient hydrolysis of the protein by trypsin (Waley & Watson, 1953; DeLange, Fambrough, Smith & Bonner, 1969a).

The sequence of this protein may be completed after purification of the large basic peptides from the thermolytic and other proteolytic digests but these peptides are difficult to purify in high yield (Greenaway, 1970). The procedure used for the purification of peptides during this study was gel filtration followed by paper chromatography and ionophoresis. These techniques separated the large basic peptides very poorly and resulted in low,

variable, yields of peptides. Ion-exchange chromatography was not extensively used during peptide purification because of the possibility of irreversible adsorption of the basic peptides on to the resin.

The sequence of this protein, when complete, will not only provide further information on protein-DNA interactions but will also provide a basis for a study of the phylogeny of histone fraction V.

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