N-Terminal Acetyl-Peptides from Two Calf Thymus Histones

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1. The same *N*-terminal peptide was isolated from two major calf thymus histone fractions, F2a1 and F2a2. 2. The structure of the peptides is *N*-acetylserylglycyl-arginine, and the yields account for most of the *N*-acetyl groups previously found in these histones. 3. Some other peptides not giving a ninhydrin reaction were also investigated.

The chief *N*-terminal amino acids of calf thymus histones were found to be alanine and proline (Phillips, 1957, 1958; Luck, Rasmussen, Satake & Tsvetkov, 1958), and they have also been found in the homologous histones from other sources (e.g. in trout liver; Palau & Butler, 1966). When the histones were fractionated, the alanine terminus was found in the 'arginine-rich' histone that we now designate F3 (Johns, Phillips, Simson & Butler, 1960) and the proline terminus in the 'slightly lysine-rich' histone fraction designated F2 (Phillips & Johns, 1959). The latter fraction was later resolved into fractions F2a and F2b (Johns, 1964) and the proline terminus found to belong to the F2b fraction only. It thus appeared that the remaining fraction F2a, and in addition the 'very lysine-rich' histone fraction F1, had unreactive or blocked *N*-terminal amino groups. It was later shown that these histones had *N*-acetyl groups that were probably *N*-terminal (Phillips, 1963b). The fraction F2a has since been further fractionated into fractions F2a1 and F2a2, both of which were found to lack reactive *N*-terminal amino groups and to contain *N*-acetyl groups (Phillips & Johns, 1965).

The present work was done to show that acetyl groups are actually *N*-terminal in these histones. A preliminary account has been given (Phillips, 1966).

MATERIALS AND METHODS

The histone fractions were prepared from calf thymus by the methods of Johns (1964) and Phillips & Johns (1965). For a better separation of the fractions F2a1 and F2a2, it was advantageous to precipitate fraction F2a1 at 35° and allow it to settle out for at least 1 hr. before centrifugation. The subsequent precipitation of fraction F2a2 was done at room temperature. An improved method of separating these two fractions has also been described by Johns (1967b).

Preparation of the peptides. Crystalline trypsin (EC 3.4.4.4) (2mg.) was dissolved in 5ml. of 0.01*N*-HCl and the solution used to dissolve 100mg. of the histone fraction. To this was added 0.2ml. of a freshly prepared solution of $m\text{-(NH}_4\text{)}_2\text{CO}_3$ or $m\text{-KHCO}_3$. The solution was kept at 38° and a precipitate was formed in the digests of fractions F2a1 and F2a2 (but not with fraction F1) within a few minutes of making the solutions alkaline. After 2hr. the digest was centrifuged, the precipitate was washed with 5ml. of a solution of the same composition as the digestion medium and the combined supernatants were freeze-dried. The precipitates, which accounted for about 20% of fractions F2a1 and F2a2, were not examined further in this work.

Fractionation. The peptides were separated on a sulphonic acid type ion-exchange resin (type A; Technicon Instruments Ltd., Chertsey, Surrey) in a column 132cm. long $\times$ 1cm. diam., with Technicon detection and recording apparatus generally similar to that used for amino acid analysis. Three successive linear buffer gradients were used to elute the peptides: (1) 1.2*M*-acetic acid–0.1*M*-pyridine grading over a 6hr. period to 0.8*M*-acetic acid–0.1*M*-pyridine (290ml. of each buffer); (2) 0.8*M*-acetic acid–0.1*M*-pyridine grading over a 25hr. period to 0.7*M*-acetic acid–0.7*M*-pyridine (1200ml. of each buffer); (3) 0.7*M*-acetic acid–0.7*M*-pyridine grading over a 20hr. period to 1.5*M*-acetic acid–2.1*M*-pyridine (1000ml. of each buffer). The elution was done at 35° at a constant rate of about 1.6ml./min. The peptide mixture derived from 100mg. of histone was applied to the column dissolved in 3ml. of the 1.2*M*-acetic acid–0.1*M*-pyridine buffer. The effluent from the column was split into three streams: (a) 0.1ml./min. was used for continuous reaction with ninhydrin reagent as in the usual amino acid analyser method; (b) 0.1ml./min. was used for continuous reaction with Sakaguchi reagents (Satake & Luck, 1958) to detect peptides containing arginine. For this purpose, the stream was mixed with a stream (0.32ml./min.) of 0.002% 8-hydroxyquinoline in 2*N*-NaOH and then, after mixing, with a stream (0.23ml./min.) of 0.1% *N*-bromosuccinimide in water. The extinction of this reaction mixture was read in a separate flow-through colorimeter with 505*m* μ filters. This reaction has only about one-third the sensitivity of the ninhydrin reaction with peptides that give both reactions. The third stream

from the column, about 1.4 ml./min., led to a time-controlled fraction collector. The time difference between the arrival of a given peptide at the collector and the recording of its ninhydrin and Sakaguchi responses (about 40 min. later) was demonstrated on the record by the periodic injection of Naphthalene Black solution (5 mg./ml. in water) into streams (a) and (b) above. The effluent corresponding to each peak was freeze-dried and the residue redissolved in 1–2 ml. of 5% acetic acid or water and stored frozen.

The pyridine in the volatile buffers used above was refluxed for 0.5 hr. with 1 g. of ninhydrin/l. and then distilled (Catravas, 1965).

Analysis. Peptides were analysed for amino acid composition in a Technicon Autoanalyser after total hydrolysis for 24 hr. at 110° in 6N-HCl. The homogeneity and electric charge of the peptides was also checked by paper chromatography in butan-1-ol-acetic acid-water (2:1:1 or 3:1:1, by vol.) and by paper electrophoresis at pH 6.4. Mixtures of peptides were resolved by the same methods with 50 cm. × 45 cm. sheets of Whatman no. 3MM filter paper. Carboxypeptidase B (EC 3.4.2.2) digestion was used to remove the basic C-terminal amino acid from some of the peptides. The enzyme (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was first dialysed against 1 mM-(NH₄)₂CO₃–1 mM-ZnSO₄ to remove contaminating amino acids. The peptide solution containing 0.1–0.6 μmole was freeze-dried and then digested in 0.05–0.1 ml. of 0.1 M-(NH₄)₂CO₃–1 mM-ZnSO₄ at 38° for 5 hr. with 7 units of enzyme activity/ml. of digest. Hydrazinolysis was used to detect acetyl groups as acetohydrazide and 2,4-dinitrophenylacetohydrazide (Phillips, 1963a), and also for releasing the C-terminal amino acid of peptides after the removal of C-terminal arginine by carboxypeptidase B. For the hydrazinolyses, the dried sample of peptide (about 0.2 μmole) was dissolved in 0.1 ml. of hydrazine ('95+ %'; Kodak Ltd., Kirkby, Liverpool) and heated in a stoppered tube at 100° for 17 hr. The contents were then freeze-dried for the minimum time, to avoid excessive sublimation of acetohydrazide. Free amino acids in the hydrazinolysate were measured in the amino acid analyser. Hydrazides were separated by chromatographing samples on Whatman no. 1 filter paper

with the solvents butan-1-ol saturated with 0.1 N-NH₃ or propan-2-ol–0.1 N-NH₃ (17:4, v/v). The hydrazides were detected on the paper by the reagent of Andreae (1958). Serine hydrazide and glycine hydrazide had the same *R_F* (0.34) when acetohydrazide had *R_F* 0.56.

The yields of the peptides from the histones were obtained from the recovery of amino acids in the sample used for total amino acid analysis. The area under the peaks recording the ninhydrin reaction of the peptides was only very approximately proportional to the quantity present.

RESULTS

As a result of the specific action of trypsin on the histones, it was expected that peptides with blocked N-terminal groups would be either arginine-containing peptides, Sakaguchi-positive but ninhydrin-negative, or lysine-containing peptides, which would be Sakaguchi-negative but still ninhydrin-positive owing to the partial reaction of the ε-amino group of lysine. Since a positive charge is removed by the blockage, these peptides are more likely to run with the acidic or neutral peptides, i.e. in the earlier part of the eluate from the column.

The properties of these peptides and of others that did not contain a free α-amino group are summarized in Table 1. Further properties of these peptides are described below.

Histone F2a2, peptide B. The amino acid ratios (except for the expected fall in lysine due to the formation of stable ε-phenylthiocarbamoyl-lysine) were unchanged after one cycle of the Edman degradation procedure, so that there was apparently no free α-amino group. Reaction with 1-fluoro-2,4-dinitrobenzene also failed to give an N-terminal amino acid. This peptide therefore

Table 1. *Peptides derived from histones F2a1 and F2a2 containing acetyl groups or not having an α-amino group*

All the peptides listed here were electrically neutral at pH 6.4. The yield of acetyl groups (Ac) from the parent histones was 1 mole/10000 g. (average) for histone F2a1 and 1 mole/18000 g. (average) for histone F2a2 (Phillips & Johns, 1965).

Histone	Peptide	Approx. elution time from column (hr.)	Amino acid composition	Ninhydrin reaction	Sakaguchi reaction	Acetyl groups	Structure	Approx. yield (g. of histone/ mole of peptide)
F2a1	K	5.9	(Arg, Glu, Gly)	—	+	—	PCA-Gly-Arg	21000
	J	6.9	(Arg, Gly, Ser)	—	+	+	Ac-Ser-Gly-Arg	14000
F2a2	B	2.8	(Glu, Gly ₂ , Lys)	+	—	—	PCA-Gly-Gly-Lys	47000
	CBA	4	(Ala, Arg, Glu, Thr)	—	+	+	Ac-Gln-Thr-Ala-Arg	500000
	DA2	6.0	(Ala ₂ , Leu, Val)	+	—	—	Not determined	230000
	D	6.1	(Arg, Gly, Ser)	—	+	+	Ac-Ser-Gly-Arg	25000–30000
	HB	10	(Arg, Glu)	—	+	—	PCA-Arg	300000

probably has a PCA* residue at the *N*-terminus and is:

PCA-Gly-Gly-Lys

The corresponding uncyclized peptide Glu-Gly-Gly-Lys was also found, being eluted 13 hr. later from the column.

Histone F2a2, peptide CBA. Hydrazinolysis gave acetohydrazide after freeze-drying five times from 0.1N-hydrochloric acid to remove any contaminating acetate. Electrophoresis at pH 6.4 showed that the Sakaguchi-positive material was neutral. Since the α -amino group is blocked, the γ -carboxyl group must be amidated to achieve neutrality. Digestion with carboxypeptidase B gave arginine and a little alanine (this enzyme preparation was known to contain a small amount of carboxypeptidase A). Hydrazinolysis of the remainder of this digest gave free alanine and threonine. The structure consistent with these observations and those in Table 1 is:

Acetyl-Gln-Thr-Ala-Arg

This peptide was obtained in very low yield and was not observed among the peptides from another preparation of histone F2a2.

Histone F2a2, peptide D. The ninhydrin peak DA is out of phase with the Sakaguchi record of peptide D and refers to other peptides described below. Peptide D was separated from peak DA material by chromatography on paper (see the Materials and Methods section). Carboxypeptidase B digestion gave free arginine and nothing else detectable with ninhydrin. Hydrazinolysis of this digest gave free glycine with a trace of serine. Another sample of the digest was run on a column of Dowex 50 (H⁺ form) ion-exchange resin. Elution with water gave a peptide which on total hydrolysis gave the analysis: (Ser₁Gly₁). The structure of this peptide is therefore:

Acetyl-Ser-Gly-Arg

The absence of a ninhydrin reaction with this peptide and the relative stability to hydrazinolysis of the acetyl group in the parent histone (Phillips, 1963b) preclude the presence of an *O*-acetyl group. An acetyl group attached to a nitrogen atom of the guanidino group would probably suppress the usual Sakaguchi reaction and prevent the exercise of both tryptic attack on the histone at this point and of carboxypeptidase B action on the peptide D.

Histone F2a2, peptide DA2. This peptide and a trace of another (peptide DA1) account for the ninhydrin reaction nearly coincident with peptide D. The presence of peptide DA2 is probably due to the action of a trace of chymotrypsin in the

trypsin used. The *C*-terminal amino acid of histone F2a2 is lysine (Phillips, 1966), so that peptide DA2 is not the *C*-terminal peptide.

Histone F2a2, peptide HB. No *N*-terminal amino acid could be obtained. This peptide, which probably has the structure:

PCA-Arg

was obtained in very low yield and was only just detectable on the record of the run with another preparation of peptides from histone F2a2.

Histone F2a1, peptide J. In its response to both carboxypeptidase B action and to hydrazinolysis this peptide gave the same results as peptide D from histone F2a2 and is therefore identical with it and has the structure:

Acetyl-Ser-Gly-Arg

Histone F2a1, peptide K. This peptide failed to give a ninhydrin reaction or an *N*-terminal amino acid with 1-fluoro-2,4-dinitrobenzene. The probable structure is:

PCA-Gly-Arg

The uncyclized form Glu-Gly-Arg was not found, but most of the arginine-containing peptides from histone F2a1 have not yet been studied.

Histone F1 peptides. On digestion with trypsin, the 'very lysine-rich' histone F1 gave about 70 peptide peaks separable under the conditions described, with many in very small yield. However, no arginine-containing ninhydrin-negative peptide was observed. On the other hand, nine of the lysine-containing peptides that are ninhydrin-positive were examined, but a peptide bearing an acetyl group has not yet been found.

DISCUSSION

The results show that two of the fractions of calf thymus histones, F2a1 and F2a2, which together amount to about 40% of the histones, have the same *N*-terminal sequence: *N*-acetyl-Ser-Gly-Arg. This accounts for the *N*-acetyl groups previously found in these fractions, and the yield of these peptides is approximately that expected from the original yield of acetyl groups (Phillips, 1963b; Phillips & Johns, 1965). The tryptic digests may contain lysine-containing peptides with an α -acetyl group, but the yields of the acetyl-peptides actually found imply that any undetected ones would be minor constituents.

The other peptides described had some properties similar to those of the acetyl-peptides, e.g. their elution character and lack of ninhydrin reaction or *N*-terminal amino acid. The minor acetyl-peptide, F2a2/CBA, occurred in very small and variable yield. The other peptides did not contain acetyl

* Abbreviation: PCA, pyrrolidonecarboxylic acid.

groups and in three cases (F2a1/K, F2a2/B and HB) appear to have PCA at the *N*-terminus. The cyclizing of glutamic acid to PCA can occur easily in weak acid and the formation of these peptides in this way is suggested by the occurrence of the uncyclized Glu-Gly-Gly-Arg and Gln-Gly-Gly-Arg among the other peptides from histone F2a2 not reported here. The yield of the peptides F2a2/B and HB also varied with the peptide preparation. With histone F2a1, the arginine-peptides have not yet been examined sufficiently to know whether the uncyclized sequence corresponding to peptide K occurs or not. The presence of minor amounts of PCA as an *N*-terminal residue in the intact proteins is possible, especially as it has been reported as the original *N*-terminal group in immunoglobulins (Porter & Press, 1965; Press, Piggot & Porter, 1966). The occurrence of the same acetyl-peptide in these two major histone fractions is not due to cross-contamination in view of the large yields obtained. These histone fractions have distinct properties and compositions and are homogeneous in polyacrylamide-gel electrophoresis (Phillips & Johns, 1965; Johns, 1967a). Moreover, the comparison of the peptide patterns and the incomplete results from analysis of the peptides suggests that only about one-third of them are identical.

The 'very lysine-rich' histone fraction F1 (lysine: arginine ratio about 15:1) also appears to lack an *N*-terminal amino acid and to contain *N*-acetyl groups (Phillips, 1963b). A terminal acetyl-peptide from this fraction has not yet been found, but it can be said that it is not in the same sequence as in the histones F2a1 and F2a2 and is not in a tryptic peptide containing arginine as the basic group.

It has been reported that acetylation *in vivo* of histones can occur after the synthesis of the proteins is complete, and it was suggested that this altered their binding capacity to DNA in the mechanism of template activity (Allfrey, Pogo, Pogo, Kleinsmith & Mirsky, 1966). However, the unacetylated peptide corresponding to peptide F2a2/D has not been found, though a small degree, such as 10%, of unmasking of α -amino or masking of ϵ -amino groups would probably not have been detected. It is not thought that the preparative

methods would separate the monoacetylated and unacetylated forms of the proteins. As isolated from calf thymus, these particular histones thus appear to be almost completely α -*N*-acetylated.

The number of proteins known to have an α -*N*-acetyl terminus is about 16, and though the number is small it seems significant that six (including the histones) have acetylserine termini.

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