Guanidination of lysine and hydroxylysine in ichthyocol

LEVY, FISHMAN AND CABRERA[1] reported recently that approx. 35–40 % of the lysine residues in tendon collagen and in ichthyocol are not available for reaction with nitrosyl chloride and they concluded that these lysine ε-amino groups are not free. Evidence for the presence in collagen of ε-amino groups in peptide linkage was provided by the isolation from partial hydrolyzates of a peptide in which the ε-amino group of lysine is bound in this manner[2].

The determination of the precise number of ε-amino groups bound in peptide linkage would be of considerable importance. The possibility that 35–40 % of the lysine residues (8–10 lysine residues per 1000 total amino acid residues) present in collagen are bound in ε-amino linkage is not in agreement with other available data for the extent of the ninhydrin reaction and the number of peptide bonds split by trypsin. Trypsin is known to split bonds only involving lysine or arginine residues. Using the quantitative ninhydrin reaction[3] on collagens from various sources[4], it is found that the equivalent weight (weight of the protein divided by the number of leucine equivalents) is 3400 ± 200. For more drastically treated gelatins the value approaches 3700–4000. An equivalent weight of 3400 is equivalent to 27 residues per thousand total amino acid residues if one takes 93 as the average amino acid residue weight for ichthyocol. This value is within the experimental error of the lysine plus hydroxylysine values found for ichthyocol and for other collagens[5]. If one assumes a maximum of 2 α-amino groups (N terminal) per thousand residues, the estimate of 8–10 lysine residues per 1000 total amino acid residues which are unavailable for reaction is not compatible with the data.

When gelatin derived from ichthyocol or calf-skin collagen is completely digested with trypsin, the average equivalent weight of the peptides formed is 1730 based on the ninhydrin reaction, and 1200 as determined by formal titration[6]. This discrepancy might be expected since the hydrolysis of Lys·Pro, Arg·Pro or the corresponding hydroxyproline-containing linkages would not be accompanied by an increase in ninhydrin color, but would yield the expected increase in the formol titration. In ichthyocol and in calf collagen there are approx. 46 residues of arginine and 24 of lysine. Thus, an equivalent weight of approx. 1300 is obtained by dividing 70 into 93,000. This is in good agreement with the formol-titration data obtained after tryptic digestion, *i.e.*, every bond involving a lysine or arginine residue is split within an error of approx. 2 residues. These data suggest that at most 2–4 lysine residues are unreactive and might have their ε-amino group in some type of linkage, presumably peptide.

The end-group methods discussed yield data which offer inferential evidence concerning the number of bound ε-amino linkages, but cannot provide conclusive proof of the quantitative aspects of this problem. For our study of the lysine residues in collagen, we have chosen the guanidination procedure of HABEEB[7] in which the active guanidinating agent is GDMP. The results of HABEEB[7] have been confirmed in that as much as 94 % of the lysine residues of serum albumin can be converted to homoarginine by this guanidination procedure. When the extent of the reaction and the mildness of the conditions employed are considered, this procedure seems to be

Abbreviation: GDMP, 1-guanyl-3,5-dimethyl pyrazole nitrate.

the one of choice in determining whether ε-amino groups of lysine might be in covalent linkage.

Ichthyocol (approx. 37 mg) and gelatin (derived from approx. 72 mg ichthyocol by heating at 53° for 1 h) were treated with 0.41 *M* GDMP (0.5 g in a final volume of 6 ml) at pH 9.5 for 7 days at 5°. Ichthyocol was soluble in the concentration of the reagent used and did not appear to undergo denaturation. In the presence of the reaction mixture, gelatin exhibited a tendency to gel at a low temperature; however, because of continuous stirring and the low concentration of protein, the gel was not firm. After allowing the reaction to proceed for 7 days, the mixtures were dialyzed for 3 days against 0.1 *M* phosphate buffer (pH 7.4) then against distilled water for 4 days with many changes of the dialyzing fluid. The proteins were hydrolyzed for 18 h at 105° with 6 *N* HCl in a sealed vial; HCl was removed *in vacuo* and amino acid analyses conducted with the automatic amino acid analyzer of SPACKMAN, MOORE AND STEIN[8]. The pertinent results are summarized in Table I. It should be noted

TABLE I

CONTENT OF CERTAIN AMINO ACIDS IN GUANIDINATED ICHTHYOCOL AND GUANIDINATED ICHTHYOCOL GELATIN COMPARED WITH ICHTHYOCOL

Amino acid	*Ichthyocol (control)*	*Guanidinated ichthyocol*	*Guanidinated ichthyocol gelatin*
	Residues per 1000 total amino acid residues		
Lysine	22.6	3.1	1.6
Homoarginine	—	21.8	24.4
Hydroxylysine	5.7	1.2	0.4
Hydroxyhomoarginine	—	3.6	2.6

that the only two amino acids which changed significantly in content as a result of the guanidination were lysine and hydroxylysine. 87 % of the lysine of ichthyocol and 94 % of the lysine of gelatin were converted to homoarginine. The hydroxylysine content also decreased and in each case a new double peak was eluted from the short column (15 cm) at a position between ammonia and arginine. The double peak is believed to be hydroxyhomoarginine and its allo form, the products that would be expected from the guanidination of hydroxylysine. It is known that hydroxylysine shows a double peak characteristic of the normal and allo forms if chromatographed on a longer column[9]. While the new double peak observed on the short column has not been definitely established as hydroxyhomoarginine and its allo form by comparison with authentic samples, it appears to account for 75 % of the loss in hydroxylysine content of ichthyocol and 87 % in the case of gelatin. The contents of other amino acids in the treated proteins were within experimental error of those for the control.

JANUS[10], employing the SAKAGUCHI method for analysis, showed that guanidination of lysine and hydroxylysine residues of bone and hide gelatins with O-methylisourea at pH 10.5 was complete. The equivalent weight per guanidino group formed can be calculated as 1100 in one case and 1250 in another, essentially in agreement with our data indicating few bound ε-amino groups.

From the data in Table I it appears that no more than 2–4 residues of lysine and

1 residue of hydroxylysine are protected from guanidination with GDMP. This is a maximum value and is in contrast to the high value of 8–10 residues calculated from the data of LEVY *et al.*[1] as being unreactive with nitrosyl chloride. It is known that the ε-amino peptide bond of lysine which is present in Bacitracin A is extremely resistant to acid hydrolysis[11,12], therefore if the unreactive ε-amino groups of lysine were bound in peptide bonds, one should have expected a greater quantity of ε-amino peptides of lysine in acid hydrolyzates of gelatin than was found by MECHANIC AND LEVY[2]. The peptide isolated by them accounted for less than 1 % of the lysine content of the protein. Similarly, the detection of a large quantity of free lysine after nitrosyl chloride treatment followed by acid hydrolysis[1] when considered with the reported stability of ε-amino peptide bonds is apparently not compatible with the presence of a large number of ε-amino peptide bonds in gelatin and collagen.

Since nitrosyl chloride gave quantitative destruction of the free amino groups of lysine in ribonuclease[1], it is surprising that so many of these residues in ichthyocol were unaffected by the reagent. This point should be investigated further to determine how the collagen structure renders the ε-amino groups of certain lysine residues inaccessible to the action of nitrosyl chloride under the experimental conditions of LEVY *et al.*[1].

It should be emphasized that while the number of bonds involving the ε-amino groups of lysine appears to be small according to our observations, these could still serve an unique role in cross-linking in collagen. These linkages should be sought by attempting to isolate peptides in the manner described by MECHANIC AND LEVY[2].

The observation that hydroxylysine can be guanidinated indicates the presence of this amino acid residue in the protein in a form in which the ε-amino group is readily accessible. Possible formation of hydroxylysine as a hydrolysis product of a cyclic structure, such as an oxazoline, appears unlikely.

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