COLLAGEN CROSSLINKING: ISOLATION OF HYDROXYALDOL-HISTIDINE, A NATURALLY-OCCURRING CROSSLINK

T. Housley and M.L. Tanzer

Department of Biochemistry
University of Connecticut Health Center
Farmington, Connecticut 06032

E. Henson and P.M. Gallop

Department of Biological Chemistry and
Children's Hospital Medical Center
Harvard Medical School
Boston, Massachusetts 02115

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SUMMARY: A new trifunctional crosslink, termed hydroxyaldol-histidine, was isolated from cow skin collagen. This compound was not reducible by sodium borohydride; it was characterized by PMR spectroscopy and by low and high resolution mass spectroscopy of volatile derivatives. This crosslink is identical to an unknown amino acid previously detected in pure collagen-derived peptides. We postulate that it arises by condensation of peptidyl allysine, hydroxyallysine and histidine. This is the first example of a non-borohydride reducible crosslink found in collagen.

INTRODUCTION: We have previously described the isolation and characterization of histidine-containing, multi-functional crosslinks from collagen (1,2). These substances were first chemically reduced by NAB^3H_4 treatment of the protein, followed by acid hydrolysis and purification of the radioactive compounds. Recently, we have omitted the chemical reduction step and have isolated ninhydrin-reactive unknowns directly from the acid hydrolysates. In this report we describe the structure and postulate the origin of hydroxyaldol-histidine (Fig. 1), a collagen crosslink related to other histidine-containing crosslinks (1,2).

MATERIALS AND METHODS: Insoluble cow skin collagen, generously supplied by Dr. G. Mechanic, and crosslinked peptides of insoluble calf skin collagen, generously supplied by Drs. R. Timpl and U. Becker, were hydrolyzed in 6N HCl for 24 hours at 108°. In some experiments, prior reduction of these

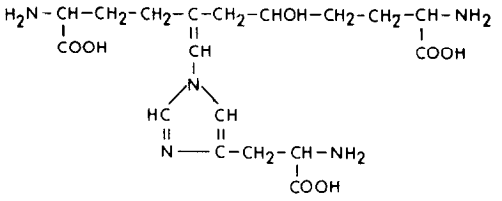

Figure 1. Postulated structure of the amino acid, hydroxyaldol-histidine.

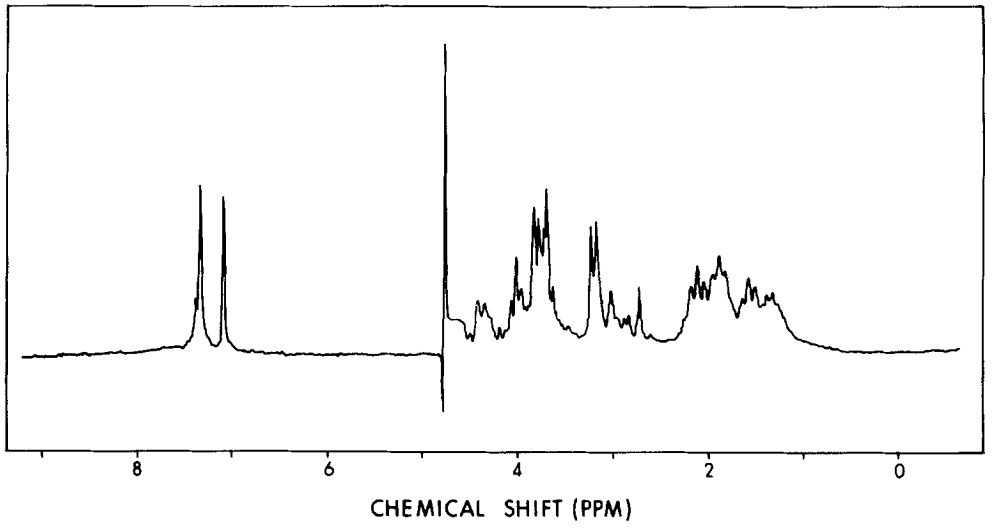

Figure 2. Proton magnetic resonance spectrum of hydroxyaldol-histidine.

substances was done with NAB^3H_4 or $NABD_4$, as previously described (2). The hydrolysates were fractionated by sequential ion-exchange chromatography and various substances were purified to homogeneity, as determined by chromatography on two different analytical columns of the amino acid analyzer (2). The isolated unknowns were characterized by PMR spectroscopy on a JEOL PFT-100 instrument, using water resonance suppression (3), and by mass spectrometry of volatile derivatives, using a Hitachi RMU-6 instrument (4), or an AEI MS-30 instrument (Shrader Analytical, Detroit, Michigan).

RESULTS: Figure 2 shows the PMR spectrum of hydroxyaldol-histidine in D_2O at pH 5.7, 29°. All chemical shifts are referenced to a TSS external standard, with an internal deuterium lock signal. The spectrum showed singlets at 7.41

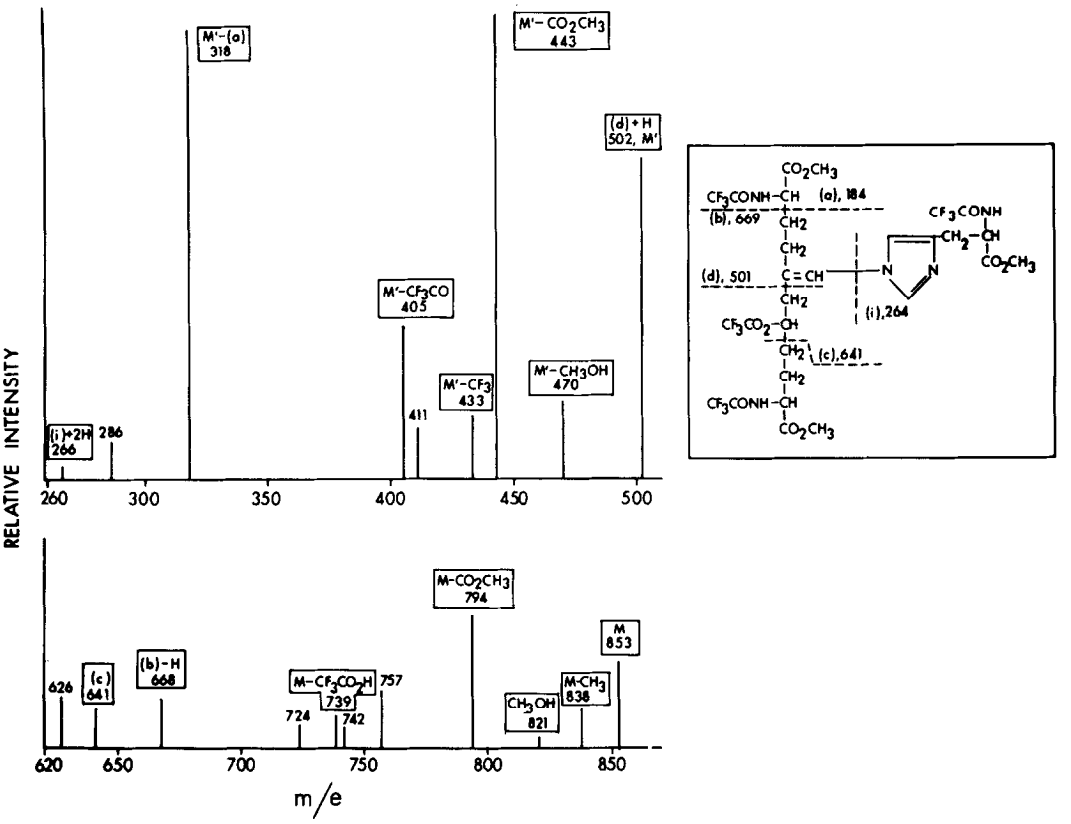

Figure 3. Mass spectrum, proposed structure and fragmentation of the trifluoroacetyl methyl ester derivative of hydroxyaldol-histidine.

(C-2, imidazole), 7.36 (vinyl), and 7.12 (C-4, imidazole). The two overlapping singlets, furthest downfield, decreased over several days; all other signals remained the same. The C-2 imidazole proton is known to slowly exchange (5,6). The other features of the spectrum are the triplet at 4.03 (α-CH, histidine), the envelope centered at 3.79 (non-histidine α-CH groups and a single CHOD group), the doublet at 3.24 and 3.19 (β-CH_2, histidine), and the remainder of the signals from 0.98 to 2.1 encompassing the protons of the β, γ and δ methylene groups. All of these assignments are based upon previous studies (1,2,6).

The mass spectrum of the trifluoroacetyl methyl ester derivative of hydroxyaldol-histidine (Fig. 3) showed a molecular ion of 853, consistent

with an elemental composition of $C_{29}H_{31}O_{11}N_5F_{12}$. The fragmentation pattern was similar to that of the same derivatives of other histidine-containing crosslinks (1,2). Virtually all of the higher mass fragments and most of the prominent fragments could be accounted for by well known losses, as detailed in Figure 3. Thus, the simple cleavages of M-CH_3 (838), M-CH_3OH (821), M-CO_2CH_3 (794) were present, as was the ion due to the loss of trifluoroacetate, M-CF_3CO_2H (739). This latter loss, in conjunction with simple cleavages, accounted for the ions at 724 (M-(CF_3CO_2H + CH_3)) and 626 (M-(CF_3CO_2H + CF_3CONH_2)). The prominent ion at 502 could be accounted for by the cleavage shown in Figure 3, with incorporation of a proton. This assignment was supported by exact mass measurements (Table I), which also corroborated the assignments of the ions derived from the 502 fragment. Table I also substantiates the imidazole fragments, ions 266 and 206, as previously detailed (1,2). Thus, all of the evidence is consistent with the proposed structure (Fig. 1) of hydroxyaldol-histidine.

Prior reduction of the collagen with NAB^3H_4 or $NABD_4$, followed by isolation of hydroxyaldol-histine showed: 1) no tritium was incorporated into this compound while previously described crosslinks were readily labeled; 2) no deuterium incorporation was detected by either PMR spectroscopy or mass spectrometry. Reduction of the isolated crosslink with $NABH_3CN$ in ^3H_2O at pH 4.0 (7) was also ineffective, as determined by radioactive and chromatographic analysis of the treated compound.

DISCUSSION: The evidence that this new amino acid serves as a crosslink in collagen, tying together three polypeptide chains, is based upon finding stoichiometric amounts of the compound in a homogeneous, collagen-derived polypeptide (8). This peptide had a structure consistent with 3 constituent chains, one arising from a region of α1-CB 5 and another from α1-CB 6. Of interest in this regard is the known location of histidine 89 in α1-CB 5 and the location of allysine in the non-collagen extension of α1-CB 6 (9). The source of the hydroxyallysine (Fig. 4) is not clear at present, but it

TABLE I

EXACT MASSES OF TRIFLUOROACETYL METHYL ESTER DERIVATIVE OF HYDROXYALDOL-HISTIDINE

m/e	Origin (Fig. 3)	Observed mass	Calculated mass	Calculated for
502	M^1	502.1231	502.1288	$C_{18}H_{20}O_6N_4F_6$
471	M^1-CH_3O	471.1112	471.1104	$C_{17}H_{17}O_5N_4F_6$
470	M^1-CH_3OH	470.1066	470.1026	$C_{17}H_{16}O_5N_4F_6$
443	$M^1-CO_2CH_3$	443.1154	443.1155	$C_{16}H_{17}O_4N_4F_6$
433	M^1-CF_3	433.1308	433.1336	$C_{17}H_{20}O_6N_4F_3$
411	$M^1-(CH_3OH + CO_2CH_3)$	411.0836	411.0892	$C_{15}H_{13}O_3N_4F_6$
405	M^1-CF_3CO	405.1367	405.1387	$C_{16}H_{20}O_5N_4F_3$
318	$M^1-(a)$	318.1068	318.1067	$C_{13}H_{15}O_3N_3F_3$
266	i+2H	266.0733	266.0753	$C_9H_{11}O_3N_3F_3$
206	$i-(CO_2CH_3)+H$	206.0520	206.0542	$C_7H_7ON_3F_3$

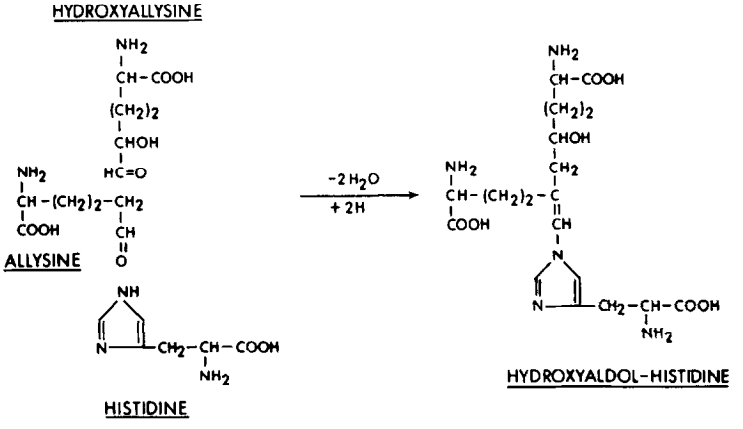

Figure 4. Postulated origin of hydroxyaldol-histidine by condensation of three amino acids present in collagen.

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could conceivably arise from an α2 chain, whose sequence is not completely known.

Thus, we postulate that hydroxyaldol-histidine could arise from condensation of allysine, hydroxyallysine and histidine as shown in Figure 4. Although the mechanistic details are not clear, one must invoke the loss of two molecules of H_2O and a reductive alkylation to obtain the final product, analogous to lysinonorleucine formation (10). The postulated structure is that of a conjugated enamine, which can be quite resistant to borohydride reduction, even at acid pH when exposed to $NABH_3CN$ (7). Therefore, the lack of reduction by the various borohydride compounds is perfectly consistent with the structure.

We have previously isolated the borohydride-reducible crosslinks, aldol-histidine and histidino-hydroxymerodesmosine from cow skin collagen (1,2) and we find that this new compound is present in relatively large amounts in the same preparation. Moreover, its abundance does not seem to change when the collagen is previously treated with $NABH_4$, suggesting that it does not become incorporated into other compounds upon reduction. Most importantly, the reservations held concerning the physiological role of histidine-containing crosslinks (11) are not valid in the case of hydroxyaldol-histidine, because it clearly forms without any chemical treatment of the collagen. This amino acid is the first example of a non-borohydride reducible, histidine-containing collagen crosslink.

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