of Sloan-Kettering Institute for partial sequencing of the final chymotrypsin product and to Anthony Mazzeo for assistance with amino acid analyses.

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Characterization of a Highly Soluble Collagenous Molecule Isolated from Chicken Hyaline Cartilage†

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ABSTRACT: Recently, we have isolated a new collagenous molecule from chicken hyaline cartilage after limited pepsin digestion. This molecule, which contains interchain disulfide bonds, has been called the high molecular weight fraction or HMW [Reese, C. A., & Mayne, R. (1981) *Biochemistry 20*, 5443-5448]. We now present a detailed model for the structure of HMW, the model being derived from analyses both of components of HMW obtained after denaturation either with or without reduction and from electron microscopic observation of replicas of HMW obtained after rotary shadowing. We propose that HMW is a typical, triple-helical collagen molecule of length 134 nm, in which one of the chains has been cleaved at a distance of 96 nm from one end of the triple helix, while the other two chains remain uncleaved. The result of this cleavage is the appearance of a recognizable kink in molecules of HMW when visualized after rotary shadowing.

We have recently reported that chicken hyaline cartilage contains two collagenous molecules which we have called the high molecular weight (HMW) and low molecular weight (LMW) fractions, these collagens comprising approximately 5% of the total soluble collagen (Reese & Mayne, 1981). HMW and LMW are solubilized after limited pepsin digestion of chicken sterna and can be fractionated from type II collagen and the native forms of the 1α, 2α, and 3α chains (Burgeson & Hollister, 1979) by differential salt precipitation performed in acidic conditions, these molecules remaining in solution at 1.2 M NaCl-0.5 M HOAc but being precipitated at 2.0 M NaCl-0.5 M HOAc. The relationship between HMW and LMW is at present uncertain, and it is not known if LMW is derived from HMW by further proteolytic cleavage (Reese

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& Mayne, 1981). HMW is very similar in amino acid composition and solubility properties to a collagenous molecule recently isolated from pepsin digests of mammalian cartilages (Shimokomaki et al., 1980; Ayad et al., 1981) but differs in being larger and in giving rise to several peptides (called C-1, C-2, and C-3) after denaturation and reduction. In the present paper, we present a detailed model for the structure of HMW, the model being derived from biochemical analyses of the peptides derived from HMW after denaturation and reduction and from electron microscopic observation of HMW by using the rotary shadowing technique.

Materials and Methods

Isolation and Fractionation of HMW. Collagen was solubilized from chicken sterna by limited pepsin digestion, and HMW together with LMW was prepared by fractional salt precipitation between 1.2–2.0 M NaCl and 0.5 M acetic acid as described previously (Reese & Mayne, 1981). Separation of HMW from LMW was achieved by molecular sieve chromatography (Bio-Gel A-5m) after denaturation (Reese & Mayne, 1981). For further characterization, HMW was reduced with 2-mercaptoethanol (0.1 M) at 37 °C and alkylated with iodoacetic acid (0.2 M) at room temperature as described (Mayne et al., 1977). The three components, C-1, C-2, and C-3, obtained after reduction of HMW were fractionated by molecular sieve chromatography on a column (1.5 × 155 cm) of agarose beads (Bio-Gel A-1.5m, 200–400 mesh). The column was eluted with 1 M $CaCl_2$–50 mM Tris-HCl, pH 7.5, at a flow rate of 7.4 mL/h. Calibration of the column was achieved with a mixture of cyanogen bromide peptides derived from chicken type II collagen.

Polyacrylamide Gel Electrophoresis. Characterization of the components C-1, C-2, and C-3 was carried out by Na$DodSO_4$–polyacrylamide gel electrophoresis with 5–7.5% gradient slab gels as described previously (Reese & Mayne, 1981). Peptides obtained after *Staphylococcus aureus* V8 protease digestion were separated with 9–15% gradient slab gels.

S. aureus V8 Protease Cleavage. The digestion conditions were as described previously (Reese & Mayne, 1981), except that the enzyme to substrate ratio was 1 to 10.

Amino Acid Analyses. Samples were hydrolyzed in 2 mL of constant-boiling HCl at 108 °C for 24 h and were analyzed with a Beckman 121 M automatic analyzer by using a dual column procedure as described previously (Butler et al., 1977).

Rotary Shadowing. Samples were dissolved in 0.1 M HOAc (10 μg/mL) to which an equal volume of glycerol was added. The samples were sprayed onto mica disks, placed in the vacuum chamber of an Edwards Model 306 vacuum coater, and evacuated to a pressure of 1×10^{-5} torr before being shadowed with platinum at an angle of 9° followed by carbon coating at 90°. The replicas were floated on water, picked up on a grid, and examined in a Siemens electron microscope (Elmiscope 102). Full details of the procedure, together with the calibration procedures for the electron microscope and the photographs, have been given elsewhere (Kühn et al., 1981). Lengths of individual molecules were determined by using a Nunomic electronic graphic calculator.

Results

Initially, HMW and LMW were isolated as the precipitate obtained after dialysis of a solution of pepsin-solubilized collagen from 1.2 M NaCl–0.5 M HOAc to 2.0 M NaCl–0.5 M HOAc. HMW was then separated from LMW by molecular sieve chromatography after denaturation (Reese & Mayne, 1981), followed by desalting and lyophilization. Figure 1 shows the separation by agarose gel filtration of the three components C-1, C-2, and C-3 obtained after the reduction and alkylation of HMW. Further purification of each component was achieved by a second passage over the agarose column, so that each component gave a single band on polyacrylamide gel electrophoresis (Figure 1, inset). Calibration of the agarose column with a mixture of CNBr peptides derived from chicken type II collagen gave molecular weight values of 87 500, 51 000, and 36 400 for C-1, C-2, and C-3, respectively. The molecular weight of C-1 was therefore the sum of the molecular weights of C-2 and C-3, suggesting that C-2 and C-3 may occasionally be joined by a nonreducible cross-link. In order to examine this possibility directly, C-1, C-2, and C-3 were separately digested with *S. aureus* V8 protease and the resulting peptides analyzed by polyacrylamide gel electrophoresis (Figure 2). Several prominent peptides were present in the digests of both C-1 and C-2, and, in addition, some peptides derived from C-3 could also be observed in the digest of C-1. It therefore seems likely that C-1 arises from the formation of a nonreducible linkage between C-2 and C-3, and the rotary shadowing observations of HMW which will be described below strongly support this suggestion.

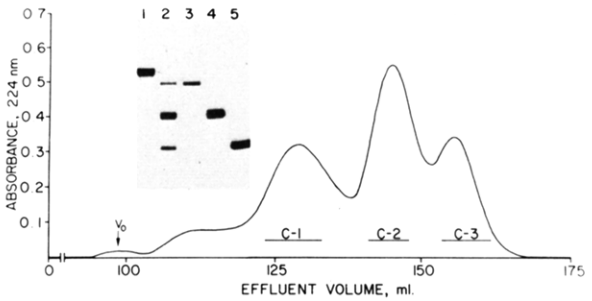

FIGURE 1: Agarose (Bio-Gel A-1.5m) molecular sieve elution pattern of HMW after reduction and alkylation. Bars indicate fractions of C-1, C-2, and C-3 which were pooled for further purification by repeated molecular sieve chromatography. Inset shows sodium dodecyl sulfate (Na$DodSO_4$)–polyacrylamide gel electrophoresis (5–7.5% gradient gel) of (lanes 1 and 2) HMW before and after reduction and (lanes 3–5) C-1, C-2, and C-3, respectively.

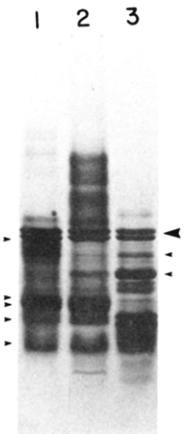

FIGURE 2: Na$DodSO_4$–polyacrylamide gel electrophoresis (9–15% gradient gel) of peptides obtained after *S. aureus* V8 protease digestion. The digestion was performed in electrophoresis sample buffer but without Na$DodSO_4$ (37 °C, 30 min, enzyme:substrate ratio = 1:10). Before electrophoresis, Na$DodSO_4$ (final concentration 0.1%) was added and the sample heated (100 °C, 30 s). Lane 1, C-2; lane 2, C-1; lane 3, C-3. Each lane was loaded with 50 μg of sample. The larger arrow indicates the doublet band of the V8 protease enzyme. The small arrows indicate bands shared by C-1 and C-2 or C-1 and C-3.

Amino acid analyses were made of C-1, C-2, and C-3 together with the component C-4 (to be introduced below) and

Table I: Amino Acid Compositions of Components Recovered after Agarose Gel Chromatography[a]

amino acid	residues/1000 C-1	C-2	C-3	C-4
3-Hyp				
4-Hyp	112	112	89	161
Asp	59	60	48	53
Thr	18	18	19	9
Ser	27	27	19	20
Glu	93	89	128	62
Pro	114	118	87	146
Gly	319	317	335	327
Ala	56	54	47	48
$^1/_2$-Cys[b]	4	3	3	0
Val	15	14	30	20
Met[c]	5	4	5	5
Ile	24	26	20	14
Leu	50	51	41	59
Tyr	2	2	5	trace
Phe	5	5	3	trace
Hyl	31	33	52	30
Lys	18	20	10	17
His	5	5	7	4
Arg	43	44	52	25

[a] Each analysis is expressed as residues/1000. No corrections were made for loss of threonine or serine, or the incomplete release of valine. [b] Determined as *S*-(carboxymethyl)cysteine. [c] Determined as the sum of methionine and methionine sulfoxide. The values are the average of closely agreeing duplicate determinations.

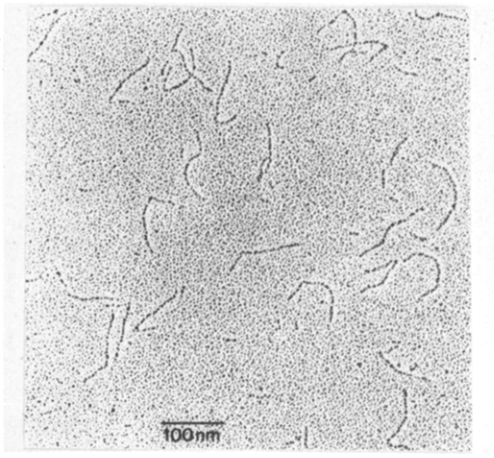

FIGURE 3: Electron micrograph of the 2.0 M NaCl–0.5 M HOAc precipitate as observed after the technique of rotary shadowing. Many of the larger molecules of HMW possess a prominent kink giving rise to a short and long arm. Smaller rods of LMW are also present.

are presented in Table I. In general, the results show that all four components are collagenous with compositions very similar to analyses for type V collagen, having the characteristically low content of alanine as discussed previously (Reese & Mayne, 1981).

The precipitate obtained at 2.0 M NaCl–0.5 M HOAc, which contained HMW and LMW in native form, was dissolved in 0.1 M HOAc, and replicas were prepared by using the rotary shadowing technique. The replicas were picked up on a grid and examined in the electron microscope (Figure 3). HMW was observed as a linear molecule in which a prominent kink was often present at one location along the molecule, giving rise to a short and a long arm. The angle between the short and the long arm was not fixed, and occasionally molecules were observed in which the short arm was apparently folded back on the long arm. Molecules of LMW were also sometimes observed as short rods. Measurement of the length of HMW gave the following values: (i) total

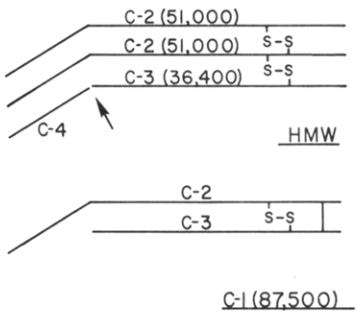

FIGURE 4: Model for the structure of HMW and for the component C-1 obtained after reduction of HMW. In this model, each chain is joined to the other chains by at least one disulfide bridge, and in some molecules, a nonreducible cross-link is present between C-2 and C-3, giving rise to C-1 after reduction. The arrow signifies the location of a probable pepsin-sensitive site. The precise location and structure of the disulfide bridges and the nonreducible cross-link in HMW are at present unknown, but both must be present in the long arm. The location of the amino and carboxy termini of the molecule is also unknown.

length = 134 nm (standard deviation ±5 nm, 117 molecules measured); (ii) length of the long arm = 96 nm (±5 nm, 41); (iii) length of the short arm = 38 nm (±3 nm, 41). It is possible to calculate the molecular weight of a collagen chain present in a molecule of HMW from the length of HMW. The calculation requires the use of the previously determined value of 0.286 nm for the residue length of a chain present in the collagen triple helix when measured parallel to the axis of the molecule (Rich & Crick, 1961) and an average molecular weight of 110 for each amino acid of HMW including possible carbohydrate residues (this is the same value which was recently used for type IV collagen; Kühn et al., 1981). The molecular weight of a chain present in the full length of HMW was calculated to be 51 500, and by a similar calculation, the molecular weight of a chain present only in the long arm of HMW was calculated to be 36 900. These values agree very closely with our previously determined values for the molecular weights of C-2 and C-3 as determined by agarose gel filtration (51 000 and 36 400, respectively) and have led us to propose a model for the structure of HMW, which is shown in Figure 4. It is suggested that the kink arises in molecules of HMW because either one of the chains has been cleaved during pepsin digestion or, less likely, cleavage has occurred during tissue processing of a precursor molecule. The other two chains then remain uncleaved. After denaturation, all three chains continue to be interconnected in the long arm by disulfide bridges, and occasionally a nonreducible cross-link is formed in the long arm between C-2 and C-3, giving rise to C-1. The model leads to several predictions which can be tested experimentally. Since the short arm of HMW is observed by rotary shadowing, it must be in a triple-helical conformation. A peptide C-4 must therefore be present in native molecules of HMW, which is not disulfide bonded to the other chains and is lost from HMW during denaturation. The molecular weight for C-4 can be calculated to be approximately the difference in molecular weights of C-2 and C-3, or 14 600. We have now isolated C-4 from HMW after denaturation without reduction (see below) and found the peptide to have an apparent molecular weight by agarose gel filtration of 14 000. Another feature of the model is the assumption that all molecules of HMW have been cleaved between C-4 and C-3, despite the fact that the kink cannot always be recognized in molecules of HMW after rotary shadowing. It can, however, be calculated from the molecular weights of C-2, C-3, and C-4 that C-4 should represent 9.2% of the total amount of undenatured HMW if all molecules are cleaved between C-3 and C-4. A further

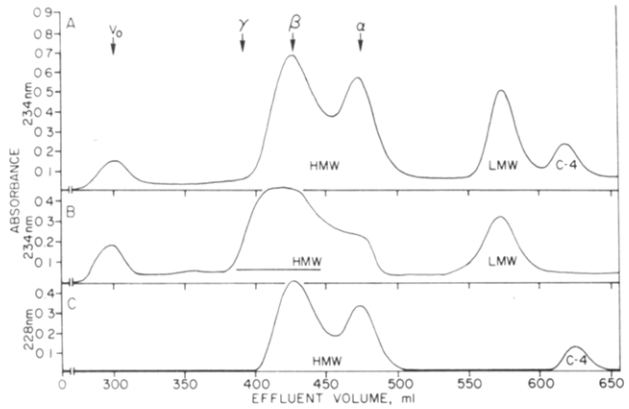

FIGURE 5: Agarose (Bio-Gel A-5m) molecular sieve elution patterns of the 2.0 M NaCl–0.5 M HOAc precipitate either with or without denaturation. (A) The sample was dissolved in elution buffer (1 M $CaCl_2$, pH 7.5) and denatured (55 °C, 20 min) prior to being applied to the column. Note the presence in addition to HMW and LMW of a small peptide labeled C-4. (B) The sample was dissolved in elution buffer but not denatured prior to application to the column. Note that C-4 was no longer present in the eluate. The bar indicates the fractions of HMW which were pooled, desalted, and lyophilized. (C) HMW which eluted from the agarose column in native form (panel B) but which was now denatured prior to being applied to the column. Note that C-4 now eluted from the column.

prediction from the model concerns the behavior of HMW after denaturation and subsequent renaturation. If HMW is denatured, the peptide C-4 will be lost. However, if the molecule is subsequently placed in acid conditions (0.1 M HOAc), the disulfide bridges present in the long arm will promote renaturation, but the renaturation should only proceed for the length of the long arm. The renatured molecules of HMW observed by rotary shadowing should therefore be approximately 96 nm in length and should lack the kink and the short arm.

Figure 5 (panel A) shows a typical separation by molecular sieve chromatography of the 2.0 M NaCl–0.5 M acetic acid precipitate after denaturation as described previously (Reese & Mayne, 1981). Characteristically, HMW separates into two peaks, followed by the elution of LMW and subsequently by a small peptide of apparent molecular weight 14000 which is labeled C-4. Experiments were next performed in which the sample was dissolved in elution buffer (1 M $CaCl_2$, pH 7.5) but not denatured prior to being passed over the molecular sieve column. Panel B shows that HMW now eluted as a rather broad peak followed by LMW and that C-4 was no longer present in the eluate. HMW, which had apparently eluted in native form, was desalted, lyophilized, and passed again over the molecular sieve column, but now after denaturation (panel C). The peptide C-4 was now observed to elute from the column at the expected location. The absorbance values for HMW and C-4 were measured from panel C, and it was calculated that C-4 represented 8.9% of the total amount of undenatured HMW. This is very close to our predicted value of 9.2%, if all of the molecules of HMW are cleaved between C-4 and C-3. Additional experiments were also performed in which molecules of HMW, which had apparently eluted from agarose gel filtration in native form (Figure 5, panel B), were examined by rotary shadowing. Both the long and the short arms were observed (R. Mayne, H. Wiedemann, and C. A. Reese, unpublished results).

Figure 6 shows an electron micrograph prepared after rotary shadowing of HMW which was initially isolated by molecular sieve chromatography after denaturation (Figure 5, panel C) and then allowed to renature in 0.1 M acetic acid. The short arm of HMW can no longer be observed. However, in many

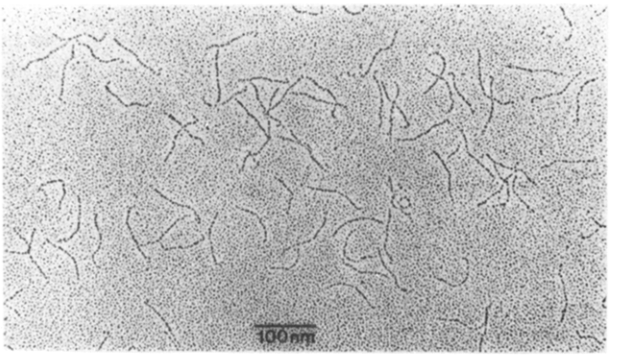

FIGURE 6: Electron micrograph after rotary shadowing of HMW isolated after denaturation and molecular sieve chromatography (Figure 5, panel C) but allowed to renature in 0.1 M acetic acid at room temperature. Note that the short arm of HMW can no longer be observed but that in many of the molecules there is a short hook at one end where the short arm is presumably located.

of the molecules, at one end a short "hook" was present which presumably represents the end of the molecule at which the short arm is located. Measurement of the average length of molecules including the hook gave a value of 98 nm (standard deviation ±8 nm; molecules measured = 67), which is very close to our previously determined length of 96 nm for the long arm of HMW. It thus appears that HMW will renature in dilute acetic acid, but with the loss of C-4 during denaturation, the short arm can no longer renature and is not observed after rotary shadowing.

The amino acid analysis of C-4 shows a very high content of proline and hydroxyproline (Table I), and this may explain why C-4 remains in a native conformation with the rest of HMW during agarose gel filtration provided the sample is not denatured prior to chromatography. It is known that proline and more especially hydroxyproline serve to increase the stability of the triple-helical conformation of collagen (Berg & Prockop, 1973; Bornstein & Traub, 1979).

Discussion

The model which we have presented for the structure of HMW appears to fit all of the biochemical and morphological observations which have so far been made. However, there are several features of the model which will require further investigation. It seems likely that the cleavage between C-3 and C-4 occurs during pepsin digestion, although we cannot exclude the possibility that some cleavage at this site may also have occurred during tissue processing of precursor forms of this collagen. Our calculations and experiments would suggest that all, or almost all, of the molecules of HMW are cleaved between C-4 and C-3 and that further cleavage of the other two chains cannot occur regardless of the pepsin concentration or length of exposure to pepsin at 4 °C. Apparently, only one of the chains of HMW contains a protease-sensitive site, which may be located in a short non-triple-helical region of one chain, as has recently been described for type IV collagen (Schuppan et al., 1980). We therefore suggest that molecules of HMW probably contain at least two different chains which are in a 2:1 proportion. However, we cannot at present eliminate the possibility that there are three different chains and that C-2 is therefore heterogeneous.

Recently, two groups have reported the isolation from mammalian cartilages of a collagenous molecule similar in solubility properties and amino acid composition to HMW (Shimokomaki et al., 1980; Ayad et al., 1981). However, the mammalian molecule appears to be smaller, with a reported molecular weight of only 110000, and gives rise to chains of

molecular weight 33 000 after reduction. We suggest from our model that this discrepancy in results between chicken and mammalian species arises because in mammals, during pepsin digestion, cleavage occurs at all three chains of HMW at the location of the kink, resulting in the isolation of only the long arm of HMW.

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Zinc-Sulfur Bonds of Aspartate Transcarbamylase Studied by X-ray Absorption Spectroscopy†

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ABSTRACT: X-ray absorption spectra have been recorded for aspartate transcarbamylase [unligated and ligated with the transition-state analogue *N*-(phosphonoacetyl)-L-aspartate] and for the model compound zinc dimethyldithiocarbamate. The spectra confirm that, in the enzyme, the zinc atom is ligated to four sulfur atoms, with a mean distance of 2.34 ± 0.03 Å. A spread in bond lengths of 0.1 ± 0.03 Å is possible, due to thermal and/or static disorder. No significant difference was found between the spectra of the ligated and unligated enzymes.

Aspartate transcarbamylase (ATCase)[1] from *Escherichia coli* [EC 2.1.3.2; reviewed by Jacobson & Stark (1973)] is a widely studied allosteric enzyme composed of six catalytic polypeptide chains (33 000 daltons each) and six regulatory chains (17 000 daltons each). The catalytic chains form two polar trimers which have only a restricted contact with each other (Monaco et al., 1978; Monaco, 1978). Instead, the principal connections between the trimers occur via the regulatory dimers, so the catalytic trimers and regulatory dimers are released when the regulatory-catalytic bonding domains are disrupted by treatment with mercurials (Gerhart & Schachman, 1965, 1968). This reaction breaks, in each regulatory chain, the bonds between a zinc atom and four cysteinyl sulfur atoms—bonds which maintain the structure of the region that binds to a catalytic trimer. Through their central position at this site, the zinc atoms must lie on or near the path of information transmission used when activation at a catalytic site on one trimer stimulates activation on the other trimer (Gibbons et al., 1976; Yang & Schachman, 1980).

Substitution of the zinc by other metals has produced changes in the optical spectrum over certain regions [250-280 nm in the circular dichroism after substitution by cadmium (Griffin et al., 1973); around 360 and 440 nm in the visible absorption spectrum after substitution by nickel (Johnson & Schachman, 1980)]. By use of the modified spectral region as a probe specific for the metal site, changes were found after activation of ATCase by carbamyl phosphate and aspartate (Griffin et al., 1973) or by PALA (Johnson & Schachman, 1980).

No crystal structure is yet available for the ligated form of ATCase, so there is no information from this source about any related structural changes, which in any case might easily be too small to be observed by this technique. However, information about metal-ligand distances in noncrystalline samples of enzymes can be obtained from extended X-ray absorption fine structure (EXAFS) measurements, permitting the detection of changes not only in the coordination number but also in bond lengths to below 0.1 Å (Shulman et al., 1978a; Stern, 1978; Cramer & Hodgson, 1979; Doniach et al., 1980). Moreover, this technique can be applied without needing to

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[1] Abbreviations: ATCase, aspartate transcarbamylase; EXAFS, extended X-ray absorption fine structure; PALA, *N*-(phosphonacetyl)-L-aspartate.

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