Molecular Pathology of Connective Tissues

edited by
Ruy Pérez-Tamayo
and
Marcos Rojkind

VOLUME 3 in THE BIOCHEMISTRY OF DISEASE

A Series of Monographs

MOLECULAR PATHOLOGY OF CONNECTIVE TISSUES

Editors

RUY PÉREZ-TAMAYO

Head, Department of Cell Biology
Instituto de Investigaciones Biomédicas
Universidad Nacional Autónoma de México
México D.F., México

and

MARCOS ROJKIND

Department of Genetics and Cell Biology
Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional
México D.F., México

MARCEL DEKKER, INC., NEW YORK 1973

MARCEL DEKKER, INC.
95 Madison Avenue, New York, New York 10016

LIBRARY OF CONGRESS CATALOG CARD NUMBER 72-86611
ISBN 0-8247-1588-8

PRINTED IN THE UNITED STATES OF AMERICA

PREFACE

It is now widely recognized that disease does not represent a qualitative, but rather a quantitative deviation from normal biological processes, usually beyond the adaptive capabilities of the individual. It is also generally agreed that there are two clearly distinguishable kinds or classes of diseases: those involve genetically determined biochemical and other characteristics, and arising from disturbances intrinsic to the individual, in the sense that they those resulting from the impact of the environment on a population that may be regarded as genetically uniform. Diseases belonging in the first category include degenerative vascular and neurological processes, autoimmune disturbances, cancer, as well as many of the infirmities that accompany senescence. The second general type of diseases refers to infections, nutritional deficiencies, intoxications, various kinds of accidental injuries produced by trauma, heat, blood loss, etc. The connective tissues appear to play a very prominent role in both categories of diseases, not only as the sites of major degenerative and immunologic disturbances (some, but not all of them included under the term *collagen diseases*), but more often as the very substrate of ominous sequelae of the many and varied pathological interactions of the individual with the environment. For these reasons, and also because in recent years there has been a very healthy increase in knowledge of various aspects of connective tissue, it has become most timely to take stock of it and explore its possible contributions to a better understanding of some abnormal processes.

The present volume represents an attempt to review several aspects of current basic research in connective tissues and to relate them to disease. Although the contents differ somewhat from that originally planned, the contributors kindly agreed to our request that their respective chapters should place emphasis on the relations, known or suspected, of recently acquired information to specific pathological processes.

It is a pleasure to record here our gratitude to our co-authors, both for the excellence of their contributions and also for their patience in finally seeing them come to light, after what probably was one of the longest gestational periods in the history of book making. Also, we wish to extend our thanks to Marcel Dekker, Inc.

RUY PÉREZ-TAMAYO
MARCOS ROJKIND
Mexico City, Mexico

CONTRIBUTORS TO THIS VOLUME

C. I. LEVENE, Scientific Staff of the Medical Research Council from the Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council, Cambridge, England

RUY PÉREZ-TAMAYO, Head, Departmento de Biología Celular, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México D.F., México

ROBERT E. PRIEST, Department of Pathology, Emory University, Atlanta, Georgia

MARCOS ROJKIND, Professor, Department of Genetics and Cell Biology, Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional, México D.F., México

JEREMIAH E. SILBERT, Connective Tissue Research Laboratory, Boston Veterans Administration Hospital, and, Department of Medicine and Biochemistry/Pharmacology, Tufts University School of Medicine and Dental Medicine, Boston, Massachusetts

MARGARITA ZEICHNER, Department of Genetics and Cell Biology, Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional, México D.F., México

CONTENTS

Chapter 1

MOLECULAR STRUCTURE OF THE FIBROUS COMPONENTS OF THE CONNECTIVE TISSUES

Marcos Rojkind

Professor
Department of Genetics and Cell Biology
Centro de Investigación y Estudios Avanzados del I. P. N.
México 14, D. F., México

I. COLLAGEN

A. Introduction

Because there are many excellent reviews for specialists on the proteins of the connective tissue, the aim of this chapter is more circumscribed. Our purpose is to introduce the general reader to the fascinating field of connective tissue and its diseases by first showing how the normal proteins are built. For this purpose a survey of the literature is presented with special attention given to the problems in which the author has both interest and personal experience.

The classic reviews by Bear (1) and Gustavson (2,3) heralded a new era in collagen research. Since those publications appeared, the chemistry, physical chemistry, and biology of collagen have been the subject of vigorous investigation, necessitating almost continuous revaluation of results. Accordingly, several specialized reviews and monographs have been published. Harrington and von Hippel (4) discussed the structure of collagen and the problems related to the collagen-fold; Veis (5) considered the physical chemistry of collagen and especially of gelatin; and Harding (6) reviewed the chemistry of the unusual links and crosslinks of collagen. The excellent review by Seifter and Gallop (7) not only covers some special aspects of the chemistry of collagen, but also of elastin and other structural proteins. The comparative biochemistry of collagen was analyzed thoroughly by Gross (8). Analytical methods used in collagen research have been assembled by Eastoe and Courts (9). Grassman (10) and Bailey (11) have published reviews that cover different aspects of the structure and function of collagen.

Selected articles on special topics have appeared in conference proceedings edited by Randall (12), Tunbridge (13), Stainsby (14), Ramanathan (15), Ramachandran (16), Hall (17), and Fitton-Jackson et al. (18). The biology and molecular biology of collagen has been discussed by Harkness (19) and Fessler (20). A complete survey on collagen biosynthesis was presented by Gould (21), and recent developments in the chemistry of crosslinking have been considered thoroughly by Piez (22). Finally, a three-volume treatise on collagen was edited by Ramachandran and Gould (23).

1. Definition

Many definitions of collagen are based on the physical and/or chemical properties of the protein. However, no one has provided all the information needed to obtain an overall picture of collagen. For this reason, the basic features of the protein are described rather than defined.

Collagen, the major protein of the extracellular connective tissue, is also the most abundant protein in the animal kingdom (8). The amount of collagen varies from tissue to tissue and from one species to another. Harkness et al. (24) have shown that 20% of the total body protein of the mouse is collagen. A similar estimate has been given by Neuberger and Richards (25) for mammalian proteins. In certain invertebrate forms, such as the Keratosa among the sponges and the sea cucumber among the echinoderms, collagen accounts for an even larger proportion of the total composition of the organism.

Collagenous tissue fibers are made of microfibrils of 0.2 to 100μ diameter. These occur in various arrangements, from a simple parallel array to a more complicated three-dimensional lattice. The nature of a specific organization is perhaps related to the function of the tissue. In the tendons, which must accomodate a high tensile strength applied along the longitudinal axis, the fibrils are closely packed in a parallel manner. At the electron microscope level, the main feature of the collagen fiber is the axial repeating period of 640 to 700 Å demonstrated by Schmitt et al. (26) and Wolpers (27) (Chap. 2, Sect. II). However, Reed and Rudall (28) have shown that collagen from earth worm cuticle has no axial periodicity, so that collagens containing a typical amino acid composition or wide angle X-ray diffraction pattern may not have a "normal" electron microscope appearance. Bear (29) and Kratky and Sekora (30) discovered the 640 Å axial repeating period of collagen using X-ray diffraction techniques. Bear (1) analyzed several vertebrate and invertebrate collagens and found a very reproducible wide-angle X-ray diffraction pattern, with some differences in the degree of orientation. The presence of a 3 Å meridional arc and of 12 Å equatorial spots that disappear upon heating of the fiber are reproducible findings for the collagen class of proteins.

Collagen also has a unique amino acid composition. One-third of the total amino acid residues is of glycine and one-fourth is of proline and hydroxyproline. The protein in devoid of tryptophan and has a very low tyrosine content; the presence of cystine, which occurs in some invertebrate collagens, has not been established for those of vertebrates.

Purified collagen in solution shows a characteristic infrared absorption spectrum. The band corresponding to the NH stretching frequency is at 3330 cm^{-1} instead of the normal 3300 cm^{-1}, suggesting a 3 Å distance for

hydrogen bonding. The specific optical rotation $[\alpha]_D$ is highly negative with values of about -350° to -400°, depending on species. Since this large negative value is related to the triple helical structure of collagen, the value drops to -110° to -120° upon heating and denaturation of the protein.

Ramachandran (31) reviewed the molecular structure of collagen and summarized six important physical and chemical parameters of the protein. He stated: "We can say that a specimen is certainly collagen if it exhibits the following characteristics:

a. One-third of the number of amino acid residues is glycine and about 20% consists of imino acid residues.

b. It exhibits the typical wide-angle X-ray diffraction pattern with the 3 Å meridional arc and the 12 Å equatorial spots.

c. It shows banded structure in the electron microscope with a spacing of 640 Å.

d. The peak corresponding to the NH stretching frequency in its infrared absorption spectrum is 3330 cm^{-1}, shifted by 30 cm^{-1} from the 3300 cm^{-1} peak of other proteins.

e. It has a negative specific rotation of about -350° in solution, which drops to -120° on heating above the transition temperature.

f. It exhibits a shrinkage phenomenon in the solid on heating, the X-ray pattern becoming amorphous above the shrinkage temperature."

A protein having the six criteria outlined above can be classified unequivocally as a collagen; however, the absence of one (particularly c. or a.) does not rule out its being a collagen.

B. Isolation and Purification Procedures

Collagen is a relatively insoluble protein with a very low metabolic turnover. Neuberger et al. (32), Neuberger and Slack (33), and Harkness et al. (34) showed that collagen from different tissues of the rat and rabbit is metabolically inert. Tompson and Ballou (35) arrived at the same conclusion, demonstrating that over 70% of the carcass collagen is relatively inert but that a small fraction has an active turnover. As is shown later, this fraction can be extracted at 4°C with a variety of media such as NaCl solutions, phosphate buffers at alkaline pH values, citrate buffers, or dilute acetic acid.

The pioneering work of Zachariades (36) and later Nageotte (37) showed that collagen from the tail-tendon of the rat could be extracted under mild conditions with dilute organic acids. The fraction thus obtained could reform fibers by suitable dialysis and was named "precollagen." Orekhovitch and co-workers (38, 39) extracted collagen with citric acid buffers from different animal species and animals of different ages within the same species. The preparation obtained was named "procollagen" to suggest that this fraction was the precursor of the insoluble collagen of the tissues. Highberger et al. (40) and Harkness et al. (34) used mildly alkaline phosphate buffers to obtain collagen, and Jackson and Fessler (41) and Gross et al. (42) employed neutral salt solutions for extraction. Each fraction was named according to the method of preparation ("alkaline-soluble collagen" and "neutral-salt-extractable collagen"), but in fact each fraction is a form of the soluble collagen precursor that gives rise to tissue fibers.

Many methods have been used to purify collagen dissolved by any of the procedures described above. Certain collagens obtained with citrate buffers at pH 3.6 are easier to purify than collagens dissolved in salt solutions. Gallop (43) prepared a highly purified collagen (ichthyocol) from the swim bladder of the carp after high-speed centrifugation of the citrate extract and dialysis against 0.02 M phosphate buffer.

Several procedures have been used to obtain pure collagen from NaCl extracts. Gross et al. (42) observed that clear collagen solutions in phosphate buffer formed precipitates when standing at room temperature for several days. Heating the extract at 37°C greatly accelerated the process, and the collagen thus obtained was quite pure as shown by analysis and by electron microscopy. Jackson (44) extracted collagen from guinea pig granulomas with 0.2 M NaCl and purified the protein after two precipitations with 20% NaCl. This procedure has been widely used to purify salt and acid-extracted collagens. Salt-extracted collagen is also purified by selective precipitation of the contaminants. Gross (45) showed that adjusting a collagen solution in phosphate buffer to pH 3.5 with 25% trichloroacetic acid (TCA), caused any hemoglobin and other proteins present to precipitate while collagen remained in solution. Collagen was then recovered by dialysis of the TCA supernate against phosphate buffer, followed by precipitation with absolute ethanol to 14% concentration.

Collagen obtained by any of these procedures is soluble in dilute acetic acid and may be reconstituted into a variety of fibrils such as native, fibrous-long-spacing (FLS) or segmented-long-spacing (SLS) (Chap. 2, Sec. II). Gross et al. (46) demonstrated that all the structures obtained are operationally interconvertible since all are composed of very thin protofibrilar particles that they named "tropocollagen particle" (from

Greek tropos, to turn). The tropocollagen unit is in fact the tropocollagen molecule, and now is frequently referred to simply as the collagen molecule.

Studies from different laboratories have shown that collagens obtained by different procedures have very similar and often identical physicochemical properties, though differing in solubility. The most important difference is the chronological age of the fraction obtained and its metabolic turnover. Jackson and Bentley (47) performed metabolic studies in guinea pigs injected with α-^{14}C-glycine. They extracted collagen serially from the skin and carrageenin-induced granulomas with different molarities of NaCl at pH 7.4, citrate buffer pH 3.6, and gelatin from the insoluble residue. After determining the specific activity of each fraction they concluded: "At any given time in developing connective tissue, there is a continuous spectrum of collagen aggregates of varying degress of strength of cross-linkage, dependent upon the time that has elapsed since their constituent molecules were synthesized. The various extraction media used remove a particular cross-section of these aggregates, depending upon their disaggregating power." In view of the close relationship between the method of extraction and the metabolic activity of the fraction obtained, one has to be cautious in comparing results obtained with collagens extracted with different methods, or even with the same method but from different tissues or animal species. Furthermore, in describing a preparation one should omit all trivial names to avoid confusion and instead one should state the source of the protein used and the exact procedure of extraction. The different methods described that render collagen soluble in the native form will only extract a small fraction of the total collagen present in a given tissue of the animal. Depending on the animal species, age of the animal, and the tissue used, the amount of collagen that can be extracted is less than 10%. The yield can be increased if very young animals are used.

Any collagen that remains after extraction with neutral-salt solutions or dilute acetic acid is called "insoluble collagen" or, euphemistically, "tissue collagen." Several methods have been devised to dissolve the insoluble residue (48-71); however, almost all will either denature or modify the native structure of the protein. A summary containing pertinent information about the methods of extraction of the insoluble collagen and some of the modification produced on the protein during extraction is presented in the Appendix.

C. α-Components

It has been known for a long time that denatured rat skin and fish collagens can be separated upon ultracentrifugation into fast (β) and slow (α) moving components. The two components can be separated from one

another by ammonium sulfate fractionation in 5 M urea (72,73). Metabolic studies performed by Orekhovitch et al. (74) demonstrated that ^{14}C-labeled glycine was first incorporated into the α fraction and that tropocollagen contains by weight a 1:1 ratio of $\alpha:\beta$ components.

Kessler et al. (75,76) fractionated rat tail tendon collagen on carboxymethyl-cellulose columns at 40°C, and obtained at least four peaks. Piez et al. (77), using the same chromatographic technique but with a different gradient system, were able to resolve the α from the β components of calf skin collagen. After modifying the chromatographic procedure, Piez et al. (78) showed that denatured rat skin collagen contained two α and two β components. The α chains are called $\alpha 1$ and $\alpha 2$, and the β components, $\beta 11$ and $\beta 12$. The $\beta 11$ dimers are made of two $\alpha 1$ chains, and the $\beta 12$ dimer contains one $\alpha 1$ chain and one $\alpha 2$ chain joined by covalent cross-links; thus β components are twice the molecular weight of α chain. Several collagen species have been analyzed by this chromatographic procedure, and almost all show the same pattern (79). Piez et al. (79) have shown that most tropocollagens are made of three α chains (two $\alpha 1$, one $\alpha 2$). When older collagens are analyzed (insoluble collagens) (Fig. 1), $\beta 22$ dimers of intermolecular origin are obtained (49,50), and also aggregates larger than β, such as the γ and δ components isolated by Veis and Anesey (48).

Most of the collagens analyzed contain two identical $\alpha 1$ chains and one $\alpha 2$ chain. However, collagen from codfish skin has three distinct chains ($\alpha 1$, $\alpha 2$, $\alpha 3$) (80). Although it has been suggested that collagen from rat skin (81), calf skin (81,82), chick embryo skin (83), and chick bone (84) also have three distinct α chains, the results must be reconsidered in view of our present knowledge of the collagen molecule. It has been shown by Kang et al. (85) that acid-extracted collagen from chick skin is heterogeneous with respect to its $\alpha 1$ components. Two $\alpha 1$ fractions were obtained after carboxymethyl-cellulose chromatography, both having almost identical amino acid composition. The difference between both chains is the absence in one of a small fraction of the N-terminal region. This partial proteolysis either may be a normal mechanism for remodeling of tissue collagen or may be due to an artifact arising from limited proteolysis during the extraction procedures.

Recently, Miller and Matukas (86) suggested the presence of two different types of collagen in chick bone cartilage, the first with normal composition of two $\alpha 1$ and one $\alpha 2$ and the second containing three identical $\alpha 1$-chains. This finding is of great interest from an evolutionary point of view, since it may represent a primitive type of collagen. It has been pointed out by Miller and Matukas (86) that the expression of an

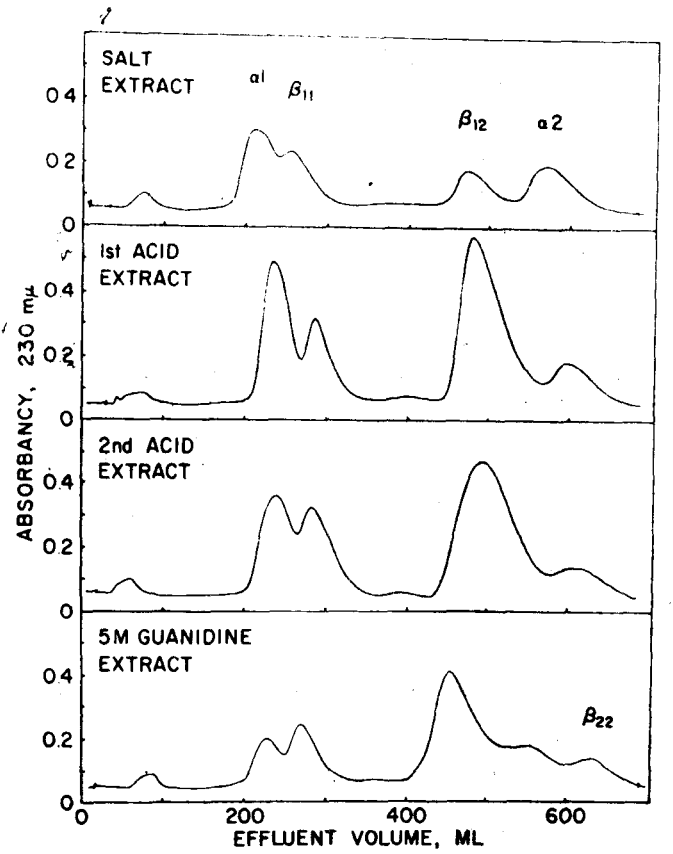

FIG. 1. Elution patterns of salt-, acid-, and guanidine-extracted collagens. Chromatography performed on carboxymethyl-cellulose at 40°. A linear gradient of ionic strength from 0.06 to 0.16 at a constant pH of 4.8 was employed [from Bornstein and Piez (49)].

additional structural gene for collagen synthesis in cartilage may be of significance, both with regard to differentiation and function of the tissue.

In view of these observations, one has to be cautious in asserting the presence of three nonidentical α-chains in any collagen. Furthermore, in those instances where urea was used to dissolve the protein, one has to rule out the possible formation of carbamyl-lysine due to the presence of cyanate.

In summary the tropocollagen is considered as a molecule with a molecular weight of about 300,000 and made up of three individual polypeptide chains (α) of 96,000 mol wt. each. Two of the α-chains appear identical in most species ($\alpha 1$) and one is different from the other two in amino acid composition ($\alpha 2$). As the protein "matures" or "ages" (Chap. 5), intramolecular cross-links take place giving rise to $\beta 11$ or $\beta 12$ components, dimers of intermolecular origin, or even to larger aggregates.

The amino acid composition of the individual components obtained from human infant skin is given in Table 1 (49). Further information regarding to the amino acid composition of different collagen species can be obtained in the article of Eastoe (23).

D. Subunits of α-Components

It has been shown in different laboratories (87-94) that collagen can be cleaved into smaller molecular weight components (designated subunits) when incubated with nucleophilic reagents under mild conditions of temperature and pH. Under those conditions, only ester or very reactive imide bonds are susceptible to nucleophilic reagents, and the name "ester-like" bonds was used thereafter (87) to include the possibility that the linkages indeed could be imide.

After incubation of ichthyocol-gelatin with 1.0 M hydroxylamine for 90 min at 39°C, the intrinsic viscosity of the protein drops from 0.4 to 0.16, the average molecular weight falls to a constant value of 27,000 (91), and about 18 moles of hydroxamic acid are formed per mole of collagen of 300,000 mol wt. The hydroxamic acid residues have been located at α- and β-carboxyl groups of aspartyl residues (90). The subunits have been purified by gel filtration chromatography into four weight classes, with molecular weights of 8000, 18,000, 34,000 and 49,000, respectively (91).

The molecular weights of the fragments obtained after cleavage of collagen or pure α-chains with hydroxylamine have been used to suggest two models for collagen subunits (92, 95, 96). The model proposed by Gallop (95) suggests that ichthyocol and other collagens (300,000 mol wt.) are made of 18 subunits, each with a molecular weight of 18,000, and of three nonidentical compositional types (A, B, C). Accordingly, each α-chain will have six subunits in proportions of three of one type, two of the second type, and one of the third type. It was also postulated that the

TABLE 1

Amino Acid Composition of Human Skin Collagen and Its Constituent Components[a]

	Residues per 1000 residues					
	Collagen[b]	α1	β11	β12	α2	β22
3-Hydroxyproline	1.1	0.8	1.0	1.0	0.9	1.0
4-Hydroxyproline	93.0	91.0	91.0	82.0	82.0	83.0
Aspartic acid	45.0	43.0	43.0	46.0	47.0	48.0
Threonine	17.5	16.5	16.3	17.9	19.2	19.3
Serine	35.6	36.8	36.7	35.2	35.1	34.1
Glutamic acid	73.0	77.0	76.0	72.0	68.0	68.0
Proline	128.0	135.0	136.0	123.0	120.0	118.0
Glycine	330.0	333.0	332.0	338.0	337.0	339.0
Alanine	110.0	115.0	116.0	112.0	105.0	104.0
Valine	24.4	20.5	20.6	28.8	33.3	31.1
Methionine	6.2	4.9	5.0	5.0	5.2	5.4
Isoleucine	9.5	6.6	6.5	11.6	14.8	13.7
Leucine	24.3	19.5	19.1	26.1	30.1	30.7
Tyrosine	2.8	2.1	2.0	3.8	4.6	4.6
Phenylalanine	12.0	12.3	12.5	12.3	11.7	12.2
Amide nitrogen	(36.9)	(37.9)	(37.2)	(44)	(45)	(45)
Hydroxylysine	5.8	4.4	4.4	6.1	7.6	8.0
Lysine	26.9	30.0	30.1	25.2	21.6	21.3
Histidine	4.8	3.0	2.0	6.3	9.7	10.7
Arginine	51.0	50.0	51.0	49.0	51.0	50.0

[a] Taken from Bornstein and Piez (49).
[b] Extracted with acetic acid after neutral salt extraction.

proportions of the three types of subunits cannot be identical in the α-components (3A + 2B + 1C; 3B + 2C + 1A; 3C + 2A + 1B) (95-97).

The second model proposed by Petruska and Hodge (96) was based on the banding pattern of SLS aggregates stained with either phosphotungstic