A CORRELATION BETWEEN THE DISTRIBUTION OF BIOLOGICAL APATITE
AND AMINO ACID SEQUENCE OF TYPE I COLLAGEN

By

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ABSTRACT

The present study was undertaken to examine the distribution of biological apatite within type I collagen fibrils of calcifying turkey leg tendons. The localization of apatite was determined by both bright field and selected-area dark field electron microscopy and was correlated with specific classes of amino acids from chick type I collagen sequence data by computer modelling. These electron microscopic techniques illustrated that apatite occurs in both the gap and overlap zones at early stages of mineralization in an asymmetric pattern which corresponds to the polarized N- to C- molecular orientation within the collagen fibril. Based on comparisons with theoretical computer images of the known amino acid sequence of collagen it was determined for early stages of mineral deposition that apatite is excluded from areas of high hydrophobic density. The gap zone is less hydrophobic than the overlap zone on average but each of these zones had areas of high density of hydrophobic amino acids which corresponded to sites of low mineral density. Conversely, regions with a high proportion of mineral were low in density of hydrophobic amino acids. The possible interactions between hydrophobic effects and the process of mineral deposition are discussed. In addition, the accommodation of collagen to the deposition of apatite in the overlap zone is discussed.

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ABBREVIATIONSAmino Acids

A	Alanine	K	Lysine	R	Arginine
C	Cysteine	L	Leucine	S	Serine
D	Aspartic Acid	M	Methionine	T	Threonine
E	Glutamic Acid	N	Asparagine	U	Hydroxylysine
F	Phenylalanine	O	Hydroxyproline	V	Valine
G	Glycine	P	Proline	W	Tryptophan
H	Histidine	Q	Glutamine	Y	Tyrosin
I	Isoleucine				

Other Abbreviations

a	"a" bands of collagen staining pattern
b	"b" bands of collagen staining pattern
c	"c" bands of collagen staining pattern
C-	Carboxy terminus of peptide chain
d	"d" band of collagen staining pattern
D	Axial period of type I collagen
e	"e" bands of collagen staining pattern
h	Hour
min	Minute
N-	Amino terminus of peptide chain
nm	nanometer
PTA	Phosphotungstic acid
SADF	Selected-area dark field electron microscopy
UA	Uranyl Acetate
μ m	Micrometer

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HISTORICAL REVIEW

Introduction

As a major component of bone, tendon and other types of loose and dense connective tissues, type I collagen is the most prevalent protein in vertebrates. Its recognized importance in a variety of biological processes has resulted in an abundance of information about this protein, but its role in mineralization remains poorly understood. The purpose of this historical review is to examine previous works about collagen molecular structure, apatite structure and their inter-relationships which have formed our current concepts of biological mineralization within type I collagen fibrils. These works have been collected from several areas of collagen research including morphology, protein structure, biochemistry, and electron microscopy in order to assess the development of ideas in this area. The volume of works on collagen and mineralization in various tissues requires that this review be selective of papers which are directly related to the thesis rather than explore the entire world of mineralization research. However, since the turkey tendon used for this study is a model of bone development, some of the similarities and differences between the two tissues will be discussed. In this thesis the use of "collagen" will refer specifically to type I collagen in order to avoid unnecessary repetition. The term

"biological apatite" is used in this thesis to emphasize the differences between hydroxyapatite, considered the best model for apatite crystals in turkey tendon, and the actual biological crystal structure. If the reader wishes further information several good reviews have been published recently (Miller, 1984; Chapman and Hulmes, 1984; Bonnucci, 1984; Kühn, 1987).

Collagen has similarities and differences from tissue to tissue. Within all connective tissues, collagen has a hierarchy of structural order from the molecular to the microscopic. Three peptide chains form the collagen molecule; several collagen molecules combine to form a microfibril; many microfibrils form a collagen fibril; and collagen fibers consist of many fibrils. Differences result from tissue-specific post-translational modifications and interactions with other molecules of the extracellular matrix, resulting in a variety of architectures to support the tissues in the body. These architectures are, in part, responsible for the biomechanical properties of the different tissues. For example, collagen fibrils are very long and may extend for microns or centimeters depending on the tissue. Fibril diameters vary from 10nm in the vitreous humour of the eye to 500nm in mature rat tail tendon (Parry et al., 1978). In bone, collagen fibrils within the extracellular matrix of osteoid are interwoven, in a poorly defined network, prior to mineralization. Following

remodelling of bone, the laminated architecture of collagen within the mineralized matrix is a reflection of compressive, shear and distractive forces. Collagen fibrils in bone are distributed in two different forms. In the "orthogonal plywood" model, fibrils are organized into distinct lamella. Within each lamella the fibrils are predominantly parallel to one another and the direction of the fibrils changes from one lamella to the next by an angle close to 90 degrees. An alternative organization of fibrils within bone, which may occur within the same specimen, is the "twisted plywood" architecture of fibrils. In this model the fibrils within each lamella are organized into a superimposed series of nested arcs. The direction of the fibrils from one lamella to another rotates continuously by a constant angle. Although these two general patterns are present, the local three-dimensional arrangement of collagen fibrils within bone is very complex. They follow a sinusoidal course, local helicoidal bundles occur and fibrils may abruptly change direction (Giraud-Guille, 1988).

In contrast to bone, tendons contain parallel, densely-packed fibrils which can sustain high tensile forces. The structure and composition of tendons permit the rapid transmission of forces generated by contractile elements of muscle. Tendons transmit these loads with a minimum of energy loss and deformation (Silver, 1987). Turkey leg tendons, which mineralize as a part of normal

growth after 9 weeks of age, have been used in electron microscopic investigations of collagen mineralization because the linear, parallel arrangement of fibrils permits analysis along individual fibrils (Nylen et al., 1960; Myers and Engström, 1965; Arsenault, 1988).

Collagen, in addition to its biomechanical role, is also intimately involved in the physiological process of mineralization in bone, dentin and turkey leg tendon. Evidence for the close relationship between collagen and biological apatite in normal bone function comes, in part, from studies of osteogenesis imperfecta, a heterogeneous group of inherited diseases. In most instances, the skeletal manifestations of osteogenesis imperfecta are due to molecular defects of type I collagen (Hollister, 1987). Despite the importance of collagen in the function of mineralized tissues very little is known about the mechanisms which give rise to this relationship.

The primary interest of this work is the localization of apatite crystals with respect to the amino acid composition of collagen and stems from an apparent need to integrate new findings with current concepts of collagen/mineral interaction. Recent information suggests a periodic distribution of apatite crystals within the overlap and gap zones of collagen fibrils (Arsenault, 1988), and a developmental gradient in the early stages of mineralization within these zones (Arsenault, 1989). As an extension of

these works the present study attempts to further analyze the periodic distribution of apatite. It is therefore necessary to review some concepts of collagen molecular structure and the properties of biological apatite crystals which have given rise to the development of this thesis.

The Collagen Molecule

The collagen molecule consists of three helical peptide chains which interact to form a triple helix (Ramachandran and Kartha, 1955; Rich and Crick, 1955; Cowan et al., 1955). These three polypeptide chains each have their individual helical coil and are coiled about each other (Rich and Crick, 1955). Piez et al. (1963) determined the relative proportions of the three peptide chains and described the collagen molecular unit as consisting of two identical 1(I) peptides and one 2(I) peptide. The size and shape of the collagen molecule was first described by Boedtker and Doty (1956) as a rigid rod 300nm in length and having a diameter of 1.25nm. Later, when the amino acid sequence was being resolved, it was found there are two non-helical portions, one at each end of the molecule. These are the N- and C-terminal portions called telopeptides. The conformations of both the N- and C- telopeptides are not known at present. These conformations are modelled using secondary structure prediction based on energy and steric calculations. Several

stereochemical models may be possible for these regions from this method (Jones and Miller, 1987).

Conceptualization of the collagen molecule as a rigid rod may not be entirely correct. The triple helical portion of the collagen molecule can also be described as a semiflexible cylinder (Kühn, 1982). Two cylindrical molecules would interact with each other via reaction edges on their surfaces depending on the position of amino acid side chains. Functional groups of amino acid side chains towards the exterior of the cylinder would determine the intermolecular interactions while the amino acid side chains within the triple helix would determine the intramolecular interactions. The composition of the edges would be dependent on the helical pitch and any alteration of the pitch will change the composition of the reaction edge. Although determination of the pitch of the collagen molecule is critical to determining the inter- and intramolecular interactions, a single value has not yet been agreed upon. The pitch of rat tail tendon collagen has been reported to be between 29 and 45 residues (Miller, 1976). The study of Fraser et al. (1983) calculated the pitch to be 30 residues or 8.68nm.

Individual collagen molecules show substantial lateral flexibility (Veis, 1985a). This can occur because segmental fluctuations are accommodated by changes in the triple helix without appreciable changes in free energy (Okuyama et al.,

1981). Flexibility may occur in two forms 1) slow flexural motions and 2) hinge regions. Veis (1984) suggested hinge regions located between residues 81-102 and 916-939 because they are devoid of proline and hydroxyproline residues which stabilize the triple helix. Proline is a major element in helix stability because the ring structure prevents rotation about the N-C bond and the rotation about the C-C=O bond is restricted. Thus, within the collagen molecule there is a semiflexible region of about 250nm length flanked by two relatively flexible regions.

Viewing the collagen molecule as a cylinder may also be somewhat simplistic. The molecular motions of the peptide backbone and side chain flexibility observed in NMR experiments (Sarkar et al., 1983; Sarkar et al., 1987) suggest that the interactions between adjacent molecules involve a myriad of conformational changes in fluid-like domains. Accommodation of these changes would cause local distortions in both the long axis and transverse axis of the triple helix. In order to explore more fully the properties of collagen molecules and fibrils in mineralization I would now like to discuss relevant issues of protein translation and post-translational modifications.

The Type I Collagen Genes

The genes for type I collagen are large and complex. They are about 38Kb in length of which 10% is a coding

sequence of about 50 exons (Kühn, 1984). The finding that many exons have an identical length of 54bp suggests that the ancestral gene arose by amplification of a single genetic unit of 54bp which in turn may have arisen from a sixfold repetition of a oligonucleotide coding for the tripeptide glycine-proline-proline. The fixation of this pattern of exon sizes may due to an intolerance to recombinational events reflected by a change in protein function (De Crombrughe et al., 1985).

Collagen shows a high degree of conservation in amino acid sequence and structure between species. Nordwig and Hayduk (1969) presented evidence for the invariance of the tertiary and quaternary structures of the type I collagen molecule based on biochemical and electron microscopic studies of collagens isolated from several species which differ in evolutionary level. Seyer and Kang (1985) used available amino acid data to compare chick type I collagen to a combined sequence from bovine and human collagen. They found 100% of the basic amino acids (R and K; see "Abbreviations" for amino acid single letter codes), 91% of the acidic amino acids (D and E) and 79% of hydrophobic amino acids in the $\alpha 1(I)$ peptide were retained. In the $\alpha 2(I)$ peptide, 96% of the basic amino acids, 87% of the acidic amino acids, and 66% of the hydrophobic amino acids were unsubstituted. Overall homology between the sequence of

the $\alpha 1(I)$ chains of chick and a combined sequence from rat and calf was found to be 93.4% (Highberger et al., 1982).

Consistency of the collagen genetic code may be a prerequisite for the normal interaction between organic and inorganic components of the extracellular matrix. Evidence for this comes not only from interspecies homology but also from genetic studies of pathological bone formation such as osteogenesis imperfecta. Small changes in the genetic code results in major biomechanical alterations. These changes may involve either the $\alpha 1$ or the $\alpha 2$ chain and be as small as a single point mutation (Hollister, 1987).

The Amino Acid Sequence

Following transcription, the procollagen peptides can be subdivided into six functional regions differing in size and sequence characteristics. These are described starting at the amino terminus. The first region is a signal peptide which enables the protein to move from the rough endoplasmic reticulum (rER). It is cleaved from the propeptide immediately following movement across the rER membrane (Kriel, 1981). The adjacent functional region is the amino propeptide. It is considered important in the molecular assembly along with carboxy propeptide, at the opposite end of the molecule, because crossbridges are formed at specific sites of the propeptides. Following the cleavage of these regions by N- and C- propeptide specific proteinases in the

extracellular matrix, no cysteine remains to form disulphide crossbridges. Adjacent to the amino propeptide, the amino telopeptide, is a short non-helical segment, 16 residues in length. The triple helical portion is 1014 amino acid residues in length. The carboxy telopeptide is 25 residues in length (Miller, 1984).

The amino acid sequence of collagen is characterized by a variety of repeating patterns which are functionally significant. The repeating D period, 234 amino acids in length (Hulmes et al., 1973), is visualized in the electron microscope following heavy metal staining and also in early stages of mineralization, in unstained tissue, due to the periodic distribution of apatite. It is considered to be the structural unit responsible for fibrillar organization. The amino acid triplet repeat with glycine in every third position of the peptide chain is responsible for the triple helical structure of the molecule (Ramachandran and Kartha, 1955). The triplet repeat of this region is often abbreviated G-X-Y where G is glycine, and X or Y can be any amino acid except cysteine or tryptophan. Jones et al. (1985) suggested that the X and Y positions differ stereochemically in the triple helix such that the Y position was more involved in intermolecular interactions while the X position was involved more in intramolecular interactions. Other potential repeating units of amino acid sequence were analysed by Hofmann et al. (1980). They found

several patterns in the two peptide chains. The repeat units D, 2D, 3D, D/3, D/2, D/13 and 4D/11 statistically are unlikely to occur by chance and are presumed to have some functional significance. The amino acid sequence has long been considered of paramount importance to the biomechanical and physiologic functions of collagen and yet there has not been an analysis of the relationship between the amino acid sequence and mineralization.

Following translation of the peptide several events take place which modify the protein. These modifications are essential for molecular assembly, for fibrillar organization and may influence biomineralization.

Post-Translational Modifications

Prior to molecular assembly, while the individual polypeptides are associated with the rough endoplasmic reticulum, a large number of segments of the unhydroxylated procollagen molecules exist in β -turn conformations (Brahmachari and Ananthanarayanan, 1978). This conformation may be the specific site of proline hydroxylation. The result of proline hydroxylation is a conformational change of the individual peptides which is necessary prior to the intertwining of the three chains (Chorpra and Ananthanarayanan, 1982). Hydroxyproline plays an important role in the stabilization not only of the triple-helical collagen molecule but also of its assembly into microfibrils

(Nemethy and Scheraga, 1986). Hydroxyproline when found is always in the Y position (Gly-X-Y) of the triple helical portion (Bornstein and Traub, 1979). It has been suggested that hydrogen bonds between triple-helices occur between hydroxyproline and a C=O group on an adjacent helix (Bansal et al., 1975). Hydroxyproline is known to be essential for the structural integrity of collagen at body temperature (Prockop et al., 1976). Ramachandran et al. (1973) has suggested the existence of specific water-bridged hydrogen bonds associated with hydroxyproline which further stabilize the triple helix.

Following peptide translation and prior to triple helix formation a variable number of the lysine residues are hydroxylated. The degree of hydroxylation of lysine is variable between tissues and ages. The importance of hydroxylysine to collagen is in the role of crosslink formation and glycosylation (Kivirikko, 1980). Hydroxylysine is formed immediately prior to triple helix formation at the rough endoplasmic reticulum (Kivirikko et al., 1973).

Intracellular post-translational modifications continue with the attachment of mono- and disaccharides to the molecule. D-galactose and D-glucose are covalently attached to the polypeptide at hydroxylysine residues via an O-glycosidic linkage (Butler and Cunningham, 1966). There appears to be a high degree of specificity for the glycosylating enzymes due to the few specific sites to which

the carbohydrates attach (Miller, 1976). To date I have found no studies directed at the function of the glycosylation of collagen in bone formation or mineral deposition.

Self assembly

Following secretion of procollagen molecules into the extracellular environment, where they behave as elongated and asymmetrical molecules in solution (Veis, 1985a), these molecules are organized into fibrils. The architecture of the collagen fibrils derived may initially be a function of the molecular geometry and solubility within the local environment (Giraud-Guille, 1988). Collagen molecules are believed to polymerize spontaneously to form fibrils in the extracellular matrix based on in vitro studies (Gross and Dirk, 1958). Furthermore, the in vitro forms of collagen, fibrous long spacing, segmented long spacing and native fibrils are interconvertible by dissolution and subsequent reaggregation under specific conditions (Schmitt et al., 1953). Thus, it has been suggested that interactions among side chains on adjacent molecules have a major role in directing collagen fibril assembly (Hulmes et al., 1973) and stabilization of collagen structure. Ionic clustering of side chains occurs due to the relative position of negative and positive charges in the G-X-Y triplet (Doyle et al, 1974). Alternating charges, positive and negative, may occur

linearly to provide stability (Piez and Torchia, 1975). In three-dimensions this would result in more than two molecules contributing to an orderly arrangement of ions and have properties of an ionic crystal. In addition, hydrophobic groups occur in periodic spacing (Piez and Trus, 1978). The aggregation of hydrophobic groups would lead to less disruption of the strong polar forces of the aqueous environment which surrounds them (Tanford, 1980). The close association of hydrophobic amino acids would therefore lead to stability in the collagen fibril prior to the formation of crosslinks. Hulmes (1983) suggested that collagen fibril growth occurs by accretion of concentric layers of collagen molecules.

Procollagen processing (Fleischmajer et al., 1983) or interactions with proteoglycans (Wood, 1960) have been suggested as factors limiting the tissue specific fibril diameter. Aminopropeptides participate in fibrillogenesis and may regulate fibril diameter (Fleischmajer et al., 1983). Procollagen aminopropeptides of type I collagen have been demonstrated collagen fibrils by immunofluorescence (Nowack et al., 1976) and immunoelectron microscopic techniques (Fleischmajer et al., 1983). Therefore, fibril formation may involve the polymerization of aminoprocollagen (Fleischmajer et al., 1985). Procollagen N-proteinase would then act on the fibrils to remove the amino propeptide. An alternative hypothesis of the control of fibril size is

derived from in vitro studies of proteoglycan/collagen interactions which have found changes in fibril diameter related to the concentration and type of proteoglycan present (Parry et al., 1982).

The striking ultrastructural feature of collagen is the cross-striated staining pattern which repeats in each D period due to the localization of charged amino acids (Bear, 1952). Early electron microscopic studies of collagen determined the length of the D period to be 64 nm (Hall et al., 1942). However, x-ray diffraction methods yield greater values (67.8nm) depending on the degree of stretch (Fraser et al., 1983). The true value for this unit remains in debate because collagen fibrils are prone to artifactual changes in D length but it may also be tissue specific or dependent on the local environment.

The electron microscopic analysis of repeating units of in vitro collagen aggregates has resulted in theories of the molecular two-dimensional packing arrangement. This electron microscopic approach led to the quarter-staggered packing model (Hodge and Schmitt, 1960). In addition, Hodge and Schmitt (1960) defined the "a, b, c, d and e" nomenclature for the collagen fibril banding which will be used in this thesis. Hodge and Petruska (1963) first suggested a model in which longitudinally adjacent molecules were separated by a space (see Figure 1).

Figure 1. The Hodge and Petruska (1963) packing model of type I collagen molecules. These molecules are depicted as linear rods staggered by 234 amino acid residues, or D , to the adjacent molecule. This model has two important consequences. First, the model predicts two zones of different densities within the repeating D unit, a densely packed overlap zone and a gap zone with potential space available. Second, the periodic localization of amino acids can be analyzed by computer modelling using the primary sequence of collagen. In this thesis the single letter codes for the amino acid sequence of collagen were organized into repeating units according to the Hodge and Petruska (1963) model.

The Hodge and Petruska (1963) model has the important consequence that it predicts two alternating zones of different densities. A closely packed overlap zone would contain tightly fitting parallel molecules. The gap zone would contain one space for every four molecules because of the longitudinal separation between molecules. The Hodge and Petruska (1963) model has been very important to the current understanding of collagen structure and histochemistry. This model permits the electron microscopic analysis of collagen fibril amino acid composition (Meek et al., 1979) which will be used in this study. Despite its utility in electron microscopic investigations the fibrillar structure of this model is oversimplified. It gives the illusion that the molecules are linear and rigid. In reality the three-dimensional structure of the collagen molecules in space is complex. There are three or four levels of coiling to the level of the fibril. The coil of the chain, the coil about the molecular axis, the coil about the microfibrillar axis and in some cases, the microfibrils are coiled within the fibril (Ruggeri et al., 1979). In addition, in Figure 1, it appears as if there are large, porous holes at the level of the gap zone because of the way Figure 1 was constructed. However, to be more to the scale of the actual molecule, in this illustration the width of the gap zone would be $5/1000 \times$ the length of the molecule (or 0.5mm). So the volume of the individual gap zones is very small based on this model.

For example, if we consider the individual gap as a box shaped space with the longest dimension 40nm and the other sides 1.5nm (Hodge and Petruska, 1963) then the total volume of one gap would be 90nm^3 assuming no other non-collagenous molecules are occupying this space. In order to explain the difference between the volume of mineral (minimum 300nm^3 to about $3,000\text{nm}^3$, see below) and the volume of individual gaps, two ideas have emerged. First, the gap zones may be aligned in three dimensions to provide sufficient space (Weiner and Traub, 1986). This theory would predict distortions in the Hodge and Petruska (1963) packing arrangement because molecules would not be in the staggered order. Second, Höhling (1980) suggested that larger spaces are built into the microfibrillar organization and these are the primary location of crystal formation and not the gap zones. Current views of the three dimensional packing arrangement do not exclude these theories but neither have they been demonstrated.

The three-dimensional packing arrangement of collagen molecules within the fibril is not known despite several attempts at defining a regular order. In cross-section fibrils exhibit crystalline qualities in some regions which support the concept of periodicity in three-dimensions. However, portions of the fibril are not consistent. In some regions the cross-section pattern is distorted over irregular areas. There may also be sharp discontinuities to

the lattice-like structure (Hulmes et al., 1985). Therefore, close packed, optimal arrangements such as the quasi-hexagonal molecular packing arrangement (Hulmes and Miller, 1979) or a microfibril arrangement (Trus and Piez, 1976) may be consistent over short distances but there are significant regions which do not conform to a perfectly regular pattern. The three-dimensional structure of collagen fibrils is considered important for the growth of apatite crystal since there needs to be the appropriate environment for crystal formation and sufficient space to have crystal growth to the biologically appropriate volume. There is some evidence that the three-dimensional organization is tissue specific. Katz and Li (1972) found the intermolecular distance to vary between mineralized and non-mineralized collagens. They found that intermolecular spaces are greater in mineralizing tissues than in non-mineralizing tissues and that mineralization may be inhibited in certain tissues for steric reasons. In addition, the geometry of packing may be different in various tissues and result in different crosslinks (Mechanic et al., 1987).

Crosslinking

Covalent crosslinks between collagen molecules are important determinants of the mechanical properties of connective tissue matrices and are an example of the tissue specific post-translational heterogeneity of type I

collagen. Crosslinking results from the reaction of specific aldehydes, in the amino and carboxy terminal nonhelical peptides, with amino groups of lysine and hydroxylysine located in the triple helical regions of molecules (Yamauchi et al., 1986). Two pathways of cross linking have been demonstrated for the fibrillar collagens - one based on lysine aldehydes which predominates in adult skin, cornea, sclera and rat tail tendon; the other based on hydroxylysine aldehyde pathway which predominates in bone, ligament and most tendons (Eyre, 1987). Histidine may also participate in crosslinks (Tanzer et al., 1973). In bone collagen, the labile reducible crosslinks are Schiff-base (aldimine) compounds (Mechanic et al., 1971). These can rearrange to form reducible stable ketoamine crosslinks (Eyre and Glimcher, 1972; Mechanic, 1974). The two principal reduced crosslinks of bone collagen are dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine. As normal bone ages DHLNL crosslinks undergo a transition to nonreducible pyridinoline (Fujii and Tanzer, 1974). The crosslinking chemistry in type I collagen fibrils initially involves residues from two collagen chains, but subsequently, residues from other chains form tri and tetra-chain links. Although it is believed that the crosslinks in the mineralized and non-mineralized collagen are different there remains some debate as to the nature of the structures involved. Banes et al. (1983) did not find pyridinoline in

the mineralized collagen of bone but it was found in the non-mineralized portion. They suggested that mineralization physically inhibits formation of pyridinoline by preventing approximation of the collagen molecules. Wu and Eyre (1988) found that the small particle size used in powdering bone for analysis resulted in protease digestion of pyridinoline crosslinks. They found no difference in pyridinoline between mineralized and non-mineralized tissue. I expect crosslinking to be of some significance in mineralization process. At this time though, there remains a need for further clarification.

Biological Apatite

Individual apatite crystals have been described either as having an irregular, thin, elongated, plate-like shape (Jackson et al., 1978; Weiner and Traub, 1986) or as a rod-like structure (Myers and Engström, 1965). The dimensions of the crystals have been measured by x-ray diffraction, bright field electron microscopy and selected-area dark field electron microscopy (SADF). Due to technical difficulties associated with studying this characteristic a wide range of values have been reported. Direct measurements by bright field electron microscopy have given a mean size between 30 to 70nm (Robinson, 1952; Robinson and Watson, 1952; Steve-Bocciarelli, 1970; Jackson et al., 1978; Landis and Glimcher, 1978; Weiner and Price,

1986). However, visual interpretation of bright field electron micrographs results in two basic problems. First, background electron density of the specimen results in differences in detection of crystals (Arsenault and Hunziker, 1988). Therefore, one would expect a biased interpretation of size due to selection of larger, more obvious crystals. Second, there may be overlap in crystal position leading to larger estimates of crystal size. Due to the small crystal cross-sectional size, even the thinnest section contains many crystals. This increases the probability of overprojection (Hunziker et al., 1989) and the perception that the cumulative electron density of many crystals is a single crystal (Arsenault and Grynpas, 1988). X-ray diffraction has produced results for crystal length from 10nm to 36nm (Myers and Engström, 1965; Jackson et al., 1978; Engström, 1972; Bonar et al., 1983; Boskey et al., 1985; Grynpas et al., 1986; Arsenault and Grynpas, 1988). The small crystal size is related to its large surface area which has been reported in the literature as between $85\text{-}265\text{m}^2/\text{g}$ (Robinson and Watson, 1952; Hodge and Petruska, 1963; Miller, 1984; Weiner and Price, 1986; Arsenault and Grynpas, 1988). The biological control of crystal size is extremely important in determining biomechanical properties and crystal chemistry.

Studies which report single mean values for crystal size may mislead the reader into assuming that there is a

narrow range in the sample. On the contrary, the large standard deviations reported are evidence for a heterogeneous population. There are several possible sources of this large range of crystal sizes. A common problem in all of the techniques is the standardization of tissue maturity. I assume that crystal size and shape would depend on the stage of mineralization and remodelling. It may also depend on the local environment such that adjacent crystals are restricted in growth at different levels. I would like to emphasize that the crystal dimensions do not seem to reflect the size of the gap zone in the Hodge and Petruska (1963) model for two-dimensional packing of collagen (Arsenault and Grynblas, 1988). For example, the width of the crystals measured are at least ten times the width of the predicted gap zones.

In electron microscopic studies the characteristics of apatite pose major technical problems. Often, in the literature, it has been suggested that changes in mineral ultrastructure are a result of the methods used for tissue preparation and specimen visualization. One such problem is associated with the solubility properties of apatite. Arsenault and Hunziker (1988) showed that conventional aqueous stains totally demineralize thin sections and this leads to ambiguities in what is being stained. In addition, apatite crystals are labile to radiation damage during electron microscopy (Arsenault, 1988). As a consequence of

these technical difficulties, methods such as quick-freezing/freeze-substitution (Arsenault and Hunziker, 1988; Arsenault et al., 1988) and anhydrous techniques (Landis et al., 1977; Landis et al; 1980) have been developed to minimize artifactual changes.

Although hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6\text{OH}$, is considered the idealized model for biological apatite there are some differences between the two. Chemical analyses reveal that bone differs considerably from the ideal formula. First, numerous impurities exist in bone including fluoride, magnesium, chloride, and carbonate (Hagen, 1973). Second, there may also be alternative stoichiometric relationships between calcium and phosphorus which result in other crystalline arrangements. These arrangements may be important at various stages of mineralization. Therefore, biological apatite crystal structure must deviate from the structure of geological or synthetic hydroxyapatite of ideal composition.

The unit cell of apatite is repeated throughout the crystal structure. It has all the characteristics, including the symmetry and chemical elements, of the crystal and has been described as a structural molecule (Hagen, 1973). In apatite, the unit cell is defined by three axes labelled a, b and c. The crystal planes are labelled according to the length of the axes, their inclination to one another, and the positions of the atoms. The unit cell of the crystal in

calcified turkey tendon is hexagonal with the dimensions $a=0.942\text{nm}$ and $c=0.688\text{nm}$ (Myers and Engström, 1965). These basic cells of crystal organization diffract electrons in a consistent manner and this makes possible the visualization of diffracted electrons from specific crystal planes in selected-area dark field electron microscopy.

Apatite/Collagen Relationships

Biological apatite crystals in mineralizing turkey leg tendon and bone are found within the collagen fibrils and within the non-collagenous extracellular matrix outside the fibrils (Glimcher, 1959; Weiner and Traub, 1986). Yet the localization of the crystals within the fibrils remains the subject of debate. Hodge and Petruska (1963) in proposing their model suggested that the gap zone would be the preferred location for mineral deposition within fibrils because of the potential space available. Since that time, certain studies have supported this view (White et al., 1977). Alternatively, Glimcher (1983) states the mineral is initially localized in the gap zone but spreads throughout the overlap zone. The primary explanation for the localization of crystals in the gap zone still remains focussed on the potential space believed to exist as a result of the staggered arrangement of collagen molecules. However, recent information suggests that early sites of mineral deposition are within both the gap and overlap zones

(Arsenault, 1988). Arsenault (1989) reported the proportion of mineral is initially greater in the gap zone than in the overlap zone but this becomes more equal as mineralization progresses. If apatite is localized in the overlap zone then the integration of apatite within the collagen fibril requires a much more complex explanation than has been previously described.

The c-axis of the apatite crystal is considered to run parallel to collagen molecules in turkey tendon (Engstrom and Zetterstrom, 1951). When apatite crystal planes are specifically viewed by selected-area dark field electron microscopy, the axial orientation of apatite crystals is observed to alternate along the fibril length (Arsenault, 1988). This is most likely due to the three-dimensional arrangement of the collagen molecules such that the rotation of apatite axial orientation reflects structural properties of the collagen fibril.

Conclusion to Historical Review

The amino acid sequence of collagen may play a role in biomineralization and the localization of mineral apatite for several reasons. First, the three-dimensional packing arrangement of collagen fibrils may result in regions of potential space because of regional flexibility or intrafibrillar spaces. Second, specific amino acids may have positive or negative effects on the nucleation or growth of

apatite crystals. Thirdly, post-translational modifications such as hydroxylation, crosslinking and glycosylation may be factors which determine the localization of apatite. Fourth, specific non-collagenous proteins involved in the process of mineralization may interact with collagen at precise locations determined by the amino acid sequence.

Considerable uncertainty regarding the mechanisms of mineralization and the role of type I collagen exists in the literature. Numerous basic questions remain unanswered. For example, where is the mineral located in relation to the collagen fibril D period? What is the relationship between the amino acid sequence of collagen and the location of apatite? Are there properties of collagen which can explain the localization of apatite in the overlap zone? In an attempt to understand some of these aspects of mineralization the following study was carried out.

INTRODUCTION

Type I collagen provides structural integrity to numerous connective tissues and is directly involved in the mineralization of bone, dentin and turkey leg tendons. The collagen molecule is a semiflexible rod, 300nm in length formed by two $\alpha 1(I)$ and one $\alpha 2(I)$ peptide chains (Piez et al., 1963; Veis, 1985a). These molecules have a triple helical portion (1014 amino acid residues) and small N- and C- terminal, nonhelical portions. Hodge and Petruska (1963) showed that the collagen molecules are longitudinally organized in a regular 67nm, D-staggered fashion which gives rise to its axial period. The molecules are linearly arranged such that there is a 35nm separation between adjacent ends and each molecule is staggered to its lateral neighbor by 67nm, corresponding to 234 amino acid residues (Hulmes et al., 1973). The repeating D period of the collagen fibril was envisioned as having two regions, a densely packed overlap region, .4D in length, and a "gap" region, .6D in length, where there would be potential space as the result of the separation between longitudinally arranged molecules. Hodge and Petruska (1963) suggested that the gap region would be the preferential location of apatite crystals during mineralization due to the space available for crystal growth.

The regular D-stagger of the collagen molecules also results in the periodic localization of amino acids with specific structural and histochemical implications (Bear, 1952). Meek et al. (1979) were able to integrate the known amino acid sequence, structural information and the staining patterns of type I collagen to model the localization of individual amino acids within the fibril. A high correlation coefficient ($r=.926$) was found between the electron-optical density of positively stained collagen and the theoretical density of their model. Subsequent studies have used the amino acid sequence data of collagen to determine the localization of electron-dense stains with specific classes of amino acids (Tzaphilidou et al., 1982a, 1982b; Chapman and Hulmes, 1984; Meek and Chapman, 1985). An analogous approach can be used to understand the impregnation of mineral into collagen fibrils by the localization of apatite crystals relative to the known amino acid composition. In this present study, in addition to heavy metal staining, the electron density and diffracted electrons of apatite are analyzed and compared to models of collagen amino acid sequences.

The role and interactions of type I collagen in the mineralization process remains an enigma. Corroborating the suggestion by Hodge and Petruska (1963) several authors have reported the occurrence of apatite in the gap zone and its exclusion from the overlap zone (White et al., 1977;

Berthet-Colominas et al., 1979). Other authors describe the initial localization of mineral to be exclusively in the gap zone followed by progressive spread throughout the overlap zone (Glimcher, 1983). Recent information indicates that the earliest detectable apatite crystals are localized within the overlap zone in addition to the gap zone (Arsenault, 1988) and that the proportion of mineral in the gap and overlap zones changes as mineralization proceeds (Arsenault, 1989). There remain many questions concerning the role of collagen structure in the mechanism of biological apatite deposition- whether the association between collagen and mineral is a passive association, an interactive process or whether an intermediary is the dominant force? Many studies have used the mineralized turkey tendon instead of bone tissue proper because of the tendon's high degree of collagen fibril alignment, thereby, permitting ease of sequential analyses of the mineralization process along individual fibrils.

The present study was undertaken to investigate the intrafibrillar localization of apatite in relation to the amino acid sequence of type I collagen. The asymmetric organization of apatite within the collagen D period was found to be related to the polarized N- to C- direction of the collagen fibril as determined by the staining pattern. A inverse relationship between the localization of apatite and the localization of hydrophobic amino acids was found to be

evident in both the gap and overlap zones. The primary conclusion of this thesis therefore, is that the hydrophobic effect, which is important to collagen structure, also serves to influence the distribution of apatite. The distribution of the crystals does not appear to be solely determined by the gap region in collagen fibrils but could be controlled by other collagenous or non-collagenous influences.

MATERIALS AND METHODS

Tissue Preparation and Visualization

Small pieces of freshly excised, non-mineralized leg tendons of 10 week old domestic turkeys were placed in a solution of 2.3% v/v glutaraldehyde, 2% w/v sucrose and 0.05M sodium cacodylate, pH 7.4, for 2h. The tissues were then rinsed with 3 changes of 0.05M sodium cacodylate and 2% sucrose for 15 min each followed by 1h post-fixation in a solution of 1% w/v osmium tetroxide, 2% sucrose and 0.05M sodium cacodylate. Dehydration was carried out by placing the tissues in increasing concentrations of methanol in distilled water (50%, 70%, 80%, 90%, 3x100%) for 15 min each followed by 3 changes of acetone. The tissues were then placed in a series of acetone:Spurr's resin (3:1, 1:1, 1:3, 100% resin) for one hour each. Following overnight infiltration of the tissues with a fresh change of 100% resin it was polymerized at 60 C for 16 h. Thin sections (gold interference color) were placed on formvar-coated 75 mesh or 200 mesh copper grids. These sections were stained with Reynolds lead citrate (Reynolds, 1963) for 6 min and 3% w/v UA in 75% methanol pH 4.2 for 6 min.

Mineralized portions of leg tendons from 10 to 14 week old domestic turkeys were excised and placed in 100% glycerol for 2h. Non-mineralized tendon becomes transparent under these conditions and so areas of interest near the

mineralization front, unmineralized tendon and heavily mineralized regions can be identified. Small pieces of glycerinated tendon were placed in a graded series of glycerol:methanol (3:1, 1:1, 1:3) followed by 3 changes of 100% methanol and 3 changes of acetone for 15 min each. The tissues were then embedded in Spurr's resin as above.

Sections were cut in longitudinal planes along the tendons with a diamond knife in different thicknesses depending on the visualization technique to be employed. Sections to be stained for electron microscopic analysis of collagen banding were gold interference color on distilled water. Sections for analysis of mineral localization and selected-area dark field were gray interference color on distilled water. These sections were quickly placed on formvar-coated 75 mesh or 600 hexagonal copper grids.

Selected-area dark field electron microscopy and bright field electron microscopy of mineralized sections were performed on unstained sections. In order to visualize collagen banding, sections were stained with 1% aqueous phosphotungstic acid (PTA) at pH 3.2 for 15 min and 1% aqueous uranyl acetate (UA) at pH 4.2 for 10 min. Although these staining methods produce the conventional banding pattern of type I collagen they were found to demineralize turkey leg tendons (Arsenault, 1988). In order to visualize the molecular orientation of collagen fibrils by analysis of their banding patterns and to maintain the native

distribution of apatite, non-mineralized and mineralized sections were stained with 1% UA in 100% methanol for 5 min. Bright field electron microscopy and SADP were performed using a Philips 300 electron microscope operating at 80 kV. The technique of SADP is described elsewhere (Jackson et al., 1978; Arsenault, 1988). This technique uses tilting of the main beam so that selected diffracted electrons are allowed to pass through the objective aperture. With the electron microscope in the diffraction mode the tilted beam is positioned so that specific electrons can be collected from the characteristic electron diffraction pattern of apatite. In this study the objective aperture was positioned to collect electrons diffracted from the c-axial lattice planes of apatite which correspond to the (002) d lattice spacing.

Computer Modelling

The system of modelling amino acid sequence in collagen fibrils used here is an adaptation of the system developed by Meek et al. (1979). The amino acid sequences for $\alpha 1(I)$ (Highberger et al., 1982) and $\alpha 2(I)$ (Boedtker et al., 1985) chick collagen were translated into single letter codes and entered each as a single string into a text editor. Chick collagen sequences were used because amino acid data for turkey is unavailable at this time. The results when using either turkey or chick data are expected to differ only

minimally because there is a large degree of sequence homology between species (Boedtker et al., 1985). These single strings were organized into repeating D units, 234 amino acids in length and 5 molecules in width. The relative positions of the two $\alpha 1(I)$ and the $\alpha 2(I)$ chains were assumed to be $\alpha 1-\alpha 2-\alpha 1$ (Chapman and Hulmes, 1984). The repeating units were then stacked to simulate four D units in length by fifty molecules in width. Some error in the localization amino acids in the telopeptides can be expected because these regions deviate the greatest from linearity and their conformations are not known. Functional classes of amino acids were then identified. In order to represent the charged amino acids responsible for the positive staining pattern of collagen D,E,R,K and H (see table of abbreviations for amino acids, p. iv) were left in their positions while all other amino acids were replaced by spaces. The resultant distribution was reduced by computer and then further reduced photographically to the equivalent size of electron microscopic images. The molecular orientation of collagen stained with alcoholic UA was determined by four alternative methods of comparison: 1) with a theoretical model of the distribution of negatively charged amino acids (D and E); 2) with a model of positively charged amino acids (K,R and H); 3) with a scaled micrograph of aqueous PTA/UA stained collagen; and 4) with data from Tzaphilidou et al. (1982b). The periodic distribution of the

hydrophobic amino acids having long side-chains, F, M, V, L and I, has been considered to be important in fibril structure (Hofmann et al., 1978) These hydrophobic amino acids were selected and then reduced to the scale of mineralized collagen fibrils for direct visual comparisons. The reverse contrast of this image represents areas low in hydrophobic groups as black and areas high in hydrophobic groups as white. Hydroxyproline and proline, which are considered to be important in molecular flexibility and helix stability were also selected in this fashion.

RESULTS

Electron microscopic examination of non-mineralized, glutaraldehyde fixed turkey tendon, post-fixed with osmium tetroxide and stained with UA and lead citrate, clearly illustrates the organization of tenocytes (fibroblast-like cells) and collagen fibrils (Figures 2a and b) which makes this an efficient system for studying the mineralization process. Tenocytes are organized into columns separated by extracellular matrix. These cells are generally elongated and are seen to have a variety of cross-sectional diameters because the section is through bodies of cells and through cell processes which extend parallel to collagen fibrils. The smallest of these processes are about 60nm in width. Intracellular organelles visualized in the tenocytes vary from cell to cell and between the cell and its processes. Rough endoplasmic reticulum, mitochondria, secretory vacuoles, filamentous structures, and microtubules are prominent features of these cells.

Within the extracellular matrix, collagen fibrils are aligned in a parallel fashion between the cells. Individual collagen fibrils can be followed longitudinally for many microns. Their diameters appear to vary from approximately 70nm to 300nm. At a relatively low magnification the pronounced axial repeat of these fibrils consists of alternating light and dark regions which are due to the

Figure 2a and b. Electron micrographs of non-mineralized tendon. Parallel columns of tenocytes (T) and their processes (CP) are separated by parallel collagen fibrils (F). Rough endoplasmic reticulum (rER), mitochondria (M), and microtubules (arrow) are intracellular organelles which are commonly observed in these cells. The parallel collagen fibrils extend longitudinally for many microns permitting analysis along individual fibrils. Glutaraldehyde/osmium tetroxide fixed, UA and lead citrate stained. Bars a=400nm, b=200; a X25,000; b X50,000.

specific staining properties of the highly ordered collagen molecules within the fibril structure. At a higher magnification (Figure 3a) each axial repeat is shown to be composed of an asymmetric series of narrow bands which have been given an "a,b,c,d,e" nomenclature (Hodge and Schmitt, 1960). Each band can be identified by its relative staining intensity, width and spatial relationships to other bands. The intensity, and therefore the fine structure, of these bands varies depending upon the staining methods used; fibrils in Figure 3a have been stained with aqueous PTA/UA which gives clear definition to each band. The morphological details of these bands are as follows: The "a" bands (a_1 , a_2 , a_3 , and a_4) are narrow, closely spaced bands which tend not to be clearly delineated; the two "b" bands (b_1 and b_2) are very distinct and well separated, also they have a well defined border between the adjacent "a" and "c" bands; for the "c" bands (c_1 , c_2 , and c_3) only c_2 is readily apparent while the others are faint; the single "d" band is an intense single band with well defined borders; the "e" bands (e_1 and e_2) are distinct lines narrowly separated from each other, e_2 and a_1 are separated by a narrow lucent space. In Figure 3 the densely stained regions are due to bands e, a and b, while c and d are within the light region. At the molecular level other studies have shown that the gap zone, the distance separating

Figure 3a. The periodic staining pattern of collagen fibrils. The D repeating unit, (D) can be labelled according to the recognizable series of bands defined by their intensity and their spatial relationships. The bands "a, b, c, d, and e" permit the determination of the N- to C-molecular orientation of the collagen fibril. 3b. A computer displayed image of positively and negatively charged amino acids is shown in the same molecular orientation as Figure 3a. This display depicts the spatial distribution and characteristic alignments of these charged amino acids; the resulting bands are labelled from "e to a" indicating that the N- to C- direction is from left to right. A computer displayed image from Figure 3b is scaled and aligned to the collagen image of Figure 3a (inset). The banding patterns of both images lie in registration. Glycerol treated, aqueous PTA and UA stained; Bar=100nm; X130,000.

3b

longitudinally arranged collagen molecules consists of $a_{3,2,1}$, $e_{2,1}$, d and c_3 while the overlap zone consists of $c_{2,1}$, $b_{2,1}$ and a_4 (Hodge and Petruska, 1963).

The banding pattern of type I collagen represents the distribution and density of charged groups of amino acids; therefore, theoretical models can be constructed from the known amino acid sequence of type I collagen. In our computer generated model (Figure 3b) charged amino acids align vertically to give the "a to e" banding pattern of native collagen fibrils. This model was then scaled and aligned to the stained collagen fibril (Figure 3a and inset). The registration and intensity of the bands derived by computer modelling correspond to the stained image and permit unambiguous determination of collagen orientation. The N- to C- direction of collagen molecular orientation correlates with the "e, d, c, b, a" presentation of this asymmetric pattern of bands (Figure 3b and insert). In this study it was found that adjacent fibrils in turkey tendon may be of the same or of opposite orientation and thus, the determination of molecular orientation within the collagen fibril is critical to the analysis of apatite localization.

Figure 4. Low magnification of glycerol treated tendon just distal to the mineralization front. The calcified regions appear white while the non-mineralized regions (N) are translucent. Mineralization proceeds distal to proximal (Min). Tissue at the boundary of visible mineralization was selected for electron microscopic analysis of early stages of apatite crystal deposition. Bar=0.5mm; X25.

Figure 5. Differing stages of mineralization present near the mineralization front in unstained sections. 5a In regions with least mineral density small localized, electron dense, regions of calcification (arrow) are evident within the otherwise electron-lucent, non-mineralized matrix. 5b. The proportion of mineralized matrix increases and periodic banding (arrowheads) within the collagen fibrils is present. 5c. Small regions of non-mineralized matrix (*) remain in a more heavily mineralized region but will be filled in by subsequent calcification. Bars=200nm; a X45,000, b X40,000, c X50,000.

Treatment of mineralized domestic turkey leg tendons with glycerol makes collagen translucent and the mineralized regions easily recognizable (Figure 4). Near to the insertion of the tendons into bone, the tendons are heavily mineralized. The degree of mineralization progressively decreases away from the insertion until it disappears to gross observation; this is representative of the region which was prepared for electron microscopic analysis of the mineralization front. When visualized in the electron microscope an individual, unstained, longitudinal section at the mineralization front has within it an apparent gradient of mineral spread. Initially small, localized regions of electron dense calcification are evident within the electron-lucent matrix (Figure 5a). As the degree of calcification increases banding occurs with a periodicity equivalent to the collagen D period (Figure 5b). In addition to the mineral which has a periodic nature there are regions which do not have a banding pattern. In this study I am only considering mineral which exhibits a periodic pattern because the non-periodic mineral deposition is most likely occurring within the non-collagenous extracellular matrix. In regions which are more heavily mineralized (Figure 5c) the banding pattern remains. The main feature at this stage is the progressive spread of mineral, seemingly in all dimensions.

At higher magnification unstained, longitudinal sections at the mineralization front of turkey leg tendons

(Figure 6a) reveal a distinct repeating pattern due to the electron density of apatite crystals. In contrast, non-mineralized regions of the same section do not demonstrate any structural detail. In regions of early mineralization, most electron density occurs in the gap zone; however, electron-dense material is also present in the overlap zone. More specifically, one observes that each zone has a pattern of electron density which is neither random nor homogeneous. The gap zone appears to be delineated, on either side, by an electron-lucent line and within the gap zone is a single asymmetrically positioned lucent area. Within the overlap zone early mineralization occurs in the mid-portion. In regions of increased mineral deposition similar features are observed but the difference in contrast between the gap and overlap zones is less marked.

Figure 6. The localization of apatite in collagen. 6a. A bright field image of an unstained, longitudinal section of mineralized turkey leg tendon showing the inherent banding pattern due to a high mineral content in gap zones (GZ) as compared to less mineral in the interspaced overlap zones (OZ). The mineral has an asymmetric distribution within gap zones demarcated by a lucent space (white arrowheads). This distribution is consistent within a given fibril. Also, the gap zones are bordered on both sides by a narrow lucent space (white arrows). Non-mineralized portion of matrix (*). 6b and c. Two representative SADf images of mineralized unstained collagen fibrils showing the specific localization of apatite crystals (c-axial lengths) which appear in the reverse contrast to bright field electron micrographs. Apatite is identified as white highlights within both the gap zones (GZ) and overlap zones (OZ) at the earliest stages of mineralization. Bars=100nm; X120,000.

SADF specifically identifies and directly visualizes apatite crystals within both gap and overlap zones (Figure 6b); these c-axially oriented crystals appear in reverse contrast to the bright field images. It is apparent that the pattern of mineral deposition within the gap and overlap zones demonstrated by SADF is the same as by bright field electron microscopy.

In order to correlate the molecular orientation of collagen with the pattern of apatite deposition it is necessary to relate the stained collagen banding pattern of non-mineralized areas with mineralized areas on the same fibril. Certain technical difficulties must be overcome because it is known that aqueous staining procedures totally demineralize sections. Also, it was found that regions adjacent to mineral deposition are not as clear in their banding as regions more distant. Therefore, to enhance the fine structure of collagen fibrils and to retain the native distribution of apatite crystals, sections were stained with alcoholic UA. The alcoholic UA staining produced a different banding pattern as compared to the aqueous PTA/UA stains. So to unambiguously determine the molecular orientation of collagen with this UA stain, a theoretical model of charged amino acids was scaled and aligned to a representative non-mineralized region of a collagen fibril (Figure 7a). The banding patterns of the stained fibril and the scaled computer model are in registration, thereby enabling the

determination of N- to C- molecular orientation which is directed from left to right. The b_1 and b_2 bands observed with alcoholic UA staining were similar in appearance to the PTA/UA stained pattern and consisted of a distinct pair of bands within the more electron-dense region of the D period. The d band also retained its characteristic intensity. However, the a, e, and c bands maintained their relative positions but were not as intensely stained as with PTA/UA. Advancing toward the mineralized portion of the fibrils the stained banding pattern is replaced by the characteristic pattern of apatite distribution (Figure 7b). The distribution of apatite in these gap and overlap zones was precisely the same as was observed for unstained mineralized section (compare Figures 7b and 6a). Within the gap zone the asymmetric distribution of mineral is created by a lucent space. The positioning of this space is consistent with the N- to C- orientation of the collagen molecules and when apparent is nearest to the N- terminus. It was observed that adjacent fibrils having opposite molecular orientations as determined by their staining pattern also have consistent lucent spaces positioned on opposite sides of the gap zone (Figure 7b).

Figure 7. Analysis of collagen orientation in mineralized turkey tendon. 7a. A non-mineralized portion of a collagen fibril stained with alcoholic UA. A computer image (7a insert) displaying the spatial distribution of charged amino acids has been scaled and aligned to determine the N- to C-molecular orientation. This staining pattern is not equivalent to the PTA/UA staining but the orientation is readily determined by the prominent b and d bands. 7b. Non-mineralized and mineralized portions of the same collagen fibril stained with alcoholic UA. The adjacent fibrils have opposite orientations as indicated by their opposite banding patterns in the non-mineralized areas. The mineralized portion shows the same periodic distribution of apatite of the unstained sections. Electron lucent spaces border the gap zone and within the gap zone lucent spaces (arrowheads) are positioned asymmetrically consistent with the opposite orientation. Bars=100nm; X120,000.

Figure 8. Electron diffraction of alcoholic UA stained mineralized (8a) and unstained mineralized tendon (8b). The characteristic pattern of apatite is observed under both conditions demonstrating that apatite is retained when sections are stained with UA in 100% methanol. Two prominent reflections are readily indentified: the inner (002) reflection, and the combined (211, 112, 300, 202, 301) reflection.

Electron diffraction analysis from these dense mineralized areas of alcoholic UA stained sections showed the characteristic maxima for biological apatite crystals and thereby confirming its presence (Figure 8a and b).

After determining the N- to C- molecular direction of mineralized collagen fibrils the nature of the apatite-collagen distribution was further analyzed by comparison to computer models selecting for various amino acid groups such as: charged (positive and/or negative), polar non-charged, aromatic, and hydrophobic groups. These classes of amino acids were selected because of their influence on collagen structure. Of these, the model having the best fit to the unstained mineralized collagen fibril was the hydrophobic amino acids displayed in normal and reverse contrast (Figure 9a). It was observed from the computer model that there are three regions with high spatial densities of hydrophobic amino acids which are displayed as white areas in the reverse contrast image. Two of these hydrophobic regions, a_4 - a_3 and c_2 - c_1 , border each side of the gap zone; the third lies asymmetrically positioned in the gap zone between a_1 and e_2 nearer the N-terminus of the molecular orientation. Regions of the collagen fibril containing low spatial densities of hydrophobic amino acids include most of the gap zone; intermediate regions lie within the overlap zone from c_1 to

a_4 , and within the gap zone from e_1 to c_2 . These computer-generated hydrophobic repeats align and correlate with micrographs of unstained mineralized collagen (Figure 9b and inset). In this set of images it is clearly shown that apatite is less dense in those areas occupied by high spatial densities of hydrophobic amino acid residues.

Analysis of the hydrophobic amino acid composition of the gap and overlap zones of the collagen D period shows that gap zone is less hydrophobic, on average, than the overlap zone. In one repeating unit, 5 molecules in width and D in length, there are 129 hydrophobic amino acids within the overlap zone and 100 hydrophobic amino acids in the gap zone. Therefore, the overlap zone has about twice (1.93) the density of hydrophobic amino acids. The ratios of the individual constituent hydrophobic amino acids varies between the two zones by at most 4%. Within the gap zone the percentage of hydrophobic amino acids are: M=9%, V=27%, L=30%, F=17%, I=17%. Within the overlap zone the percentages are: M=11%, V=25%, L=34%, F=16%, I=14%. Using figures for the additional free energy of transfer from water to an organic solvent as a measure of amino acid hydrophobicity (see Tanford, 1980, p. 140) the differences in amino acid composition vary the overall hydrophobicity by about 0.1%. Thus, the differences in average hydrophobicity throughout these two zones relate to differences in density and not to differences in composition.

The proportions of gap and overlap zones relative to the D unit were calculated by counting the amino acids for each zone; 101 for the overlap zone and 133 for the gap zone. This results in the predicted proportion of the D unit occupied by the overlap zone to be .432D and the gap zone to be .568D assuming that the lengths occupied by amino acids are equivalent. These figures could then be used to predict the density of amino acids per molecule and to standardize for length of each zone.

Hydroxyproline and proline are periodically localized along the D period (Figure 10). The density of these residues are a reflection of stability of the molecular triple helix. There are 359 hydroxyproline and proline residues within the overlap zone compared to 335 within the gap zone. Therefore, there is a greater proportional density of these residues in the overlap zone as compared to the gap zone (1.13:1). Two regions, one at each end of the gap zone have very few proline or hydroxyproline residues. These regions may represent more flexible portions of the D unit.

Figure 9. A comparison of the distribution of hydrophobic amino acids and mineral apatite. 9a. A computer image of hydrophobic amino acid groups displayed in normal and reverse contrast; the N- to C- molecular orientation is from left to right. Periodic regions of the collagen fibril contain high densities of hydrophobic amino acids depicted in the top image as black and in the reverse contrast image as white. 9b. An unstained mineralized collagen fibril is shown with an aligned and scaled reverse contrast computer image of hydrophobic amino acids (inset). The registration and densities of the two images correspond. The asymmetrical localized mineral (density) corresponds to regions low in hydrophobic amino acids; while lucent areas, with less mineral density, correspond to regions high in hydrophobicity. Bar=100nm; X120,000.

Figure 10. A computer image of proline and hydroxyproline distribution. These amino acids confer stability to the structure of collagen molecules. This image indicates that there is a periodic localization to these residues which may have implications on the adaptation of collagen molecules to apatite crystal growth. Regions with fewer proline and hydroxyproline residues are considered to be more flexible and may accomodate to changes in conformation.

$$N \rightarrow C$$

DISCUSSION

The ordered distribution of apatite crystals in type I collagen has been an important subject of study because of the interactive role of mineral and collagen in producing the biomechanical properties of skeletal tissues. This distribution has long been viewed to reflect the nature of collagen structure. However, at this time our knowledge of how apatite crystals may interact with collagen is very limited. To date, theories of mineral/collagen relationships have focussed on the potential space presumed to be available within the gap zone as a result of the D-stagger of collagen molecules (Hodge and Petruska, 1963; Glimcher, 1983). Therefore, apatite has been believed to be excluded from the overlap zones during the early stages of the mineralization process. Evidence to support this viewpoint comes from electron microscopic (Glimcher, 1959), electron diffraction (Engström, 1966), neutron diffraction and x-ray diffraction studies (White et al., 1977). A recent study on the specific and direct visualization of apatite by SADP imaging has shown crystals to be present in both gap and overlap zones at the earliest detectable stages of mineralization (Arsenault, 1988). As an extension of these recent findings the present study compares apatite localization to the molecular orientation and amino acid sequence of collagen; and consequently offers a hypothesis

of collagen influence on bone formation. It was observed by both bright field and selected-area dark field imaging that early stages of intrafibrillar mineralization are characterized by an asymmetric and periodic distribution of apatite crystals which corresponds to specific regions along the N- to C- molecular orientation of the collagen fibril. Areas which had greater density of mineral consisted of most of the gap zone and the midportion of the overlap zone. Areas of least mineral density were discrete regions on either side of the gap zone and an area within the gap zone invariably positioned nearer the N-terminal direction of the fibril. It was concluded from these observations that the heterogeneous distribution of apatite crystals within the gap and overlap zones most likely reflects periodic properties of the collagen fibril within both zones.

Regions of the D period which have least mineral density correlate in position to the periodic density of hydrophobic amino acids as depicted by computer modelling. Conversely, regions which have the greatest mineralization correlate in position to regions of less hydrophobicity. These findings provide evidence for a more complicated distribution of apatite crystals in collagen than simple confinement to the gap zones. Thus, hydrophobicity may be involved in the mechanism of collagen mineralization as a result of apatite exclusion from these domains. This work has a number of implications on the interrelationship

between the mechanism of collagen mineralization and the architecture of crystal distribution as defined by the molecular ordering of collagen.

Specific regions localized along the collagen fibril are high in hydrophobic amino acids (a_4 - a_3 , c_2 - c_1 and a_1 - e_2) and correlate to areas which may inhibit crystal formation or prevent crystal growth into the area. Conversely, mineral distribution can be expected to occur in regions with less spatial density of hydrophobic amino acids. We are speculating that the hydrophobic effect may influence either of two processes. First, the initial site of nucleation may be determined by a specific environment isolated from the hydrophobic areas. For example, the diffusion and localization of various ions including calcium and phosphate will be affected by the close association of hydrophobic groups. Local concentration gradients of these ions are likely to be critical for the nucleation of biological apatite. Also, properties of the collagen fibril may be instrumental in the localization of non-collagenous molecules which have negative or positive control on crystal growth. Macromolecules, such as proteoglycans (Scott and Haigh, 1985) have specific sites of association with collagen. Various macromolecules such as phosphoproteins (Veis, 1985b), γ - carboxyglutamic acid (Glimcher et al., 1979) and osteocalcin (Hauschka et al., 1975) have been associated with collagen-rich mineralizing tissues.

Secondly, apatite crystal size and growth may be influenced by space and energy requirements. The dissociation of hydrophobic groups during the mineralization process will require a net energy which will affect the distribution of mineral. The result being that areas of hydrophobicity may compartmentalize regions of optimal crystal growth. Both of these two processes, whether local environmental conditions or energy considerations, occur as a result of the favorable free energy state achieved by minimizing the interaction of hydrophobic groups with the aqueous medium (Tanford, 1980); and neither is mutually exclusive. In addition, these potential processes do not exclude concurrent influences.

Although there does seem to be exclusion of mineral from hydrophobic regions there are also points along these regions where mineral is present (Figure 6). In addition, some D periods visualized do not exhibit the a_1 - e_2 line within the gap zone. One must ask the question "Why is the periodic distribution of apatite in collagen in the unstained, mineralized sections not more defined and consistent?" In other words, one must examine the mineral crystals which represent exceptions to this proposed hypothesis. Several potential explanations are possible. First, the computer images (Figure 9) show that the amino acid composition is not homogeneous within the hydrophobic regions. Rather than a perfectly linear arrangement, this class of amino acids is depicted more as clumps. This

concept is compatible with thermodynamic concepts of hydrophobicity in that the hydrophobic amino acids tend to aggregate. Therefore, it may be more accurate to describe these regions as differing in the density of hydrophobic amino acids. There may be sufficient non-hydrophobic amino acids (in a three-dimensional packing arrangement) to permit some crystal formation within each of these hydrophobic regions. Second, the three-dimensional packing arrangement of collagen is somewhat distorted and discontinuous (Hulmes et al., 1985). The Hodge-Petruska (1963) model could not be expected to hold true for the relationship between all collagen molecules visualized in a section which is approximately 70nm thick. Therefore, sufficient molecules of a 70nm thick section may be offset to result in crystals forming outside of the general pattern. However, there is no means to predict the degree of this distortion. Third, the thicker the section, the more likely the electron lucent regions within the gap zone will not be visualized. This is because the space (in this case the proposed hydrophobic regions) between crystals are small relative to the thickness of the section. Incident electrons entering the space at 0° would pass through but if the specimen were tilted the space would appear smaller as the angle became greater. At a certain angle of incidence the space would no longer permit the passage of electrons. The predicted size of the hydrophobic regions based on the computer models

($a_4 - a_3 = .12D$ or about 7.8nm; $c_2 - c_1 = .14D$ or about 9.4nm; and $a_1 - e_2 = .07D$ or about 4.7nm) and an estimated specimen thickness (70nm) gives a critical incident angle at which the space would no longer be detectable. Assuming that the mineral within the gap zone are blocks, equal to the specimen in thickness, positioned in parallel, the $a_1 - e_2$ region within the gap zone would disappear at an electron incident angle of 3.5° . The largest of these predicted spaces, the $c_2 - c_1$ region, would no longer be visible at an angle of 7.6° . Therefore, the non-mineralized regions bordering the gap zone would be detected much more frequently than the region within the gap zone. The angle of sectioning becomes critically important as does the normal change in direction of collagen within the tendon. And so this pattern would be more difficult to image in bone because of its more complex structural organization of collagen fibrils.

Electron microscopic imaging of calcified tissues represents a technical challenge to maintain the mineral component while visualizing the associated organic matrix. The staining of collagen with UA in alcohol is an alternative approach to the demineralizing effects of aqueous PTA/UA stains but produces a different collagen banding pattern. While the positioning and registration of the bands "a to e" are the same due to the interaction of UA with charged groups, the relative intensities and

delineations of the bands are not as clear. An explanation of the observed pattern is not readily apparent for two reasons. First, although early works of UA staining consider UA to be a cationic stain it actually stains both negatively and positively charged amino acids depending on concentration and pH variables (Tzaphilidou et al., 1982b). Secondly, the effects of alcohol as a solvent in the staining of collagen has not been studied and so a comparison between water and alcohol as solvents in this procedure has not been done.

The visual interpretation of repeating patterns in collagen fibrils is a preliminary to more precise correlational methods. A mathematical correlation between density of mineral and the degree of hydrophobicity within each region would help to substantiate the proposed relationship. However, despite the availability of several hydrophobic scales, hydroxyproline has not been assigned a value on these scales, thus making calculations in the standard method impossible for collagen molecules. The limitation of visual methods is in estimating the strength of the relationship because there is a subjective interpretation of the degree of fit.

The periodic localization of apatite crystals within the overlap zones of collagen fibrils (Arsenault, 1988) is evidence which contradicts theories exclusively localizing crystals within gap spaces (eg. Weiner and Traub, 1986) or

spaces within microfibrils (Höhling, 1980). Other evidence comes from studies that have shown that crystal size is variable and not determined by the size of the gap space (Arsenault and Grynblas, 1988). Although these potential spaces may be influential in crystal growth or the localization of non-collagenous materials they do not explain these observations. Consideration must therefore be given to the discrete environment which gives rise to heterogeneity of crystal size and heterogeneity of crystal deposition within the entire D unit. So how does the apatite mineral become deposited in the densely packed overlap zone?

Collagen peptides can be conceptualized as semiflexible cylinders which intertwine to form a higher order semiflexible cylinder, the collagen molecule (Veis, 1985a). These molecules have a high degree of backbone and side chain flexibility (Sarkar et al., 1987) because the triple helix can accommodate fluctuations without appreciable changes in free energy (Okuyama et al., 1981). These molecules intertwine to form collagen fibrils which also have longitudinal and lateral flexibility according to electron microscopic and biomechanical studies (Viidik, 1980; Giraud-Guille, 1988). The flexibility of collagen (and its stability) are dependent on: 1) the G-X-Y triplet; 2) proline and hydroxyproline localization; 3) crosslinking; 4) charged amino acid side chain interactions; and

5) hydrophobic amino acid side chain interactions. Thus, the collagen molecules exhibit flexible and inflexible regions (Veis, 1985a) which can be built into repeating units and depicted by computer modelling (Figure 10). From this modelling it can be observed that there are periodic flexible regions and that the gap zone is more flexible than the overlap zone. It is possible that mineral is accommodated into the collagen overlap zones by the semiflexibility of collagen molecules within the fibrils. Some of the difference between mineral density in gap and overlap zones may be explained by differences in localized collagen flexibility. The gap zone is bounded by regions with very low densities of proline and hydroxyproline (a_3-a_2 and c_3-c_2). These regions correspond to regions with a low density of hydrophobic amino acids and are adjacent to the regions of high hydrophobicity discussed above.

Conclusions

In an observational, correlational study such as this, it would be improper to suggest that a cause and effect relationship exists between the variables under examination. Based on previous studies the relationships described in this thesis are biologically plausible but alternative experimental designs are required to substantiate and clarify the potential role of hydrophobicity and flexibility in mineralization. In conclusion, the asymmetric and

periodic distributions of apatite crystals within the gap and overlap zones of collagen fibrils were observed to correspond to collagen molecular orientation. This mineral distribution has a reverse correlation to the position of hydrophobic groups as determined by computer modelling and may reflect intrinsic properties of collagen structure throughout the gap and overlap zones. In addition, it was found that periodic differences in intrafibrillar flexibility determined by density of proline and hydroxyproline, may explain preferential accommodation of apatite into specific regions. Therefore, to understand the role of collagen during mineralization, consideration should be given to the discrete properties of collagen as they vary throughout the D period rather than focussing on the potential space within the gap zone. Further study of the association between the localization of apatite and properties of collagen fibrils may enhance our understanding of the mechanisms of biomineralization and the mechanics of mineralized tissues.

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