Collagen II Containing a Cys Substitution for Arg-α1−519: Abnormal Interactions of the Mutated Molecules with Collagen IX†

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ABSTRACT: Single amino acid substitutions in collagen II cause heterogeneous cartilage disorders including some chondrodysplasias and certain forms of heritable osteoarthritis. In this study, we examined molecular interactions between normal collagen II and collagen IX, and the effect of a Cys substitution for Arg-α1−519 in collagen II on these interactions. Binding assays showed that the association equilibrium constant of collagen IX−collagen II interaction is 15×10^6 M^{-1}. Specificity of the interaction was analyzed by the binding of collagen IX to recombinant collagen II variants lacking fragments of 234 amino acids corresponding to particular D-periods. The results indicated that the C-terminal half of collagen II, which includes the D3 and D4 periods, has a high affinity for collagen IX, and that the nontriple helical telopeptides of collagen II are not essential for the specific binding of collagen IX. Computer analysis of the surface of the mutated collagen II and binding assays showed that a Cys substitution for Arg-α1−519 changes electrostatic properties around the mutation site, increases the affinity of mutant collagen II for collagen IX, and possibly alters the specificity of the interaction. Thus, the results indicate that interactions between collagen II and collagen IX are site specific and that single amino acid substitutions in collagen II may change the molecular interactions with collagen IX that could destabilize the cartilaginous matrix.

Collagen fibrils are the major structural component of cartilage. These fibrils are heterotypic assemblies of collagens II, IX, and XI (*1*) that are organized into a continuous network-like structure. The main core of a fibril is formed during a self-assembly process of collagen II monomers. It was recently shown that collagen XI nucleates self-assembly and limits lateral growth of the collagen fibrils in cartilage (*2*). In contrast, the role of collagen IX is less understood. It was suggested that collagen IX mediates interactions between collagen fibrils and glycosaminoglycans (*3*), thus contributing to the long-term structural and functional stability of cartilage (*4*). Binding of collagen IX to collagen II fibrils appears highly specific. Collagen IX is deposited on the surface of the collagen fibril in an antiparallel fashion (*5*), and it interacts with it through its NC1, COL1, NC2, and COL2 domains (*5, 6*). The collagen IX molecules are evenly distributed on the D-periodic[1] intervals of the collagen fibril (*7*) and are covalently bound to collagen II through cross-linking bonds (*5*−*7*). The lysine-derived cross-links are formed between the triple helical COL2 domain of collagen IX and the N- and C-terminal nontriple helical telopeptides of collagen II (*5, 6*). The distance between these cross-links is 137 amino acid residues or about 40 nm (*5*). This distance corresponds to the length of a gap region (0.6D) in the collagen fibrils (*8*). Therefore, one collagen IX molecule is cross-linked to two collagen II monomers, and the domain that has direct contact with collagen II is about 110 nm in length (*7*) and most likely extends through D0.4, D4, and part of the D3 period of the C-telopeptide-linked collagen II molecule. While there has been progress in characterizing collagen II−collagen IX interactions, the molecular basis that is critical for the specific binding and precise alignment of these molecules is not known.

Mutations in the genes encoding collagen II (COL2A1) and collagen IX cause heterogeneous cartilage disorders including several forms of chondrodysplasia and heritable osteoarthritis (*9*−*15*). One class of mutations found in the human COL2A1 is single base substitutions that convert codons for Arg residues in the -Y- position of the repeating Gly-X-Y- sequence to codons for Cys. The first identified Cys for Arg substitution was at α1−519[1] in a family with an early onset of generalized osteoarthritis with features of mild chondrodysplasia (*16*). Subsequently, nonrelated probands

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[1] Amino acid positions of the α1(II) chain are numbered from the first glycine in the major triple helix; F, full-length collagen II molecule; residues 1−1014, D1, D2, D3, and D4, consecutive fragments of 234 amino acids from the amino terminus of the triple helical domain of procollagen II.

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with the same Cys for Arg-α1−519 substitution were also reported (*17*, *18*). The Cys for Arg substitutions at the -Y-position of the Gly-X-Y triplet were also found at α1−75 (*12*, *19*) and α1−789 (*20*). In all occurrences, the mutated collagen monomers were incorporated into cartilaginous extracellular matrix as determined by the presence of disulfide-bonded α1(II) chains extracted from the cartilage of the probands (*20*, *21*) or by the presence of abnormal collagen fibrils (*12*). In vitro studies of recombinant collagen II containing Cys at α1−519 demonstrated that the mutant α chains fold into a stable triple helix, and do not form intramolecular disulfide bonds (*22*). We have also reported that mutant collagen II does not self-assemble into normal fibrils. Instead, mutant collagen monomers form amorphous aggregates (*23*). When present in a mixture with normal collagen II, mutated monomers alter fibril formation by changing the kinetics of assembly and morphology of fibrils (*22*). Moreover, as suggested by Adachi et al., mutated monomers present on the surface alter the topography of the collagen II fibril (*23*).

In this study, we have extended the analysis of the consequences of the Cys substitution for Arg-α1−519 by studying the interaction of mutant collagen II monomers with collagen IX. We have tested the hypothesis that binding between collagen II and collagen IX is site-specific, and that Cys for Arg-α1−519 substitution alters this interaction. Using recombinant collagen II variants lacking specific D-periods, we conducted binding studies, and demonstrated that collagen IX interacts preferentially with the C-terminal region of the normal collagen II monomer. We also found that the Cys for Arg-α1−519 substitution changed the affinity of collagen II for collagen IX and, possibly, altered the specificity of binding.

EXPERIMENTAL PROCEDURES

Expression of Normal and Mutated COL2A1 Genes. To express normal and mutated human COL2A1 genes, the DNA constructs were stably transfected into HT-1080 cells by calcium phosphate precipitation, and clones expressing the cotransfected neomycin resistance gene were selected with G418 (*22*, *24*). The selected clones that secreted procollagen II were cultured under standard conditions without G418. To harvest the recombinant procollagen, the cells were cultured in Dulbecco's modified Eagle's medium supplemented with L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako; Osaka, Japan). In some experiments, 0.2 μCi/mL of a uniformly ^{14}C-labeled amino acid mixture (DuPont NEN) was added to the culture medium.

Procollagen II DNA Cassette System. Genetically engineered variants of procollagen II lacking consecutive fragments of 234 amino acids, defined here as D-periods, were expressed in HT-1080 cells as described in detail by Arnold et al. (*25*, *26*).

Purification of Recombinant Procollagen II. Recombinant normal and mutant procollagen II were purified from culture media according to the method described by Fertala et al. (*24*). The procollagen variants with specifically deleted D-periods were obtained from cell culture media using a modified procedure described by Arnold et al. (*25*). In brief, for each cell line producing procollagen II, approximately 4 L of medium harvested from each 24 h period was filtered through a 1.6 μm glass-fiber filter (Millipore) and supplemented with the following reagents at the indicated concentrations: 0.1 M Tris-HCl buffer, 0.4 M NaCl, 25 mM EDTA, and 0.02% NaN_3 adjusted to pH 7.4. Because of a concern of decreased stability, the procollagen II variants with deleted D-periods were concentrated and precipitated in the presence of 10 mM *N*-ethylmaleimide and 1 mM *p*-aminobenzamidine (*25*). High molecular weight proteins in the medium were concentrated approximately 10-fold at 4 °C by the use of cartridges with a 100 kDa molecular mass cutoff (Prep/Scale-TFF filter; Millipore). Proteins in the concentrated media were precipitated overnight at 4 °C with 175 mg/mL ammonium sulfate and collected by centrifugation at 15000*g* for 1 h at 4 °C. Procollagen II was purified using three-step ion exchange chromatography as described by Fertala et al. (*24*). Procollagen peak fractions were pooled and dialyzed against a storage buffer (0.1 M Tris-HCl buffer, pH 7.4, with 0.4 M NaCl and 25 mM EDTA). Finally, the purified procollagen was concentrated by ultrafiltration on a membrane filter (YM-100; Amicon) and stored at −80 °C.

Cleavage of Procollagen II with Procollagen N- and C-Proteinases. To generate collagen II with intact telopeptides, procollagen propeptides were enzymatically removed by cleavage with procollagen N-proteinase (EC 3.4.24.14) (*27*) and procollagen C-proteinase (EC 3.4.24.19) (*28*) purified from chick embryo tendons. Enzymatic digestion was carried out in 25 mM Tris-HCl buffer, pH 7.5, containing 7 mM $CaCl_2$, 0.1 M NaCl, 0.015% Brij, and 0.02% NaN_3. The reaction mixture contained approximately 200 μg of procollagen, 100 units of N-proteinase, and 100 units of C-proteinase. One unit of each of these enzymes is defined as the amount of enzyme needed to cleave 1 μg of substrate in 1 h at 35 °C. The reaction was carried out at 35 °C for 4 h. The enzymes were then inactivated by the addition of EDTA to a final concentration of 10 mM.

Purification of Collagen. The product of the cleavage of procollagen II by procollagen N-proteinase and C-proteinase was purified by size exclusion chromatography. The sample was passed through a 21.5 mm × 60 cm size exclusion column (G 3000 SW, Tosohaas, Japan) equilibrated with 0.1 M Tris-HCl buffer, pH 7.5, containing 0.4 M NaCl, 5 mM EDTA, 0.02% NaN_3 and connected to an HPLC system (Dynamax, Rainin). Chromatography fractions containing collagen II were combined and then dialyzed against phosphate-buffered saline (PBS).

Purification of Recombinant Collagen II Variants. To isolate D-period-deficient collagen II variants from cell culture media, the ammonium sulfate precipitates from six 24 h collections were pooled and dissolved in 0.1 M HCl at 4 °C. Subsequently, noncollagenous proteins were digested with pepsin (Boehringer Mannheim) at a concentration of 100 μg/mL for 24 h at 4 °C. Pepsin-resistant collagen II variants were then precipitated by addition of NaCl to a final concentration of 0.7 M. Precipitated proteins were collected by centrifugation at 15000*g* for 1 h at 4 °C. The pellets were washed twice with cold distilled water and dissolved in 0.1 M HCl. The concentration of collagen II was determined by amino acid analysis (Wistar Protein Microsequencing Facility; Philadelphia, PA) or by collagen-specific colorimetric assay (Sirol Assay Kit; Biocolor Inc., Ireland).

Quantitation of Free Sulfhydryl Groups in Mutant Collagen. Purified procollagen was converted to collagen by digestion of globular propeptides with pepsin. Subsequently, collagen was purified by size exclusion chromatography. Free sulfhydryl groups in mutant collagen II were analyzed by the use of Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] (DTNB; Pierce) (*29*). In brief, 150 μg of native or heat-denatured mutant or normal collagen II was added to a reaction mixture that contained 50 μL of 0.1 M DTNB in 0.1 M phosphate buffer, pH 8.0, and 1 mL of 0.1 M phosphate buffer, pH 8.0. The samples were incubated at room temperature for 15 min, and then measured for absorbance of a yellow-colored product at 412 nm. The content of free sulfhydryl groups in collagen samples was determined from a standard curve derived from the measurements of absorbance of samples containing known concentrations of a cysteine standard (Cys-HCl monohydrate; Pierce).

Purification of Collagen IX. Collagen IX was expressed in insect cells as described by Pihlajamaa et al. (*30*) (a crude protein precipitate containing collagen IX was a generous gift from Dr. Leena Ala-Kokko, Department of Medical Biochemistry, University of Oulu, Finland). Collagen IX was purified according to the method described previously (*30*). In brief, the protein precipitate was dissolved in 0.05 M Tris-HCl buffer (pH 7.4), containing 0.5 M urea and 0.2 M NaCl. Collagen IX was purified by gel filtration through Superdex 75 (Amersham Pharmacia Biotech) using a 0.05 M Tris-HCl buffer (pH 7.4), containing 0.2 M NaCl as the elution buffer. The sample was then dialyzed against a 0.05 M PIPES buffer (pH 6.5) containing 2 M urea, 0.05 M NaCl, 10 mM EDTA, and 0.02% NaN_3. Subsequently, the sample was loaded on an ion exchange column (S HyperD 10, Beckman) and eluted with a 0−0.05 M continuous gradient of NaCl. Purified collagen IX was analyzed by electrophoresis in 7.5% SDS−polyacrylamide gels. The concentration of purified collagen IX was determined by amino acid analysis (AAA Laboratory; Mercer Island, WA).

Binding of Collagen IX to Normal and Mutant Collagen II. Collagen IX−collagen II interaction assays were carried out on an optical biosensor (Iasys; Affinity Sensors, U.K.). Collagen II or collagen IX was immobilized onto the surface of carboxyl chips (Iasys; Affinity Sensors, U.K.). The biosensor surface was activated by an injection of a 1:1 mixture of 0.1 M *N*-hydroxysuccinimide and 0.4 M *N*-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce). Recombinant proteins at a concentration of 100 μg/mL dissolved in PBS were then allowed to bind to the activated surface until a response plateau was reached. The residual active groups were blocked by an injection of 200 μL of 1 M ethanolamine hydrochloride (pH 8.5). Nonspecific binding sites were blocked by the injection of 200 μL of a 1% solution of bovine serum albumin (BSA) dissolved in PBS. Excess BSA was removed by washing of the cuvette with PBS containing 0.05% Tween-20 (PBST), followed by three consecutive washes with 0.1 M HCl. After reequilibration with PBST, the cuvettes were ready for the binding assays. In addition, a control cuvette with immobilized BSA was prepared using the same procedure.

The following interactions were analyzed: (a) between normal or mutant collagen II and collagen IX, (b) between collagen IX monomers, and (c) between variants of collagen II lacking particular D-periods and collagen IX. Cuvettes with immobilized collagens were primed with PBST at 25 °C for 15 min. A 200 μL sample containing free collagen dissolved in PBST was added to the cuvette, and proteins were allowed to interact for 10 min (association phase). Subsequently, the sample was removed, and PBST without collagen was added to the cuvette for an additional 5 min (dissociation phase). After each assay, the surface of a cuvette was regenerated by washing with 0.1 M HCl, followed by equilibration with PBST. To analyze kinetics, the collagens in solution were added at concentrations ranging from 1×10^{-8} to 8×10^{-8} M. In collagen IX−collagen IX binding assays, free collagen IX was used at concentrations ranging from 2×10^{-5} to 8×10^{-5} M. Data from the biosensor were analyzed by the global fitting method described by Myszka and Morton (*31*). For each assay, the k_{on} and k_{off} were obtained, and the K_d values were calculated from a ratio of k_{off}/k_{on}. Subsequently, the K_a values were derived as a K_d inverse.

Analysis of Disulfide Bond Formation. ^{14}C-Labeled mutant collagen II at a concentration of 150 μg/mL was incubated for 24 h with recombinant collagen IX at the same concentration. The proteins were then analyzed for the presence of disulfide-bonded α chains by electrophoresis on a 7% polyacrylamide gel with SDS. The gels were stained with colloidal Brilliant Blue G (Sigma), destained, and dried. The same gels were also assayed with a phosphostorage plate (Phosphoimager Storm; Molecular Dynamics) for the presence of disulfide-bonded α1(II)-α1(II) or α1(II)-α(IX) chains. This assay made it possible to detect as little as 0.1 μg of the α1(II) chains (*22*).

Surface Properties of Mutant Collagen II. To determine how the Cys for Arg-α1−519 substitution changes the properties of the surface of collagen II, computer analysis was performed employing a modeling program (Sybyl 6.6; Tripos, Inc., St. Louis, MO) installed on an Octane computer station (Silicon Graphics, Inc.). The model of the collagen II fragment spanning Arg-α1−519 was generated as described earlier (*22*, *32*), and the electron density surface, which represents an isosurface of electron densities, was calculated (*33*). The electrostatic potential [EP; expressed as kcal/(mol·e)] of the surface was then evaluated. The surface area that had a similar EP as the mutation site was also calculated.

RESULTS

Recombinant Proteins. Recombinant normal collagen II, mutant collagen II with the Cys for Arg-α1−519 substitution (Figures 1 and 3), and the D-period-deficient collagen II variants (Figure 1) were expressed in HT-1080 cells (*22*, *24*, *25*). As described earlier, the proteins had normal thermal stability and correct amino acid and carbohydrate composition (*22*, *24*, *25*). To analyze the role of the nontriple helical telopeptides of collagen II in interaction with collagen IX, purified procollagen II was enzymatically cleaved by procollagen N-proteinase and procollagen C-proteinase. As previously shown by N-terminal amino acid sequencing and determination of the molecular mass of the products, the enzymes specifically process both the natural and recombinant fibrillar procollagens (*24*, *25*, *28*, *34*). As shown in Figure 1, the size of the fragment generated by digestion with procollagen N-proteinase was consistent with the prediction that the cleavage occurs only at the single

Table 1: Kinetics of Binding of Collagen II to Collagen IX and of Collagen IX to Collagen IX[a]

interaction	k_{on} ($M^{-1}\,s^{-1}$)	k_{off} (s^{-1})	K_d (M)	K_a (M^{-1})
normal collagen II−collagen IX[b]	3.9×10^4	2.5×10^{-3}	6.5×10^{-8}	15.4×10^6
normal collagen II−collagen IX[c]	3.4×10^4	2.8×10^{-3}	8.2×10^{-8}	12.1×10^6
-D1−collagen IX	3.3×10^4	2.3×10^{-3}	7.0×10^{-8}	14.3×10^6
-D2−collagen IX	3.8×10^4	2.5×10^{-3}	6.6×10^{-8}	15.2×10^6
-D3−collagen IX	1.7×10^4	4.8×10^{-3}	2.8×10^{-7}	3.5×10^6
-D4−collagen IX	1.5×10^4	5.1×10^{-3}	3.4×10^{-7}	2.9×10^6
mutant collagen II−collagen IX	8.1×10^4	2.7×10^{-3}	3.3×10^{-8}	30.0×10^6
collagen IX−collagen IX	4.0×10^2	3.4×10^{-2}	8.5×10^{-5}	11.7×10^3

[a] Symbols: -D1, -D2, -D3, and -D4, collagen II variants with deleted D1, D2, D3, and D4 periods; k_{on}, association rate constant; k_{off}, dissociation rate constant; K_d, equilibrium dissociation constant; K_a, equilibrium association constant. [b] Collagen II was obtained by the pepsin digestion of procollagen II; telopeptides are not present. [c] Collagen II was obtained by the specific cleavage of the procollagen II propeptides by procollagen N- and C-proteinases; telopeptides are intact.

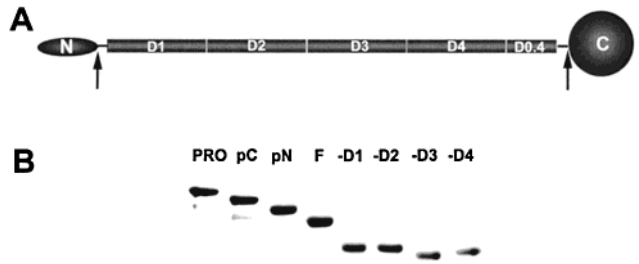

FIGURE 1: Recombinant proteins used in the binding assays. Upper Panel: Drawing of recombinant procollagen II. The subdivisions of procollagen indicate the D-periods that were deleted from the full-length protein. Arrows indicate sites of cleavage of the procollagen propeptides by procollagen N- and C-proteinases. Note: After the cleavage, the telopeptides remain part of the collagen. Lower Panel: Polyacrylamide gel electrophoresis of normal procollagen II, pC-collagen II, pN-collagen II, and recombinant variants of collagen II. Symbols: N, procollagen N-terminal propeptide; C, procollagen C-terminal propeptide; D1, D2, etc., homologous sequences of 234 amino acids each of the collagen II monomer; PRO, procollagen II; pC, procollagen in which N-propeptide was enzymatically removed; pN, procollagen in which C-propeptide was enzymatically removed; F, full-length collagen II generated by the cleavage of procollagen II with procollagen N- and C-proteinases; -D1, -D2, -D3, and -D4, collagen II variants with deleted D1, D2, D3, and D4 periods.

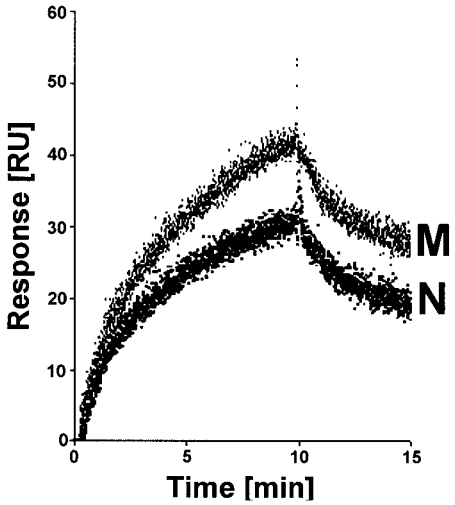

FIGURE 2: Sensogram showing binding of normal and mutant collagen II to immobilized collagen IX. The interaction of normal (N) or mutant (M) collagen II with collagen IX was analyzed on an optical biosensor. The response of the instrument (RU) was recorded and plotted against time. The presented graphs are derived from binding of collagen II at a concentration of 3×10^{-8} M.

N-proteinase-cleavage site so that pC-collagen was generated. The size of the fragments generated by digestion with the C-proteinase was also consistent with the prediction that the cleavage occurs at the single C-proteinase cleavage site in the protein so that pN-collagen was generated. Recombinant collagen IX expressed in insect cells (*30*) had the expected 1:1:1 ratio of α chains (Figure 3). Moreover, amino acid analysis of the purified protein indicated a purity of over 95%.

Quantitation of Free Sulfhydryl Groups in Mutant Collagen II. The Ellman's method was employed to quantitate the content of free sulfhydryl groups in samples containing mutant collagen II. The determined molar ratio of collagen to cysteine sulfhydryl groups was 1:3, which is in agreement with a theoretical prediction. There were no detectable sulfhydryl groups in control collagen samples. Similar values for the content of sulfhydryl groups were obtained with the heat-denatured collagens.

Interaction of Collagen IX with Normal and Mutant Collagen II. An optical biosensor was used to determine the kinetics of binding of collagen IX to normal collagen II and to mutant collagen II, and to map the collagen II regions most critical for this interaction. Binding assays were also used to analyze collagen IX−collagen IX interactions. The results summarized in Table 1 show that the affinity of collagen IX for collagen II is about 1000 times higher than the affinity of collagen IX for collagen IX. The results also indicate that the K_a value for the interaction of collagen IX with mutant collagen II is 2 times higher than the value for the interaction with normal collagen II. An analysis of the kinetics of binding indicates that this change of affinity was due to a faster on rate. There was a decrease in the response signal due to a buffer change and consequential dissociation of the interacting collagens (Figure 2). These results show that binding of collagen IX to both normal and mutant collagen II was a reversible process. In addition, it was further established that there was no formation of disulfide bonds between mutant collagen II and collagen IX (Figure 3). A small number of intermolecular disulfide bonds (Figure 3) were formed only between mutant α1(II) chains (see also *22*).

As there have been previous reports on a uniform D-periodic distribution of collagen IX on the surface of collagen II fibrils (*7*), we analyzed whether collagen II contains domains that are critical for specific binding of collagen IX. We determined that binding of collagen IX to collagen II does not depend on the presence of the nontriple helical telopeptides. The kinetics of binding and affinity of collagen IX to collagen II with intact telopeptides were the same as those for binding to the collagen II lacking

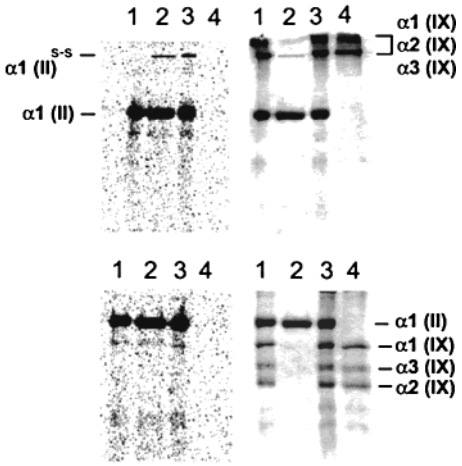

FIGURE 3: Analysis of disulfide bonds. ^{14}C-Labeled normal or mutant collagen II at a concentration of 150 μg/mL was incubated with nonlabeled recombinant collagen IX at the same concentration. The proteins were then separated in a 7% SDS−polyacrylamide gel under reducing and nonreducing conditions. The gels were stained with colloidal Brilliant Blue G, destained, and dried. The same gels were also analyzed with a phosphostorage plate for the presence of the ^{14}C-labeled, disulfide-bonded α1(II) chains. Only a small amount of α1(II)-α1(II) dimers (S−S) was formed, whereas α1(II)-α(IX) dimers were not detected. Left Column: Autoradiography of the proteins; only collagen II bands are apparent. Right Column: Colloidal Brilliant Blue G-stained proteins. Upper Row: Proteins separated under nonreducing conditions. Lower Row: Proteins separated under reducing conditions. Lanes: 1, normal collagen II incubated with collagen IX; 2, mutant collagen II; 3, mutant collagen II incubated with collagen IX; 4, collagen IX. The migration of α chains of collagen II and collagen IX is indicated.

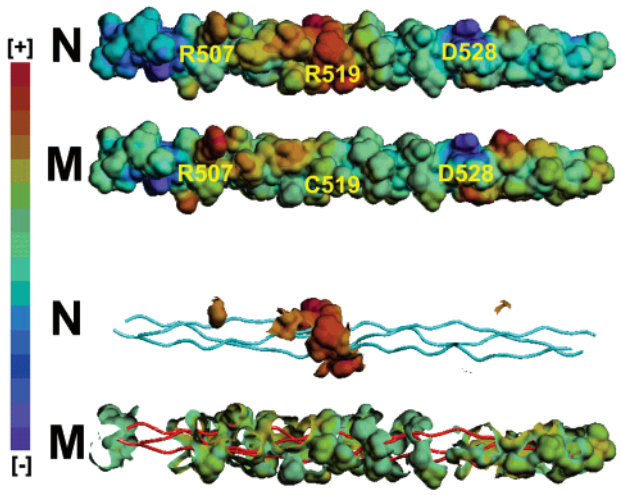

FIGURE 4: Electrostatic potential (EP) of the surface of collagen II with the Cys for Arg-α1−519 substitution. Electron density surfaces were generated using a computer-modeling program, and the electrostatic potential of the analyzed collagen II fragment was calculated. The narrow region encompassing Arg-α1−519 is characterized by a highly positive EP. In contrast, in the mutant collagen, the region adjoining the Cys-α1−519 site has significantly lower EP, and the EP of a similar value is characteristic of a broad region. Therefore, the Cys substitution for Arg-α1−519 significantly changes the unique electrostatic properties of the collagen II region encompassing the mutation site. Upper Panels: EP of the collagen II fragment neighboring amino acid positions α1−519. Lower Panels: Surface area with the EP value, which is similar to the value at amino acid positions α1−519. Symbols: N, normal collagen II; M, mutant collagen II with Cys at position α1−519. Legend: Color code used to indicate differences in the EP: red, highly positive EP; blue, highly negative EP.

telopeptides (see Table 1). Consequently, the binding of the D1, D2, D3, or D4 period-deficient collagen II to collagen IX was studied. The binding to the collagen variants lacking the D1 or the D2 period was similar to the binding of collagen IX to full-length collagen II. In contrast, interaction between collagen IX and collagen II variants lacking the D3 or the D4 period was characterized by a significant decrease of affinity (Table 1). Neither collagen II nor collagen IX was bound to the BSA-coated control sensor (not shown).

Surface Properties of Mutant Collagen II. Electrostatic interactions are the main component of the interaction energy between molecules. Therefore, we analyzed how the Cys substitution for Arg-α1−519 changes the EP of the collagen region encompassing the mutation site. The total area of the analyzed surface was 7200 Å^2, and the EP ranged from −126 to +181 kcal/(mol·e). Analysis of the collagen II region encompassing Arg-α1−519 indicated a highly positive EP ranging from +110 to +181 kcal/(mol·e). However, the high EP value was limited only to a narrow area of 750 Å^2 (Figure 4). Because of the Cys for Arg-α1−519 substitution, the EP of the region encompassing Cys-α1−519 significantly decreased and ranged from −16 to +25 kcal/(mol·e). The surface area that had a comparable EP increased significantly to a value of 3800 Å^2 (Figure 4). In contrast, there was no change in the hydrophilic potential of the analyzed collagen fragment (data not shown).

DISCUSSION

As demonstrated earlier, mutant collagen II molecules with Cys for Arg-α1−519 substitution are incorporated into cartilaginous extracellular matrix of a patient with an early onset of generalized osteoarthritis (*21*). In vitro fibril formation studies demonstrated that recombinant mutant collagen II does not self-assemble into correct fibrils (*22*, *23*). In addition, the presence of mutant protein in mixture with normal collagen II significantly altered both the kinetics of fibril assembly and the morphology of fibrils (*22*, *23*). The research here extends previous results by analyzing the interactions between mutant collagen II and collagen IX. The in vitro binding studies we performed collectively suggest the existence of a putative domain in collagen II that specifically interacts with collagen IX. A significantly smaller affinity of collagen IX to collagen II variants lacking the D3 or D4 period suggests that the most critical binding regions are located at the C-terminus of collagen II. Of interest was the observation that collagen II telopeptides are not critical for binding of collagen IX. Therefore, the collagen II telopeptides play a role in integrating the heterotypic fibrils by participating in the formation of covalent cross-links, while the triple helical D3 and D4 regions of collagen II serve as a docking site for collagen IX.

Although the data were obtained with the use of collagen II monomers rather than collagen fibrils, it is predicted that collagen II molecules forming the superficial layer of fibril have the same binding characteristics; as has been shown for decorin (*35*, *36*) and heparin (*37*), the binding sites on free procollagen I molecules are identical to the binding sites identified in the fibril-incorporated collagen I molecules.

We have shown that the association equilibrium constant (K_a) for the interaction between normal collagen II and

FIGURE 5: Model of the possible interactions between mutated collagen II fibrils and collagen IX. N: Binding of collagen IX to normal collagen II; interaction of collagen IX with domains in the D3 and D4 periods allows formation of covalent cross-links (X) between the COL2 domain of collagen IX and the N- and C-telopeptides of collagen II. M: Binding of collagen IX to mutant collagen II; change of EP of the collagen II region encompassing the α1−519 site probably allows nonspecific, higher-affinity binding of collagen IX. Symbols: COL1, COL2, and COL3, collagenous domains of collagen IX; D1, D2, D3, and D4, collagen II regions that correspond to the D-staggering of monomers in the collagen fibril.

collagen IX is 15×10^6 M^{-1}, whereas the K_a for the interaction between collagen IX monomers is only 12×10^3 M^{-1}. It is not clear how this significant difference between analyzed interactions in vitro could affect the process of fibril formation in vivo. Although, collagen IX is not critical in limiting the diameter of the collagen II fibril (*38*, *39*), we hypothesize that the low affinity of collagen IX for collagen IX facilitates maintenance of interfibrillar spacing by limiting lateral aggregation of collagen II fibrils decorated with collagen IX.

Our results indicate that the Cys for Arg-α1−519 substitution changed the interaction between collagen IX and mutant collagen II. The affinity of collagen IX for mutant collagen II increased 2-fold to a value of 30×10^6 M^{-1}. Even though the sulfhydryl groups in mutant collagen II were not chemically blocked, we determined that the observed affinity change is not a result of intermolecular disulfide bond formation between collagen IX and mutant collagen II. Therefore, these results support previous observations (*22*) that the ability of collagen II with the Cys-α1−519 substitution to form intermolecular bonds is limited. Mutations in another cartilage matrix macromolecule, cartilage oligomeric matrix protein, and their role in disrupting interactions with collagen IX were recently described by Holden et al. (*40*). Hence, changes of the interactions between extracellular molecules caused by the amino acid substitutions may represent a common mechanism in the pathogenesis of heritable diseases in cartilage.

To determine how the change of arginine to cysteine in the D3 period could modify the binding properties of collagen II, we analyzed the electrostatic potential of a collagen II fragment encompassing the mutation site. As determined by computer modeling, the Cys substitution for Arg-α1−519 resulted in a loss of the distinctive physicochemical characteristics of the site. Although part of the D3 period plays an important role in the specific binding of collagen IX, it is unlikely that in normal collagen II Arg-α1−519 takes direct part in this interaction (see Figure 5). Because of the length of the collagen IX domain that interacts with the collagen II fibril, the mutation site is located only about 25 nm from the C-terminal end of collagen IX. As demonstrated by Vogel et al. (*41*), a substitution of Cys for Gly-α1−748 in collagen I caused structural changes that altered the procollagen N-proteinase cleavage site located over 225 nm away. Hence, it is possible that the Cys substitution for Arg-α1−519 changes the local structure of collagen II, alters repulsive forces between the α1−519 region of collagen II and collagen IX, or perhaps changes water-mediated intermolecular interactions and, consequently, causes nonspecific binding of collagen IX to collagen II (Figure 5). Therefore, we hypothesize that the nonspecific binding of collagen IX to collagen II fibrils containing mutated monomers is likely to have deleterious effects on the structure of heterotypic fibrils.

In summary, we propose that binding of collagen IX to collagen II is site-specific and depends on recognition of the C-terminal region of the collagen II triple helix. We also postulate that in addition to changes in the morphology and kinetics of collagen fibril assembly, the Cys substitution for Arg-α1−519 in collagen II alters the interactions of collagen II with collagen IX. These substructural changes could result in disorganization of the architecture and perhaps could alter the stability of the heterotypic fibrils, leading to degradation of cartilaginous matrix.

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