Human matrix metalloproteinase-2 (MMP-2) contains an array of three fibronectin type II (FII) modules postulated to interact with gelatin (denatured collagen). Here, we verify that the NMR solution structure of the third FII repeat (COL-3) is similar to that of the second FII repeat (COL-2); characterize its ligand-binding properties; and derive dynamics properties and relative orientation in solution for the two domains of the COL-23 fragment, a construct comprising COL-2 and COL-3 in tandem, with each domain possessing a putative collagen-binding site. Interaction of the synthetic gelatin-like octadecapeptide (Pro-Pro-Gly)\(_6\) (PPG6) with COL-3 is weaker than with COL-2. We found that a synthetic peptide comprising segment 33–42 (peptide 33–42) from the MMP-2 prodomain interacts with COL-3 and, albeit with lower affinity, with COL-2 in a way that mimics PPG6 binding. COL-3 strongly prefers peptide 33–42 over 65–74, which suggests that intramolecular interactions with the prodomain could modulate binding of pro-MMP-2 to its gelatin substrate. In COL-23, the two modules retain their structural individuality and tumbling independently. Overall, the NMR data indicate that the relative orientation of the modules in COL-23 is not fixed in solution, that the modules do not interact with one another, and that COL-23 is rather flexible. The binding sites face opposite each other, and their responses to, and normalized affinities for, the longer ligand PPG12 are virtually identical to those of the individual domains for PPG6, thus precluding cooperativity, although they may interact simultaneously with multiple sites of the extracellular matrix.

Matrix metalloproteinase-2 (MMP-2),\(^1\) also known as gelatinase A or 72-kDa type IV collagenase (EC 3.4.24.24), plays an important role in processes involving degradation of the extracellular matrix (ECM): development, inflammation, tissue repair, tumor invasion, metastasis, etc. (reviewed in Ref. 1). Besides a catalytic domain, MMP-2 and the closely related MMP-9 (gelatinase B, 92-kDa type IV collagenase) contain a hemopexin-like domain (2) and, unique among the metalloproteinases, three in-tandem fibronectin type II (FII) modules, which are inserted in the catalytic domain in the vicinity of the active site. In its latent form, the prodomain folds over the active-site cleft and contributes a cysteine thiol group, which coordinates the catalytic zinc ion and, as indicated by the recent x-ray crystallographic model (3), inserts the side chain of Phe\(^{37}\) into the hydrophobic pocket of the third FII domain. This interaction can be disrupted by proteolysis. Once the active site is free, MMP-2 undergoes autolytic cleavage, resulting in loss of the prodomain (1).

The FII modules account for the affinity of MMP-2 for gelatin, type I and IV collagens, elastin, and laminin (4–9). A number of residues involved in binding of small hydrophobic ligands to the related second FII module of the bovine seminal fluid protein PDC-109 (PDC-109/b) were inferred from \(^1\)H NMR studies (10). In addition, several residues that are important for interaction with gelatin have been identified, via site-directed mutagenesis, in the second FII modules from MMP-2 (11) and MMP-9 (12). However, little is known as to how tandem arrays of FII domains interact with other molecules. Fragments containing two or three consecutive FII modules from MMP-2 exhibit significantly higher apparent affinities for immobilized gelatin than any of the single modules (5). Modeling studies based on the structures of two FII modules from human fibronectin (13) as well as a recent NMR study of this pair (14) indicate that the binding sites of two consecutive domains do not get close to each other. This indeed seems to be the case for the three FII domains in pro-MMP-2 (3).

The reported x-ray study of MMP-2 (3) was performed on the ligand-free protein. Lingering questions are the nature of the interaction of FII domains with collagen-type ligands and whether the interaction of the propeptide with the third FII repeat reflects a specific affinity for the N-terminal domain. The latter is relevant in the context of identifying MMP-2 peptide ligands, as the binding molecule could serve as a template for the design of potential peptidomimetic anticancer...
drugs. Finally, contrary to the picture one may derive from the rigid crystallographic structure (3), FII domains are joined by flexible polypeptide linkers; hence, one is led to wonder as to the extent to which these modules act independently when in solution since binding assays indicate that the three modules in tandem possess at least two binding sites that can be occupied simultaneously by two collagen molecules (9). We have reported elsewhere (15) on the solution structure and ligand-binding surface of the second FII module (COL-2) (see Fig. 1a) of MMP-2, which was mapped on the basis of $^1$H and $^{15}$N NMR perturbations induced by the synthetic gelatin-like octadecapeptide (Pro-Pro-Gly)$_6$, henceforth denoted as PPG6, a mimic of gelatin. Here, we present the NMR solution structure of the third FII module (COL-3) (see Fig. 1b); analyze its ligand-binding properties; and describe the structure, function, and dynamics of a construct comprising the second and third FII domains in tandem (COL-23) (see Fig. 1c).

**MATERIALS AND METHODS**

**Protein Expression and Purification**—COL-2 belonged to a previous batch (15). COL-3 and COL-23 (residues 337–394 and 278–394, respectively, for human MMP-2) were expressed in E. coli expression vectors as described (4). The 34–35-amino-acid long N-terminal tails, derived from the β-galactosidase moiety of the expression vector, were partially removed by limited trypsin digestion (15). The digests were purified on a gelatin-Sepharose 4B column using a 0–8 M urea gradient. The digests were desalted and lyophilized. Sequence analyses with an ABI 472A Pulsed Liquid Phase Protein Peptide Sequencer were used to determine the N-terminal sequences of the digested proteins. $^{13}$N-Labeled proteins, expressed as described elsewhere (15), were isolated and cleaved as indicated above for the unlabeled material. The sequences of the truncated recombinant type II modules are shown in Fig. 1.

Electrospray ionization mass spectrometry and amino acid analysis revealed a discrepancy between the expected and actual composition of COL-3 samples, whether unlabeled or $^{13}$N-labeled. It was verified by DNA sequencing that the codon for Glu 11 (GAA) in the COL-3 expression plasmid changed to GGA (Gly) during plasmid propagation, which was confirmed by the NMR analysis. Fortunately, the effect of the E11G mutation on the overall conformation of COL-3 is negligible: $^1$H and $^{15}$N chemical shifts of the mutated protein and of wild-type COL-3 within the COL-23 construct are virtually identical.

**NMR Spectroscopy of COL-3 and COL-23**—NMR data were acquired at 25 °C on a Bruker Avance DMX-500 spectrometer equipped with a 5-mm triple-resonance three-axis gradient probe. Spectra were processed and analyzed with the program Felix 95 (Molecular Simulations, Inc., San Diego, CA) on a Silicon Graphics Indy R-5000 workstation. The base line was corrected with a model-free algorithm developed by Friedrichs (18). Proton chemical shifts were referenced using $p$-dioxane (δ 3.75 ppm) as an internal standard (19). $^{13}$N chemical shifts were referenced indirectly (20).

**Sequential assignments of COL-3 were initially obtained for a sample of 0.5 mM unlabeled COL-3 in 90% H$_2$O and 10% D$_2$O, pH 5.4, based on two-dimensional homonuclear COSY (21), magic-angle-gradient double-quantum filtered COSY (22), TOCSY (23) with DIPI-p mixing sequence (24, 25) (mixing time of 70 ms), and NOESY (26) (mixing times of 60 and 200 ms) spectra.**

**Sequential assignments of COL-3 were initially obtained for a sample of 0.5 mM unlabeled COL-3 in 90% H$_2$O and 10% D$_2$O, pH 5.4, based on two-dimensional homonuclear COSY (21), magic-angle-gradient double-quantum filtered COSY (22), TOCSY (23) with DIPI-p mixing sequence (24, 25) (mixing time of 70 ms), and NOESY (26) (mixing times of 60 and 200 ms) spectra. The experiments were recorded with standard pulse sequences and phase cycles (XWIN-NMR Version 2.0, Bruker, Karlsruhe, Germany). Solvent suppression in COSY was achieved via selective low power irradiation (presaturation) during the relaxation delay, whereas in TOCSY and NOESY, the WATERGATE sequence (27, 28) was used. COSY, TOCSY (mixing time of 70 ms), and NOESY (mixing time of 120 ms) experiments were also acquired for a sample of 0.5 mM COL-3 in 99.995% D$_2$O, pH 5.4 (uncorrected pH glass electrode readings). The assignments were confirmed and extended based on two-dimensional $^1$H-$^{15}$N HSQC (29–31), three-dimensional $^{15}$N-edited TOCSY (mixing time of 70 ms), three-dimensional $^{15}$N-edited NOESY (mixing time of 200 ms) (32–35), three-dimensionalHNHB (36, 37), and steady-state $^1$H-$^{15}$N nuclear Overhauser effect (X-NOE) (38) experiments, which were recorded for a sample of 1.8 mM $^{15}$N-labeled COL-23 in 90% H$_2$O and 10% D$_2$O, pH 6.0, based on two-dimensional $^1$H-$^{15}$N HSQC (29–31), three-dimensional $^{15}$N-edited TOCSY (mixing time of 75 ms), three-dimensional $^{15}$N-edited NOESY (mixing time of 150 ms) (32–35), and three-dimensional HNHB (36, 37) experiments.

**Calculation of COL-3 Structure**—Cross-peak volumes from two-dimensional NOESY (mixing time of 60 ms), after correction for the WATERGATE excitation profile, were converted to interproton distances with the program Felix 95. To calibrate the interproton distances, we used the program FlexX (4) and the 34–35-amino-acid long N-terminal tails, derived from the expression vector. Gly$^{14}$ in COL-3 is a mutation (see "Materials and Methods").

**Ligand Binding Studies**—Peptides (Pro-Pro-Gly)$_6$ (PGP6). (Pro-Pro-
Gly\textsubscript{12} (PPG12), and acetyl-Pro-Ile-Ile-Lys-Phe-Pro-Gly-Asp-Val-Ala-amide (p33–42) were synthesized on a Model 431A peptide synthesizer (Applied Biosystems-Perkin Elmer, Foster City, CA) using Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. The peptides were weighed and dissolved in H\textsubscript{2}O, and pH of the solution was adjusted to 7.0. Insoluble material in the PPG12 sample was collected by centrifugation and dissolved in H\textsubscript{2}O, pH 3.0. \textsuperscript{1}H NMR signal intensity indicated that the stock solution retained \textasciitilde 90\% of all peptide material. Concentrations of COL-2, COL-3, and COL-23 were determined spectrophotometrically at 280 nm using absorption coefficients calculated according to (45). Small aliquots of the peptide stock solution were added to samples of 0.35 mM \textsuperscript{15}N-labeled COL-2, COL-3, or COL-23 in 90\% H\textsubscript{2}O and 10\% D\textsubscript{2}O, pH FIG. 2. NMR and x-ray crystallographic structures of COL-2 and COL-3. Shown are superposed backbone traces of residues 3–58 of 50 calculated COL-3 NMR structures (a); COL-3 solution (red) and crystal (3) (blue) structures (b); and COL-3 solution (red), COL-2 solution (15) (green), and COL-2 crystal (3) (light blue) structures (c). The orientation of the molecules in a–c is the same.

![Figure 2](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Structure statistics for COL-3</th>
<th>Ensemble</th>
<th>Selected structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>r.m.s.d.\textsuperscript{a} from experimental restraints</td>
<td>0.080 ± 0.005</td>
<td>0.076</td>
</tr>
<tr>
<td>NOE distance restraints (Å)</td>
<td>0.8 ± 2.6</td>
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</tr>
<tr>
<td>No. of experimental restraint violations</td>
<td>2.1 ± 1.0</td>
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<tr>
<td>NOE violations &gt; 0.5 Å</td>
<td>0.2 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>Dihedral angle violations &gt; 5°</td>
<td>0.0062 ± 0.0003</td>
<td>0.0061</td>
</tr>
<tr>
<td>r.m.s.d. from idealized geometry\textsuperscript{b}</td>
<td>0.8 ± 1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Bonds (Å)</td>
<td>0.7 ± 0.1</td>
<td>0.6</td>
</tr>
<tr>
<td>Angles (degrees)</td>
<td>0.6 ± 0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Improper (degrees)</td>
<td>0.9 ± 0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>r.m.s.d. of residues 3–58 from mean coordinates\textsuperscript{c}</td>
<td>71.8</td>
<td>80.0</td>
</tr>
<tr>
<td>Backbone atoms (N, C\textsubscript{a}, C) (Å)</td>
<td>26.5</td>
<td>20.0</td>
</tr>
<tr>
<td>Heavy atoms (Å)</td>
<td>1.6</td>
<td>0.0</td>
</tr>
<tr>
<td>Distribution of ϕ/ψ dihedral angles of residues 3–58 in Ramachandran plot\textsuperscript{d}</td>
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<td>0.0</td>
</tr>
<tr>
<td>Most favored regions (%)</td>
<td>71.8</td>
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<td>Additional allowed regions (%)</td>
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<td>Generously allowed regions (%)</td>
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</tr>
<tr>
<td>Disallowed regions (%)</td>
<td>0.2</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Root mean square deviation.

\textsuperscript{b} Idealized covalent geometry is based on the parallhdg5.0.pro force field (42).

\textsuperscript{c} Mean coordinates were obtained by averaging coordinates of the 50 calculated structures, which were first superposed using backbone atoms (N, C\textsubscript{a}, and C) of residues 3–58.

\textsuperscript{d} Ref. 43.

![Figure 3](image)

**FIG. 3.** Ribbon representation of COL-3 (residues 3–58): secondary structure and aromatic cluster. Front (a) and side (b) views are shown. β-Sheets are depicted as purple arrows; an α-helical turn is in red; and disulfide bridges are in yellow. Aromatic side chains in the front view are colored blue. Phe\textsuperscript{17}, which faces the backside, was omitted for clarity.
7.0. 1H-15N HSQC experiment was recorded at each step to monitor the ligand-induced resonance shifts. The equilibrium association constant was determined by a combination of linear and nonlinear least-squares fitting of the chemical shift changes as described previously (46, 47). The peptides were assumed to be monomeric in solution (48).

Dynamics of COL-23—
X-NOEs and 15N longitudinal ($R_1$) and transverse ($R_2$) relaxation rates were determined from two-dimensional heteronuclear NMR experiments (38). The program Felix 97 (Molecular Simulations, Inc.) was used to measure peak heights, fit relaxation decays, and estimate uncertainties.

$R_2/R_1$ relaxation rate ratios were analyzed for each module separately with the program TENSOR (49). Only N–HN vectors with well defined orientation in the ensemble of calculated NMR structures and with $R_1$ and $R_2$ values that satisfied the criteria outlined in Ref. 50 were included in the fitting. This comprised data for 29 residues in COL-23/2 and 31 residues in COL-23/3. TENSOR was also used to calculate the moments of inertia for COL-23.

RESULTS AND DISCUSSION

Solution Structure of COL-3—Virtually complete 1H and 15N NMR assignments of COL-3 were obtained as reported for COL-2 (15). Restraints for 654 interproton distances (140 long-range, 5 $\leq |i-j|$; 47 medium-range, 2 $\leq |i-j|$ $\leq$ 4; 120 sequential; and 347 intraresidual), 12 hydrogen bond distances, and 28 $\psi$ and 28 $\chi_1$ dihedral angles were derived, as described under “Materials and Methods.” 50 structures were calculated using these restraints (Fig. 2a); statistics for the ensemble are summarized in Table I. Overall, the structures agree well with the experimental data and exhibit good covalent geometry. A model closest to the mean (Table I) was selected for illustrations and the following discussion.

The module consists of two short double-stranded antiparallel $\beta$-sheets (Phe$^{106}$-Phe$^{21}$/Asn$^{24}$-Tyr$^{36}$ and Trp$^{40}$-Ala$^{42}$/Trp$^{53}$-Phe$^{55}$) arranged approximately perpendicular to each other and three large irregular loops (Fig. 3). The first loop includes
the amino-terminal segment preceding the first disulfide bridge. The second loop links the two β-sheets. The third loop connects the strands of the second β-sheet and contains an α-helical turn (Tyr^{47}–Asp^{50}). The first β-sheet and the loops are arranged around the second β-sheet, thus forming a large cavity filled with aromatic side chains of Phe^{17}, Phe^{19}, Phe^{21}, Tyr^{26}, Trp^{40}, Tyr^{47}, Trp^{53}, and Phe^{55}. An extended hydrophobic surface is formed by Phe^{21}, Tyr^{26}, Trp^{40}, Tyr^{47}, Trp^{53}, and Phe^{55}, whereas Phe^{19} is buried, and Phe^{17} faces the opposite side of the module (Fig. 4, a and b). The hydrophobic areas are surrounded by residues with charged side chain groups (Fig. 4, c and d). The Cys^{15}–Cys^{41} and Cys^{29}–Cys^{56} disulfide bridges and both the N and C termini are located at the back of the second β-sheet, opposite the aromatic cluster (Fig. 3). NMR (15) and x-ray (3) structures of COL-2 and COL-3 are compared in Fig. 2 (b and c). Pairwise root mean square deviations of superposed backbone atoms of residues 3–58 are as follows: 1.34 Å for COL-2 NMR versus COL-2 x-ray; 1.57 Å for COL-2 NMR versus COL-3 NMR; 1.11 Å for COL-3 NMR versus COL-2 NMR x-ray; and the same, 1.1 Å, for COL-3 NMR versus COL-3 x-ray. The excellent agreement between the solution and crystal structures of COL-3 confirms that the E11G mutation in COL-3 is essentially inconsequential for the conformation of the module.

Interaction of COL-3 with a Gelatin-like Peptide—We have shown previously that PPG6, a synthetic peptide with collagen consensus sequence, interacts with COL-2 (15). This peptide afforded a valuable probe for mapping of the COL-2 gelatin-binding surface. To elucidate the interaction of gelatin with COL-3, a similar approach was taken. COL-3 chemical shift changes induced by PPG6 binding were monitored in 1H-15N HSQC spectra. From ligand titration experiments (Fig. 5), it was determined that the peptide interacts with COL-3 with $K_{d} \approx 0.10 \pm 0.02 \text{ mM}^{-1}$. Thus, the binding of PPG6 to COL-3 is weaker than to COL-2 ($K_{d} \approx 0.36 \pm 0.02 \text{ mM}^{-1}$), in line with the relative apparent affinities of the domains for gelatin ($K_{a} \approx 2.3 \pm 0.5 \times 10^{5}$ and $1.2 \times 10^{5}$ for COL-2 and COL-3, respectively (5)). A rough picture of the COL-3 binding surface can be generated by localizing the ligand-induced spectral perturbations on the module’s three-dimensional structure (Fig. 6, a and b). Noteworthy, residues with perturbed backbone amide resonances surround the central depression on the front side of the module and comprise the aromatic cluster (Phe^{21}, Tyr^{26}, Trp^{40}, Tyr^{47}, Trp^{53}, and Phe^{55}), its right-hand rim (Ser^{31}, Ala^{32}, Gly^{33}, and Arg^{34}), and its upper left boundary (Leu^{32} and Asp^{35}). Backbone amide resonances of Arg^{34} and Gly^{33} are the most affected (Arg^{34} > Gly^{33} > Tyr^{47} > Ala^{32} > Asp^{35} > Phe^{55} > Phe^{21} > Thr^{43} > Trp^{40} > Leu^{32} > Ser^{31} > Ala^{42} > Lys^{52} > Trp^{53} > Tyr^{26} > Thr^{20}). In contrast, backbone amide resonances of residues at the back of COL-3 are negligibly perturbed upon PPG6 binding, the only apparent exceptions being Thr^{43} and, to a lesser extent, Lys^{52}. However, since their amides are buried in proximity to the aromatic cluster, they are likely to echo ligand-induced perturbation of the latter. Overall, the data suggest that the peptide interacts with the exposed aromatic side chains on the front side of COL-3 while leaning against the rim configured by the Ala^{32}–Arg^{34} stretch.

There are differences in the pattern of COL-3 backbone amide chemical shift changes induced by PPG6 binding when compared against those observed for COL-2 (15). Most apparent, residues with affected backbone amide resonances define an area that is shorter and wider in COL-3 than in COL-2. In particular, the backbone amides of Asp^{35}, Gly^{33}, and Lys^{38} at the lower right and Thr^{20} and Gly^{23} at the top are significantly less perturbed by PPG6 binding in COL-3 than in COL-2, whereas those of Ser^{31} and Ala^{42} at the upper right and Asp^{35} at the upper left are sensitive in COL-3, but negligibly so in COL-2. Lys^{38} is likely to be responsible for the curtailed binding surface and lower ligand affinity of COL-3. The Y58A mutation in COL-2 was shown previously to impair gelatin binding (11), and a substitution of Tyr^{38} with lysine can be expected to have a similar effect.

Interaction of COL-3 with p33–42 from the Prodomain—In the pro-MMP-2 crystal structure (3), the third FII module is involved in an intramolecular interaction with the prodomain: Phe^{37} inserts itself into the hydrophobic pocket of COL-3, whereas Ile^{39} and Asp^{40} form a hydrogen bond and a salt bridge with COL-3 Gly^{33} and Arg^{34}, respectively. These characteris-

![Figure 6](http://www.jbc.org/)

FIG. 6. Contact surface of COL-3 (residues 3–58) colored according to backbone amide chemical shift changes induced by PPG6 (a and b) and p33–42 (c and d) binding. Front (a and c) and back (b and d) views are shown. Color intensity is proportional to the sum of median-normalized 1H and 15N amide chemical shift changes of the individual residues and is scaled to achieve a balanced distribution. Maximum intensity is used for residues with a sum $\geq 8$ (a and b) or $\geq 6$ (c and d). Intensities for Pro^{14}, Pro^{18}, Ser^{25}, and Pro^{57} were obtained by averaging values of the neighboring residues.
bations induced by PPG6 binding to COL-3, in particular with surrounding residues (Gly 8, Gln22, Thr31, Gly33, Arg34, Asp36, and the aromatic residues (Fig. 6, a and b). Thus, it would appear that the interaction of the prodomain with COL-3 mimics gelatin binding, as previously suggested (3).

To examine the apparent parallelism between gelatin and prodomain binding to COL-3, the interaction of COL-3 with a peptide corresponding to segment 33–42 of human pro-MMP-2 (p33–42) was investigated and compared to the interaction with PPG6. From ligand titration experiments, it was determined that COL-3 displays higher affinity for p33–42 ($K_a = 0.058 \pm 0.02 \text{mM}^{-1}$) than for PPG6 ($K_a = 0.10 \pm 0.02 \text{mM}^{-1}$). Residues with backbone amide resonances perturbed by p33–42 binding are limited to the front side of the module and include the aromatic cluster (Phe21, Trp40, Tyr47, and Phe55), its right-hand rim (Ser31, Gly33, Arg34, Asp36, and Gly37), and its lower left boundary (Asn9, Arg34, and Lys52) (Fig. 6, c and d). Indeed, this pattern of resonance shifts resembles the one observed upon PPG6 binding (Fig. 6, a and b), consistent with PPG6 and p33–42 interacting with COL-3 in analogous fashions. Not all amide resonances, however, are perturbed to the same extent by the two ligands. In particular, the backbone amides of Asn9, Asp36, Gly37, Arg34, and Lys52 at the right-hand rim and the lower left boundary of the hydrophobic pocket are affected mainly by p33–42 binding, whereas those of Leu22 and Asp34 at the upper left are relatively more perturbed upon interaction with PPG6. The $\epsilon$-NH resonance of Arg34, which exhibits a large shift upon PPG6 binding, is virtually insensitive to interaction of COL-3 with p33–42 (data not shown). This apparent discrepancy from what one would expect from the crystal structure may arise from obvious structural differences between the flexible p33–42 and the intact prodomain ligand.

CO2-2 is also capable of binding to p33–42. As determined from ligand titration experiments, COL-2 interacts with p33–42 with $K_a = 0.058 \pm 0.004 \text{mM}^{-1}$, 10 times less strongly than COL-3. COL-2 residues whose amides are perturbed by p33–42 binding are analogous to those in COL-3 and include the aromatic cluster (Phe21, Trp40, Tyr47, and Phe55) and the surrounding residues (Gly8, Gln22, Thr31, Gly33, Arg34, Asp36, Gly37, Lys51, and Lys52) (data not shown). The $\epsilon$-NH resonance of Arg34 is affected by both PPG6 (15) and p33–42 binding to similar extents (data not shown).

p33–42 contains 2 prolines and a number of hydrophobic residues. Hence, it is conceivable that the gelatin-binding pocket of FII modules is endowed with an inherent affinity for this region of the prodomain. However, relative affinities of FII modules differ significantly: although COL-2 binds gelatin the most avidly among the homologous repeats in MMP-2 (6), it interacts only marginally with p33–42. COL-3, in contrast, shows a preference for p33–42 over PPG6.

The intramolecular interaction of the prodomain with COL-3 is likely to be of functional significance, hindering gelatin binding to the proenzyme. Removal of the prodomain during activation will not only liberate the active site, but also release COL-3 and provide the enzyme with full gelatin binding capability. Consistent with this model, active MMP-2 exhibits significantly higher affinity for gelatin than does pro-MMP-2 (11).

**COL-23 Structure and Dynamics**—Identification of the $^1$H and $^{15}$N NMR signals of COL-23 was straightforward from the spectral assignments of COL-2 (15) and COL-3 (this study). The large difference between the $^{15}$N backbone amide chemical shifts of residue 11 in COL-3 and module 3 of COL-23 (COL-23/3) (112.8 and 116.8 ppm, respectively), together with smaller differences in backbone amide chemical shifts of the surrounding residues, reflects the fortuitous E11G mutation in COL-3. Otherwise, the $^1$H and $^{15}$N backbone amide chemical shifts of the separate domains are well conserved in COL-23, except for the linking segment (Thr59 and Ala60) of module 2 of COL-23 (COL-23/2) and Met3 and Ser4 of COL-23/3) and the carboxyl terminus of COL-23 (Glu58 and Gly60 of COL-23/3). The overall spectral similarity of COL-23 to COL-2 and COL-3 supports the view that the two modules retain their individuality within COL-23 and that their structures in COL-23 are identical to those of the separate domains.

The ratios of the $^{15}$N transverse ($R_2$) and longitudinal ($R_1$) magnetic relaxation rates ($R_2/R_1$) vary significantly for the backbone amides of COL-23 (Fig. 7a). Analysis of $R_2/R_1$ ratios as a function of N–H bond vector orientation suggests that tumbling of each module is best described by an anisotropic rotational diffusion tensor (Table II). The longest axes of the tensors, $D_x$, run approximately parallel to the lines which connect the centers of COL-23/2 or COL-23/3 with the linking segment (Fig. 8). Consistent results were obtained whether the coordinates used for the fitting stemmed from a selected NMR, the mean NMR, or the x-ray crystallographic structure. The calculated rotational correlation times of 7.2 and 7.0 ns for COL-23/2 and COL-23/3, respectively, are in excellent agreement with those of 7.1 and 7.2 ns determined for a pair of independently tumbling FII modules from fibronectin (14). Relative moments of inertia of COL-23, derived from the x-ray crystallographic coordinates of pro-MMP-2 (3), are 4.268: 4.108:1. This indicates that in the crystal structure, COL-23 is well approximated by a prolate ellipsoid. Diffusion anisotropy of an ellipsoid can be estimated from the relationship $I_x = I_y = (I_z/2)^{1/2}$, where $I_x$, $I_y$, and $I_z$ are the moments of inertia about the $x$, $y$, and $z$ principal axes (52). For a rigid COL-23 fragment, the expected diffusion anisotropy would be $2.75$; however, $D(D_z/D_x)$ is found to be only $1.4$ and $1.5$ for COL-23/2 and COL-23/3, respectively (Table II). Hence, our NMR data are not consistent with a rigid model of COL-23. Instead, the rotational diffusion tensor is likely to represent an average over an ensemble of rapidly interconverting extended and bent conformations. In line with the above results, residues within the linking peptide segment of COL-23 (Glu58-Thr59-Ala60-Met3-Ser4-

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Interaction of COL-23 with Gelatin-like Peptides—To characterize how two consecutive FII modules bind gelatin, the interaction of COL-23 with PPG6 was investigated. Chemical shift changes induced by PPG6 binding were monitored in 1H-15N HSQC spectra (Fig. 9). The 1H and 15N amide resonance shifts of the component modules of COL-23 are essentially the same as those observed in COL-2 (15) and COL-3 (15). From ligand titration experiments (Fig. 5), it was determined that the peptide interacts with two domains of COL-23 with different affinities: $K_a = 0.38 \pm 0.01 \text{ and } 0.11 \pm 0.01 \text{ mM}^{-1}$ for COL-2/2 and COL-2/3, respectively (Table III). Hence, COL-23 appears to possess two independent binding sites, whose outline and affinity for PPG6 are virtually identical to those of the isolated domains: $K_a = 0.36 \pm 0.02$ and $0.10 \pm 0.02 \text{ mM}^{-1}$ for COL-2, COL-2/2, and COL-2/3, respectively (Fig. 5).

Interaction of COL-2 and COL-23 with a longer synthetic peptide, PPG12, was also investigated, and consistent results were obtained: $K_a = 0.70 \pm 0.02$, $0.71 \pm 0.10$, and $0.22 \pm 0.01 \text{ mM}^{-1}$ for COL-2, COL-2/2, and COL-2/3, respectively (Fig. 5). The apparent affinities of FII domains for PPG12 are $\approx 2$-fold higher than those for PPG6 (Table III), in line with the doubled number (on a molar basis) of PPG units available for binding in PPG12 relative to PPG6. The resonance shifts observed in 1H-15N HSQC spectra of COL-2 and COL-23 upon interaction with PPG12 are otherwise practically indistinguishable from those induced by PPG6 binding (data not shown).

Therefore, our results do not support binding cooperativity between the two component modules of COL-23 (Table III). This concurs with the NMR structural data: the position of the N and C termini in COL-2 (15) and COL-3 (Fig. 3) implies that the two consecutive modules are connected side to back so that their binding sites face opposite from one another. Preliminary experiments indicate that the three domains in tandem also contain distinct binding sites for PPG6, whose affinities are essentially the same as those of the separate modules.3 This is in agreement with the recently solved x-ray structure of the intact pro-MMP-2, where the gelatin-binding surfaces of the three COL domains point outward in a divergent fashion reminiscent of a “three-pronged fishhook” (3). Hence, it seems unlikely that two or multiple FII modules could form a single continuous binding surface. Prima facie, our data contrast the cooperativity observed for binding of multiple FII modules to gelatin-Sepharose (5). However, it is conceivable that in these earlier experiments, which more closely approached the high protein density conditions prevalent in the extracellular milieu, several gelatin molecules attached to the same bead interacted simultaneously with the consecutive FII domains in a cooperative fashion.

Conclusions—Jointly with plasmin (53), a trypsin-like serine proteinase of narrow substrate specificity, the proteolytic activity of MMP-2 contributes to both the removal of ECM barriers that limit cell movement and the modulation of cell adhesion (54), migration (55), proliferation and differentiation (56). In the selectivity for macromolecules, the FII domains of MMP-2 play a crucial role. From our study, it is apparent that although COL-2 and COL-3 exhibit close structural related-

## Table II

<table>
<thead>
<tr>
<th>Coordinates</th>
<th>$D_x^a$</th>
<th>$D_y$</th>
<th>$D_z$</th>
<th>$\tau_\alpha$</th>
<th>$D/D_z$</th>
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</thead>
<tbody>
<tr>
<td>COL-2 mean NMR$^b$</td>
<td>1.87</td>
<td>2.18</td>
<td>2.88</td>
<td>7.20</td>
<td>1.42</td>
</tr>
<tr>
<td>COL-2 selected NMR$^b$</td>
<td>1.88</td>
<td>2.15</td>
<td>2.91</td>
<td>7.20</td>
<td>1.44</td>
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<tr>
<td>COL-2 x-ray$^b$</td>
<td>1.94</td>
<td>2.19</td>
<td>2.81</td>
<td>7.20</td>
<td>1.36</td>
</tr>
<tr>
<td>COL-3 mean NMR</td>
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<td>2.25</td>
<td>3.01</td>
<td>7.03</td>
<td>1.47</td>
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<tr>
<td>COL-3 selected NMR</td>
<td>1.88</td>
<td>2.23</td>
<td>3.01</td>
<td>7.03</td>
<td>1.46</td>
</tr>
<tr>
<td>COL-3 x-ray$^b$</td>
<td>1.94</td>
<td>2.11</td>
<td>3.08</td>
<td>7.02</td>
<td>1.53</td>
</tr>
</tbody>
</table>

$^a$ $D_x$, $D_y$, and $D_z$, components of the rotational diffusion tensor; $\tau_\alpha$, rotational correlation time, $\tau_\alpha = 1/(2D_x + D_y + D_z)$; $D_5$ and $D_z$, parallel and perpendicular components, respectively, of the rotational diffusion tensor relative to the unique axis in an axially symmetric model approximation, $D/D_z = 2D_y/(D_x + D_z)$.

$^b$ Ref. 15.

$^c$ Ref. 3.
ness, judging from their ligand preferences, they differ in their functional properties. Thus, by providing an anchoring site for the prodomain, COL-3 would stabilize the pro-MMP-2 in a compact conformation; in contrast, the main role of COL-2 may be that of promoting the binding interaction with the gelatin substrate. This is reminiscent of what has been observed for plasminogen, the proenzyme of plasmin, where the five kringle domains, which exhibit various degrees of affinity for lysine-containing peptides (putative anchoring sites in the plasminogen-fibrin interaction), also differ in their affinities for the plasminogen N-terminal peptide, suggesting that they may selectively regulate the compact folding of the macromolecule (47, 57). Following activation to plasmin, the N-terminal peptide is autolytically cleaved off, causing plasminogen to assume an “open” conformation (Ref. 58 and references therein). Such transformation thus would mirror the transition of MMP-2 from its pro to its active form.

In line with what is observed for the plasminogen kringle domains, the interaction of COL-2 and COL-3 modules with the tested peptide ligands is relatively weak. In the case of p33–42, the low affinity may be rationalized on the basis of entropic effects since (a) the peptide, being linear and flexible, is unlikely to assume the conformation of the native segment within the intact prodomain; and (b) the intramolecular interaction is likely to be favored in the intact protein where segment 33–42, tethered relative to COL-3, might be placed in a more favorable configuration for binding relative to the free peptide in solution. As to the low affinities measured for PPG6, it is significant that the $K_a$ values appear to double upon going to PPG12.

**FIG. 9.** $^1$H-$^15$N HSQC of COL-23: effect of PPG6 binding. Spectra of ligand-free COL-23 (black) and COL-23 in the presence of an ~50-fold molar excess of PPG6 (red) are superimposed; the most conspicuous cross-peak shifts are indicated with arrows. Residues of COL-23/3 are marked with a prime.

**TABLE III**

Affinity of type II modules for gelatin-like peptides

<table>
<thead>
<tr>
<th>Domain</th>
<th>$K_a$ PPG6</th>
<th>$K_a$ PPG12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{mM}^{-1}$</td>
<td>$\text{mM}^{-1}$</td>
</tr>
<tr>
<td>COL-2</td>
<td>0.36 ± 0.02</td>
<td>0.70 ± 0.02</td>
</tr>
<tr>
<td>COL-3</td>
<td>0.10 ± 0.02</td>
<td>ND</td>
</tr>
<tr>
<td>COL-23/2</td>
<td>0.38 ± 0.01</td>
<td>0.71 ± 0.10</td>
</tr>
<tr>
<td>COL-23/3</td>
<td>0.11 ± 0.01</td>
<td>0.22 ± 0.01</td>
</tr>
</tbody>
</table>

*a Taken from Ref. 15.

*b Not determined.
Extrapolating to the situation in the ECM, this suggests that while MMP-2 interacts with native or denatured collagen fibrils, the effective $K_d$ of the COL modules may be drastically amplified. On the other hand, weak binding to single sites on the substrate implies fast on/off association with the ECM scaffold. Akin to plasmin, which interacts dynamically with fibrin, a polymeric macromolecule, it may not be advantageous for MMP-2 to remain localized at a single site in the ECM environment. To perform its function efficiently, it has to diffuse throughout the mesh it degrades. Thus, the exposure of fibrin, a polymeric macromolecule, it may not be advantageous amplified. On the other hand, weak binding to single sites on the substrate implies fast on/off association with the ECM scaffold. Akin to plasmin, which interacts dynamically with fibrin, a polymeric macromolecule, it may not be advantageous for MMP-2 to remain localized at a single site in the ECM environment. To perform its function efficiently, it has to have a large diffusion mobility while remaining in close proximity to its tissue targets.

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Gelatin-binding Region of Human Matrix Metalloproteinase-2: SOLUTION STRUCTURE, DYNAMICS, AND FUNCTION OF THE COL-23 TWO-DOMAIN CONSTRUCT
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