The Effect of Matrix Metalloproteinase Complex Formation on the Conformational Mobility of Tissue Inhibitor of Metalloproteinases-2 (TIMP-2)

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The backbone mobility of the N-terminal domain of tissue inhibitor of metalloproteinases-2 (N-TIMP-2) was determined both for the free protein and when bound to the catalytic domain of matrix metalloproteinase-3 (N-MMP-3). Regions of the protein with internal motion were identified by comparison of the $T_1$ and $T_2$ relaxation times and $^{1}H,^{15}N$ nuclear Overhauser effect values for the backbone amide $^{15}N$ signals for each residue in the sequence. This analysis revealed rapid internal motion on the picosecond to nanosecond time scale for several regions of free N-TIMP-2, including the extended $\beta$-hairpin between $\beta$-strands A and B, which forms part of the MMP binding site. Evidence of relatively slow motion indicative of exchange between two or more local conformations on a microsecond to millisecond time scale was also found in the free protein, and two other regions of the MMP binding site (the CD and EF loops). On formation of a tight N-TIMP-2:N-MMP-3 complex, the rapid internal motion of the $\beta$-hairpin was largely abolished, a change consistent with tight binding of this region to the MMP-3 catalytic domain. The extended $\beta$-hairpin is not a feature of all members of the TIMP family; therefore, the binding of this highly mobile region to a site distant from the catalytic cleft of the MMPs suggests a key role in TIMP-2 binding specificity.

Breakdown of the extracellular matrix is an important event in many normal and pathological processes, such as growth, wound repair, tumor metastasis, and arthritis (1–3). A large family of zinc-dependent proteinases, the matrix metalloproteinases (MMPs), are thought to be primarily responsible for this matrix catabolism. The activities of the MMP family in the extracellular matrix are highly regulated by transcriptional control, zymogen activation, and inhibition by a family of specific protein inhibitors, the tissue inhibitors of metalloproteinases or TIMPs (4). The TIMPs bind tightly to the active proteinases to form an inactive TIMP-MMP complex (5). Four mammalian TIMP proteins have now been identified (TIMP-1 to -4) (6–9), and their high degree of sequence similarity and conservation of 12 Cys residues suggests that each consists of the same basic fold but with some variations in loop structures and glycosylation. The location of the MMP inhibitory site on the TIMP molecule has been shown to reside predominantly in the N-terminal two-thirds of the protein, defined by the first three disulfide bonds. This domain (N-TIMP) can be expressed independently to generate a fully folded, stable, and active inhibitor (10, 11).

High resolution three-dimensional structures are now available for both the active N-terminal domain of TIMP-2 (12) and for the full-length inhibitor (13). Crystal structures have also been published for full-length TIMP-1 and TIMP-2 in complexes with the catalytic domains of MMP-3 (N-MMP-3) and MT1-MMP, respectively (14, 15). The structures of the TIMP-MMP complexes, together with NMR data on chemical shift perturbation seen for N-TIMP-2 on complex formation with N-MMP-3 (16), have identified the key features of the TIMP inhibitory binding site. It is now clear that the N terminus of TIMP (residues 1–5), together with the two loops with which it is disulfide-bonded, form a “wedge”-like structure that interacts with the active site cleft of the proteinase. A further region in the N-terminal domain of TIMP that interacts with the proteinase is the loop between $\beta$-strands A and B. In TIMP-2, this region is extended by 7 residues compared with TIMP-1 and is therefore capable of making more extensive interactions with the proteinase. The involvement of the extended $\beta$-hairpin in TIMP-2:MMP interactions was first proposed on the basis of chemical shift changes observed on binding of N-TIMP-2 to N-MMP-3 (16), and further confirmed in the crystal structure for the TIMP-2:MT1-MMP complex (15).

NMR studies of N-TIMP-2 provided the first three-dimensional structure for the inhibitor and allowed it to be identified as a member of the OB (oligonucleotide/oligosaccharide)-fold protein family (17). This work was later extended to provide a high resolution structure of N-TIMP-2 using heteronuclear NMR-based methods (12). These studies provided some insights into the dynamics of N-TIMP-2, particularly for the $\beta$-hairpin, which we proposed to be a highly flexible structure due to the relatively narrow line widths seen for signals in this region and the lack of long range NOEs. Furthermore, we suggested that another region of the N-TIMP-2 binding site (Ser$^{195}$–Cys$^{197}$) was in relatively slow exchange between multiple conformations interconverting on a millisecond time scale,
as the backbone amide resonances for these residues were missing from NMR spectra (16).

To provide a more detailed picture of the backbone mobility of N-TIMP-2 and to assess how MMP complex formation affects these motions, the values of $T_1$, $T_2$, and heteronuclear $^{1}H$-$^{15}N$ NOE have now been determined for the backbone amide $^{15}N$ signals of N-TIMP-2 when free in solution and when bound to N-MMP-3. These studies show that several regions of N-TIMP-2 have significant local motility on time scales both slower or faster than the overall tumbling time of the protein (estimated to be 10 ns). In particular, rapid motion was seen for the AB β-hairpin of free N-TIMP-2, and this motility was lost on complex formation with N-MMP-3, suggesting that the extended AB loop is a general and important feature of the TIMP-2 binding site.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation—**$^{15}N$-Labeled human N-TIMP-2 was expressed in *Escherichia coli* and refolded from intracellular inclusion bodies as described previously (16, 18). The expression vector for N-MMP-3 was kindly provided by A. Marcy (Merck), and the recombinant protein expressed and purified as described by Marcy et al. (19). N-MMP-3 was activated by p-aminophenylmercuric acetate and incubated with $^{15}N$-labeled N-TIMP-2 to produce the N-TIMP-2-N-MMP-3 complex (16). The complex was purified from any free N-TIMP-2 or N-MMP-3 by gel filtration chromatography on Sephacryl S-100 (16). NMR experiments were carried out on 0.3-ml samples of 1.5 mM $^{15}N$-labeled N-TIMP-2 (25 mM sodium phosphate buffer, 100 mM NaCl, pH 6.5) or 0.5 mM $^{15}N$-labeled N-TIMP-2-N-MMP-3 complex (5 mM deuterated-imidazole/HCl, 100 mM NaCl, 5 mM CaCl$_2$, pH 6.5) in 5-mm Shigemi tubes. D$_2$O was added to the NMR samples to a final concentration of 10% (v/v).

**NMR Spectroscopy—**The NMR experiments were carried out at 35 °C on a Varian INOVA spectrometer operating at 1 H frequency of 600 MHz. The spectra used to determine $T_1$, $T_2$, and $^{1}H$-$^{15}N$ NOE values for the backbone amide $^{15}N$ signals of both free and N-MMP-3 bound N-TIMP-2 were recorded with acquisition times of 16.8 ms in $T_1$ and 142 or 148 ms in $T_2$ using the sensitivity enhanced pulse sequences described by Farrow et al. (20). The $^1H$ carrier was centered at the water frequency for the $T_1$ and $T_2$ measurements and at the center of the amide region for the heteronuclear $^{1}H$-$^{15}N$ NOE experiments. The $^{15}N$ carrier was placed in the center of the amide region.

In the case of free N-TIMP-2, spectra were used to determine $T_1$ values were acquired with 16 transients per increment and a relaxation delay of 4.4 s, $T_2$ values with 32 transients per increment and a relaxation delay of 3 s, and $^{1}H$-$^{15}N$ NOEs with 128 transients per increment and a relaxation delay of 8 s. The $T_1$ values were calculated from a series of spectra recorded with $T_1$ relaxation delays of 10.8, 64.9, 259.9 (s), 389.8, 563.1 (s), 660.2, and 1234.4 ms. Similarly, to determine $T_2$ values for the free N-TIMP-2 spectra were acquired with $T_2$ relaxation delays of 15.7, 31.5 (s), 47.2, 62.9, 94.4 (s), 110.1, and 141.6 ms. The magnitude of the $^{1}H$-$^{15}N$ NOE for the backbone amide $^{15}N$ signals was calculated from a pair of spectra recorded either with or without presaturation of backbone amide $^{1}H$ resonances during the relaxation delay.

For N-TIMP-2 bound to N-MMP-3, spectra were used to determine backbone amide $T_1$ values were recorded with 56 transients per increment and a relaxation delay of 5 s, $T_2$ values with 80 transients per increment and a relaxation delay of 3 s, and $^{1}H$-$^{15}N$ NOEs with 128 increments per transient and a relaxation delay of 8 s. The $T_1$ values were calculated from a series of spectra acquired with $T_1$ relaxation delays of 11.1, 68.3, 254.2 (s), 375.8, 552.7 (s), 662.2, and 1258.0 ms. In the case of $T_2$ values were determined from spectra recorded with $T_2$ relaxation delays of 15.7, 31.5 (s), 47.2, 62.9, 94.4 (s), 110.1, and 141.7 ms. The size of the heteronuclear $^{1}H$-$^{15}N$ NOE for the backbone amide $^{15}N$ resonances of N-TIMP-2 bound to N-MMP-3 was determined as described for the free protein.

**Data Processing and Analysis—**The NMR spectra were processed on a Silicon Graphics Indigo 2 workstation using the program NMRPipe (21). To improve the resolution in the final spectra the number of data points in $F_1$ was extended 2-fold by linear prediction, and both $F_1$ and $F_2$ were zero-filled once. The spectra were examined and peak heights determined using the program XEASY (22).

The $T_1$ and $T_2$ values for backbone amide $^{15}N$ signals were calculated by nonlinear, least-squares fitting of the observed changes in peak heights to appropriate single exponential functions (23, 24), using the program SigmaPlot. The values of steady-state $^{1}H$-$^{15}N$ NOEs were determined from the peak heights measured in spectra recorded either with ($I_o$) or without ($I_s$) presaturation of backbone amide $^{1}H$ resonances during the relaxation delay, according to the formula NOE = ($I_o$ - $I_s$)/$I_o$ (25).

**RESULTS**

**Relaxation Measurements—**This study provides a detailed picture of the backbone dynamics of the inhibitory domain of TIMP-2 (N-TIMP-2), and identifies those regions of the protein that undergo substantial changes in backbone mobility on formation of a stable complex with the MMP-3 catalytic domain (N-MMP-3). $T_1$, $T_2$, and heteronuclear $^{1}H$-$^{15}N$ NOE measurements were obtained for the majority of the backbone amide $^{15}N$ signals of free N-TIMP-2 (102/121) and for N-TIMP-2 in the complex (98/121). The residues for which no relaxation data could be obtained were Cys$^2$–Cys$^4$, Val$^5$, Cys$^7$, Asp$^{16}$, Ser$^{68}$–Val$^{11}$, Asp$^{77}$, Lys$^8$, Gly$^{83}$, Phe$^{103}$, Thr$^{113}$, Ser$^{117}$, His$^{120}$, Arg$^{121}$, and Met$^{124}$ in free N-TIMP-2, and Cys$^1$–Val$^{4}$, Asn$^{14}$, Ile$^{20}$, Lys$^3$–Ser$^{6}$–Gly$^7$, Ser$^{7}$, Lys$^{8}$, Gly$^{32}$, Asp$^{77}$, Cys$^{103}$, Thr$^{113}$, Thr$^{115}$, Tyr$^{122}$, and Arg$^{121}$ in N-TIMP-2 bound to N-MMP-3 (12, 16). $^{1}H$ relaxation data for the remaining residues could not be determined due to peak overlap in the spectra or very broad signals that prevented accurate measurement of peak heights. For residues Lys$^{27}$, Val$^3$, and Thr$^{112}$ of free N-TIMP-2, and Tyr$^{122}$, Gin$^{39}$, Glu$^{83}$, and Tyr$^{122}$ of N-TIMP-2 in the complex, it was possible to determine the size of the $^{1}H$-$^{15}N$ NOE but reliable estimates of $T_1$ and $T_2$ could not be obtained.

The $T_2$ values for backbone amide $^{15}N$ signals of proteins are sensitive to both fast and slow local motions of the polypeptide backbone. NMR signals with $T_2$ values significantly longer than the mean are indicative of rapid local motion on a time scale (picosecond to nanosecond) significantly shorter than the overall tumbling time ($\tau_m$) of the protein, and have relatively narrow line widths. In contrast, signals with $T_2$ values significantly shorter than the mean have relatively broad line widths and arise from exchange between two or more states (conformations) with different local environments. To significantly broaden NMR signals and decrease $T_2$ values, this exchange must be on a time scale (microsecond to millisecond) similar to the chemical shift difference between the two states (so-called intermediate exchange). If the chemical shift difference between the two states is considerable (>100 Hz), then intermediate exchange will broaden the NMR signals to such an extent that they can no longer be detected. Conformational exchange on time scales significantly longer that the chemical shift difference will result in the different states being detected as separate signals, while conformational exchange on time scales significantly shorter than the chemical shift difference will result in sharp signals at an intermediate frequency.

The size of the $^{1}H$-$^{15}N$ NOE for backbone amide signals is also sensitive to local motility of the peptide chain on a picosecond to nanosecond time scale (i.e. significantly faster than the overall tumbling rate of the protein). Regions of the protein backbone with rapid local motion are characterized by large negative $^{1}H$-$^{15}N$ NOEs.

**Free N-TIMP-2—**The $T_2$ relaxation times for the backbone amide $^{15}N$ signals of free N-TIMP-2 are shown in Fig. 1A. The mean and standard deviation (indicated on the chart) were calculated after omitting the data for 3 residues which were judged to have unusually long $T_2$ values (Gly$^{32}$, Gly$^{82}$, and Glu$^{127}$). Fig. 1A clearly shows five regions where the $T_2$ values...
are significantly longer than the mean. The N terminus (residues Ser31-Ile85, Gly125–Glu127), the end of b-strand A, the AB loop and the beginning of strand B (the AB b-hairpin, residues Ser31–Lys41), the region between the Cys1–Cys72 disulfide bond and strand D (residues Asp77 and Lys82) and strands D and E (Gly92–Asp93) and the C terminus of the protein (residues Gly125–Glu127). Residue Lys58 (in the loop between strands B and C) is also raised. These elevated T2 values suggest that these residues experience rapid internal motion on a picosecond to nanosecond time scale. The 1H-15N NOE results (Fig. 1B) identified very similar regions of N-TIMP-2 as having rapid local mobility. Large negative NOEs were recorded for residues near the N and C termini (Ser4, His7, and Gly125–Glu127), in the AB b-hairpin (Ser31–Lys41), and in the loops between strands B and C (Lys30, the Cys41–Cys72 disulfide bond and strand D (Asp77 and Lys82) and strands D and E (Gly92–Asp93). The T2 and 1H-15N NOE data for free N-TIMP-2 are summarized on the loop diagram in Fig. 2A.

The T2 data for free N-TIMP-2 also identified several regions of the molecule with significantly shorter T2 relaxation times than the mean, suggesting exchange between two or more conformations on the microsecond to millisecond time scale (Fig. 1A). Residues Gln49–Lys51 near the end of strand B, residue Ile60 in the loop between strands B and C, and residues His97–Cys101 in strand E and the EF loop all have T2 values below a threshold of 1 standard deviation from the mean (Figs. 1A and 2A).

The T2 values for the backbone amide 15N signals of free N-TIMP-2 showed very little variation with sequence (data not shown). The average T1 was found to be 0.807 with a standard deviation of 0.088. The T1/T2 ratios for N-TIMP-2 were used to estimate the overall rotational correlation time (τm) of the molecule (26). A value of 10 ns (at 35 °C) was obtained, which is comparable to the τm values reported for several other proteins on the basis of T1 and T2 data (26–28).

N-TIMP-2-N-MMP-3 Complex—The T2 data for N-TIMP-2 bound to N-MMP-3 are shown in Fig. 1C. Significantly elevated T2 values were found for Cys13 in helix 1, Lys22 in strand A, Glu57–Lys58 in the BC loop, Gly79–Gly80 in the loop between the Cys1–Cys72 disulfide bond and strand D, Gly92 in the DE loop, Ser117 in helix 2, and Gly123–Glu127 at the C terminus. In addition, the T2 values for Gly32–Asn33 in the AB b-hairpin were also marginally above the threshold (mean plus 1 standard deviation) considered to be significant (Fig. 1C). As observed for free N-TIMP-2, these regions of rapid internal motion were similarly identified in the 1H-15N NOE experiment (Fig. 1D). Large negative 1H-15N NOEs were recorded for His7, Lys122, Gly123–Asn125, Gly78–Lys79, Gly122, and Tyr125–Glu127. The T2 and 1H-15N NOE data for N-TIMP-2 in the complex with N-MMP-3 are summarized in Fig. 2B.

Residues with significantly shorter T2 values in N-TIMP-2

![Backbone amide 15N relaxation data for both free (A and B) and bound (C and D) N-TIMP-2. A, T2 relaxation times for free N-TIMP-2. The mean is indicated by the solid line (0.077), and the mean ± 6 S.D. (0.013) by the dashed lines (these values were calculated after first removing the exceptional T2 data for residues 32, 92, and 127). B, heteronuclear 1H-15N NOE values for free N-TIMP-2. The threshold of ~0.34 used to classify residues with large negative NOEs is indicated by the solid line. C, T2 relaxation times for the N-TIMP-2-N-MMP-3 complex. The mean (0.045) and the mean ± S.D. (0.008) are shown by the solid and dashed lines, respectively. The unusually long T2 values for residues 13, 22, 92, and 127 were omitted from the calculation. D, heteronuclear 1H-15N NOE values for the N-TIMP-2-N-MMP-3 complex. The threshold of ~0.34 is indicated as for B. Error bars show the standard error of the exponential fit (A and C); standard deviation of the baseplane noise as described by Stone et al. (24) (B); and the half-range for duplicate measurements (D).](image-url)
bound to N-MMP-3 (Fig. 1C) were Ile$^{19}$ and Thr$^{21}$ in strand A, Ile$^{80}$, Lys$^{51}$, and Phe$^{53}$ at the C-terminal end of strand B, and His$^{87}$, Ile$^{88}$, and Leu$^{100}$ in strand E and the EF loop. The $T_1$ data suggest that these residues exist in two or more conformations with interconversion on the microsecond to millisecond time scale. The location of these residues are shown in Fig. 2B.

The $T_1$ data obtained for the complex showed the same general trends in mobility as identified by the more sensitive $T_2$ and $^1$H-$^1$H NOE data (data not shown). The clearest trend was a decrease in $T_1$ toward the C terminus of the protein (Tyr$^{122}$–Glu$^{127}$) indicative of rapid internal motion on a nanosecond time scale. Depressed values of $T_1$ were also seen near the N terminus (His$^{7}$ and Gln$^{10}$), and for two glycine residues in the loop between the Cys$^{1}$–Cys$^{72}$ disulfide bond and strand D (Gly$^{79}$ and Gly$^{80}$). The average $T_1$ value for N-TIMP-2 was
found to increase from 0.81 s for the free protein to 1.48 s in the complex. This increase is consistent with the change in molecular size of the system on complex formation resulting in a longer overall correlation time ($\tau_m$). The variation in $T_1$ values across the sequence for N-TIMP-2 in the complex was found to be substantially greater than that observed for the free molecule (standard deviations of 0.288 and 0.088, respectively). This greater range of $T_1$ values is thought to reflect the increased anisotropy of the N-TIMP-2 molecule when bound to the protease.

The most dramatic mobility difference seen for N-TIMP-2 on N-MMP-3 binding is the loss of rapid local motion in the AB $\beta$-hairpin (Fig. 2, compare A and B). Residues Asp$^{34}$, Tyr$^{36}$–Asn$^{38}$, and Lys$^{41}$ no longer show elevated $T_2$ values and large negative $^{1}$H$^{-15}$N NOEs.

**DISCUSSION**

**Backbone Mobility in Free N-TIMP-2**—In free N-TIMP-2, the regions of protein backbone that showed greatest internal motion on a rapid picosecond to nanosecond time scale were the AB $\beta$-hairpin (Ser$^{31}$–Lys$^{41}$), the tight turn between strands D and E (Gly$^{39}$–Asp$^{69}$), and the C terminus of the protein, which shows increasing mobility from residue Gly$^{125}$ onwards (Fig. 2A). In addition, several other regions showed a more moderate degree of rapid internal motion including the N-terminal region of the protein (Ser$^4$ and His$^5$), the loop between strands B and C (Lys$^{68}$), and the loop between the Cys$^1$–Cys$^{72}$ disulfide bond and strand D (Asp$^{79}$–Lys$^{82}$). The core $\beta$-barrel and the two helices of N-TIMP-2 were all found to be comparatively rigid on a picosecond to nanosecond time scale. This picture of N-TIMP-2 is consistent with that seen for other proteins, where the core $\beta$-barrel is not rigid but able to flex slightly at this point where strand B coils tightly to form the $\beta$-barrel structure (17). Interestingly, residues Glu$^{49}$–Lys$^{51}$ are immediately adjacent to Thr$^{21}$ across the strands of the $\beta$-barrel, and two variants of TIMP-2 are known with either Ala (considered to be the wild-type sequence) or Thr (the protein used in this study) at this position (30). The Thr$^{21}$ variant is known to be less stable than the wild-type protein (11), and this may contribute to the conformational heterogeneity seen at Glu$^{49}$–Lys$^{51}$. A similar picture is seen for N-TIMP-2 in the complex, but in this case the region of slow internal motion was somewhat larger and includes Ile$^{19}$ and Thr$^{21}$, suggesting that the flexibility of the $\beta$-barrel is greater when N-TIMP-2 is bound to the enzyme.

**Backbone Mobility of the Binding Site for N-MMP-3**—The binding site on TIMP for the catalytic domain of the MMPs has now been mapped by both NMR spectroscopy and x-ray crystallography (12, 14–16). These studies have identified a binding site on N-TIMP-2 comprising residues Cys$^1$–Pro$^5$ at the N-terminus of the protein (Ser$^4$ and His$^5$), the loop between strands B and C (Lys$^{68}$), and the loop between the Cys$^1$–Cys$^{72}$ disulfide bond and strand D (Asp$^{79}$–Lys$^{82}$). The core $\beta$-barrel and the two helices of N-TIMP-2 were all found to be comparatively rigid on a picosecond to nanosecond time scale. This picture of N-TIMP-2 is consistent with that seen for other proteins, where the core $\beta$-barrel is not rigid but able to flex slightly at this point where strand B coils tightly to form the $\beta$-barrel structure (17). Interestingly, residues Glu$^{49}$–Lys$^{51}$ are immediately adjacent to Thr$^{21}$ across the strands of the $\beta$-barrel, and two variants of TIMP-2 are known with either Ala (considered to be the wild-type sequence) or Thr (the protein used in this study) at this position (30). The Thr$^{21}$ variant is known to be less stable than the wild-type protein (11), and this may contribute to the conformational heterogeneity seen at Glu$^{49}$–Lys$^{51}$. A similar picture is seen for N-TIMP-2 in the complex, but in this case the region of slow internal motion was somewhat larger and includes Ile$^{19}$ and Thr$^{21}$, suggesting that the flexibility of the $\beta$-barrel is greater when N-TIMP-2 is bound to the enzyme.

**Backbone Mobility of N-TIMP-2**—In N-TIMP-2, the regions of protein backbone that showed greatest internal motion on a rapid picosecond to nanosecond time scale were the AB $\beta$-hairpin (Ser$^{31}$–Lys$^{41}$), the tight turn between strands D and E (Gly$^{19}$–Asp$^{39}$), and the C terminus of the protein, which shows increasing mobility from residue Gly$^{125}$ onwards (Fig. 2A). In addition, several other regions showed a more moderate degree of rapid internal motion including the N-terminal region of the protein (Ser$^4$ and His$^5$), the loop between strands B and C (Lys$^{68}$), and the loop between the Cys$^1$–Cys$^{72}$ disulfide bond and strand D (Asp$^{79}$–Lys$^{82}$). The core $\beta$-barrel and the two helices of N-TIMP-2 were all found to be comparatively rigid on a picosecond to nanosecond time scale. This picture of N-TIMP-2 is consistent with that seen for other proteins, where the core $\beta$-barrel is not rigid but able to flex slightly at this point where strand B coils tightly to form the $\beta$-barrel structure (17). Interestingly, residues Glu$^{49}$–Lys$^{51}$ are immediately adjacent to Thr$^{21}$ across the strands of the $\beta$-barrel, and two variants of TIMP-2 are known with either Ala (considered to be the wild-type sequence) or Thr (the protein used in this study) at this position (30). The Thr$^{21}$ variant is known to be less stable than the wild-type protein (11), and this may contribute to the conformational heterogeneity seen at Glu$^{49}$–Lys$^{51}$. A similar picture is seen for N-TIMP-2 in the complex, but in this case the region of slow internal motion was somewhat larger and includes Ile$^{19}$ and Thr$^{21}$, suggesting that the flexibility of the $\beta$-barrel is greater when N-TIMP-2 is bound to the enzyme.
terminus (site 1), Ala^{66}–Cys^{72} in the CD loop (site 2), Leu^{100}–Cys^{101} in the EF loop (site 3), and Ile^{35}–Arg^{42} in the AB \( \beta \)-hairpin (site 4). The first two sites have been shown to contribute 60% of all intermolecular contacts in the interaction of TIMP-2 with MT1-MMP (15). If the relaxation data for these regions of N-TIMP-2 are examined, it is clear that several of them show significant mobility on either a slow or rapid time scale.

**Site 1 (N Terminus)**—\(^{15}N \) relaxation data could only be obtained for Ser\(^4\), which clearly experiences rapid local mobility. The backbone amide signal of Ser\(^2\) has never been detected in NMR spectra, and we have previously argued that this suggests that they are significantly broadened by exchange between several conformational states on a microsecond to millisecond time scale (12, 16) (Fig. 2). Furthermore, the backbone amide line widths for Val\(^71\) and Cys\(^{72}\) are very broad (hence their omission from the relaxation analysis). A finding consistent with the view that this entire region is in conformational exchange. This flexibility may play an important role in facilitating the large conformational change suggested for this region on MMP binding by a comparison of the structures available for both free and bound TIMP (12, 14).

**Site 3 (EF Loop)**—The \( T_2 \) values determined for Leu\(^{100}\) and Cys\(^{101}\) (Figs. 1A and 2A) suggest that this region, like site 2, is also in slow exchange between two or more conformational states on a microsecond to millisecond time scale.

**Site 4 (AB Loop)**—The backbone atoms of this region (Ile\(^{35}\)–Arg\(^{42}\)) were found to be highly mobile on a rapid picosecond to nanosecond time scale.

The NMR data clearly show that the binding site for the catalytic domain of MMP-3 on N-TIMP-2 is substantially more mobile than the protein as a whole. This property has been reported for a number of protein and nucleic acid binding sites on proteins (31, 32) and may be an important factor controlling the specificity of the binding interaction. It has been shown by Kay and co-workers (33, 34) that the flexible region of the peptide binding site in the phospholipase C-\( \gamma \) SH2 domain has a much wider ligand binding specificity than the more rigid phosphotyrosine binding site.

**Mobility Changes Seen on Complex Formation with N-MMP-3**—The binding of N-TIMP-2 to N-MMP-3 resulted in a dramatic change in the mobility of the AB \( \beta \)-hairpin (Ser\(^{31}–\)Lys\(^{41}\)), with the majority of this region losing the rapid motion seen in the free protein. Only residues Ser\(^{31}–\)Asn\(^{33}\) still retain significant mobility in the complex; these residues, however, form a \( \beta \)-bulge in strand A that may act as a flexible hinge for the AB \( \beta \)-hairpin. The highly mobile regions around Lys\(^{68}\), Gly\(^{92}\), and the C terminus of N-TIMP-2 are distant from the MMP binding site and, as expected, retain rapid motion in the complex. The loss of rapid local motion for the majority of the AB \( \beta \)-hairpin on binding to N-MMP-3 suggests that this region interacts with the enzyme, and this result strongly supports our earlier NMR chemical shift perturbation studies that showed a change in the environment of the backbone amide resonances of Glu\(^{28}–\)Lys\(^{41}\) on binding to N-MMP-3 (12, 16). There is currently no high resolution structure for the TIMP-2-MMP-3 complex, but our NMR results clearly show that the AB \( \beta \)-hairpin plays an important role in complex formation.

The formation of a tight N-TIMP-2-N-MMP-3 complex did not have a dramatic effect on the regions of N-TIMP-2 that are not in exchange between several conformations on a microsecond to millisecond time scale. \( T_2 \) values for the EF loop, which forms part of the interaction site, remained low in the complex, suggesting that this region is not “frozen” into a single conformation by interaction with N-MMP-3. Similarly, the backbone amide signals for Ser\(^{68}–\)Cys\(^{72}\) were not detected in spectra of the complex, suggesting that this region is also not locked into a single conformation by its interaction with the enzyme, even though it is thought to undergo a substantial change in conformation on binding (12, 13).

**Binding Interactions Made by the AB \( \beta \)-Hairpin**—The role of the AB \( \beta \)-hairpin in TIMP-2/MMP binding is of considerable interest. X-ray crystallography revealed that this region makes specific interactions with the catalytic domain of MT1-MMP, particularly the side chain of Tyr\(^{36}\), which binds in a hydrophobic groove formed from the MT-loop, a unique feature of the membrane-bound MMPs (15). These findings have been recently confirmed by kinetic analysis of a mutant form of N-TIMP-2, where Tyr\(^{36}\) was substituted by glycine (35). This mutant shows a 100-fold decrease in the rate of binding (\( k_{\text{on}} \)) and a 40-fold increase in the inhibition constant (\( K_{\text{app}} \)) for MT1-MMP, but showed binding kinetics and inhibition constants essentially unchanged to those of the wild-type for several other MMPs (MMP-2, -3, -7, and -13). TIMP-1 lacks the extended AB \( \beta \)-hairpin structure present in TIMP-2 and is a comparatively poor inhibitor of MT1-MMP (36). It is clear that the AB \( \beta \)-hairpin contributes considerably to the binding of TIMP-2 to MT1-MMP, but otherwise TIMP-1 shows no dramatic difference in MMP specificity and thus far the extended AB \( \beta \)-hairpin has not been shown to be critical in other TIMP-2 inhibitory interactions. Interestingly, TIMP-3, which also lacks the AB \( \beta \)-hairpin, binds to MT1-MMP with similar affinity to TIMP-2 (37) and must, therefore, make additional binding interactions with the enzyme that in some way compensate for the loss of the extended AB \( \beta \)-hairpin structure.

The positioning of the extended AB \( \beta \)-hairpin at one end of the inhibitory wedge of TIMP-2 suggests that this structure will interact with all MMP catalytic domains on complex formation. On binding to an MMP catalytic domain, the AB \( \beta \)-hairpin must move away from the core \( \beta \)-barrel of the molecule to allow the proteinase sufficient access to interact at the TIMP inhibitory site. This movement is quite considerable (up to 8 Å in the case of TIMP-2 binding to MT1-MMP; Ref. 13) and may be facilitated by the \( \beta \)-bulge at Ser\(^{31}–\)Gly\(^{32}\) in strand A that could act as a hinge. This role is supported by the \( ^{15} \)N relaxation data, which show that this putative hinge region has high mobility in both free and bound forms of the inhibitor.

The nature of the interaction of the AB \( \beta \)-hairpin with MMP catalytic domains will have a significant effect on the overall
binding energy and kinetics of inhibition. The high mobility observed for this region may be essential for it to adopt complementory conformations with the wide range of MMP catalytic domains. The contribution of this interaction to the overall binding energy will depend on the nature of the interactions formed, but in order to increase the overall binding energy the strength of favorable contacts will first have to overcome the loss of entropy on binding and it is clear that the contribution of the extended AB β-hairpin will vary from case to case. For some MMPs, like MT1-MMP, specific interactions mediated by this region (e.g. Tyr30) will help to offset the loss of entropy, and hence overall this region contributes positively to complex formation. In other cases, the nature of the close contacts may simply balance the loss of entropy resulting in little or no effect on the binding affinity, or indeed, may act to reduce the overall binding energy by making non-favorable interactions with the proteinase. The extended β-hairpin of TIMP-2 may also have an important role in controlling the rate of TIMP-2/MMP interactions by affecting the way the molecules orientate to one another before contact or by restricting access of the incoming MMP to the TIMP inhibitory site. The precise contribution of the AB β-hairpin to the binding kinetics and affinity of TIMP-2/MMP interactions will require careful future studies on engineered forms of TIMP-2 where this region is excised or modified by site-directed mutagenesis.

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