High Resolution Structure of the N-terminal Domain of Tissue Inhibitor of Metalloproteinases-2 and Characterization of Its Interaction Site with Matrix Metalloproteinase-3*

(Received for publication, March 16, 1998, and in revised form, May 26, 1998)

Frederick W. Muskett‡, Tom A. Frenkiel§, James Feeney¶, Robert B. Freedman†, Mark D. Carr‡,§, and Richard A. Williamson¶

From the ‡Department of Biosciences, University of Kent, Canterbury, Kent, CT2 7NJ, United Kingdom and the ¶Medical Research Council Biomedical NMR Centre and §Division of Molecular Structure, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

The high resolution structure of the N-terminal domain of tissue inhibitor of metalloproteinases-2 (N-TIMP-2) in solution has been determined using multidimensional heteronuclear NMR spectroscopy, with the structural calculations based on an extensive set of constraints, including 3132 nuclear Overhauser effect-based distance constraints, 56 hydrogen bond constraints, and 220 torsion angle constraints (an average of 26.9 constraints/residue). The core of the protein consists of a five-stranded β-barrel that is homologous to the β-barrel found in the oligosaccharide/oligonucleotide binding protein fold. The binding site for the catalytic domain of matrix metalloproteinases-3 (N-MMP-3) on N-TIMP-2 has been mapped by determining the changes in chemical shifts on complex formation for TIMP-2 (Gomis-Ruth, F.-X., Maskos, F., Gueiros-Filho, R., Nagase, H., Bourne, G. P., Bartunik, H. & Upton, NY. 1997 Nature 389, 77–80) revealed that the core β-barrels are very similar in topology but that the loop connecting β-strands CD (P67–C72) would need to undergo a large conformational change for TIMP-2 to bind in a similar manner to TIMP-1.

Remodeling of connective tissue is an important event in many normal and pathological processes such as growth, wound healing, tumor invasion, and rheumatoid and osteoarthritis. The matrix metalloproteinases (MMPs)1 are a group of enzymes thought to be primarily responsible for this catabolism (1, 2). As expected, MMP activity is highly regulated and includes inhibition by a family of specific protein inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). Four TIMPs have now been identified (TIMP-1 to TIMP-4), which are about 190 amino acids in length and share 40–50% sequence identity (3–6). The TIMPs inhibit activated MMPs by forming tight ($K_d < 1$ nM), 1:1, noncovalent complexes and do not undergo cleavage by the enzymes (7). The TIMPs show relatively little selectivity in binding to the MMPs, but substantial differences have been reported for their binding to members of the recently discovered membrane-bound MMP family (8).

The TIMPs appear to consist of two domains, although only the N-terminal one has been shown to form a stable, autonomously folded unit (N-TIMP). This domain, encompassing about two-thirds of the molecule and including three of the six disulfide bonds, retains full inhibitory activity and is able to form stable tight complexes with MMP catalytic domains (9). Previously, we were able to determine a low resolution solution structure for N-TIMP-2 (127 residues) using only two- and three-dimensional 1H NMR data (10); this revealed the backbone topology of the molecule and surprisingly identified it as a member of the oligosaccharide/oligonucleotide binding protein fold family (11). These studies were carried out using recombinant N-TIMP-2 produced in mammalian cells. To obtain a high resolution solution structure for N-TIMP-2, it was necessary to produce uniformly 15N- and 15N/13C-labeled N-TIMP-2. This was recently achieved by expression of the protein in Escherichia coli grown on labeled minimal medium followed by refolding of the labeled N-TIMP-2 from inclusion bodies (12). In this paper we report both the high resolution solution structure of N-TIMP-2 and the mapping of its binding site for the catalytic domain of human stromelysin-1 (N-MMP-3), both of which were achieved using double and triple-resonance heteronuclear NMR-based methods. Recently, a crystal structure for the complex formed between TIMP-1 and N-MMP-3 has been reported (13). The coordinates of this model have been made available by the authors and a comparison with the high resolution structure reported here for N-TIMP-2 reveals several notable differences between the structures for bound TIMP-1 and free N-TIMP-2. For example, the extended β-hairpin formed from strands A and B in N-TIMP-2 allows this region to make extensive interactions with N-MMP-3, which...
are simply not possible for TIMP-1 and may form the basis for the selectivity reported for membrane bound MMPs (8). In addition, the well defined loop connecting b-strands C and D in N-TIMP-2 would need to undergo a substantial conformational change on binding to N-MMP-3 if TIMP-2 is to interact with the catalytic domain in a manner analogous to TIMP-1.

**EXPERIMENTAL PROCEDURES**

Sample Preparation—The following protein samples were prepared as described previously: nonisotopically enriched and uniformly 15N- or 13C/15N-labeled N-TIMP-2, N-MMP-3, and complexes formed between N-TIMP-2 and N-MMP-3 (10, 12, 14, 16). The NMR experiments were carried out on 0.6-ml samples of 1.5-3.0 mM nonisotopically enriched, 1.7 mM 15N-labeled and 1.4 mM 13C/15N-labeled N-TIMP-2 dissolved in 25 mM sodium phosphate and 100 mM potassium chloride at pH 6.7. In the case of the 15N/13C-labeled N-TIMP-2-unlabeled N-MMP-3 complex, spectra were recorded from 0.9 ml samples of 0.6 mM complex in 5 mM deuterated imidazole, 100 mM sodium chloride, and 5 mM calcium chloride at pH 6.7 using 8-mm Shigemi tubes. The NMR samples were prepared in either 100% D2O or 90% H2O, 10% D2O as appropriate.

**NMR Spectroscopy**—All NMR experiments were performed on Varian Unity and UnityPlus spectrometers operating at 500 and 600 MHz, with all the data collected in phase-sensitive mode using the method of States and Bax at the temperature of 35 or 4°C. Detailed descriptions of the two- and three-dimensional 1H, 13N/1H, 15N/1H, and 13C/15N/1H experiments used to obtain nearly complete sequence-specific 1H, 15N, and 13C resonance assignments for N-TIMP-2 have been reported previously (10, 14). In addition, three-dimensional 15N/1H NOESY-HSQC (18) and 15N/1H HMQC-NOE (19, 20) spectra were acquired with an NOE mixing time of 125 ms and three-dimensional 15N/1H HNHA (21) and HNHB (22) spectra recorded. A series of 10 two-dimensional 15N/1H HSQC spectra (23–25) were also acquired over 4 h from a sample of 15N-labeled N-TIMP-2 freshly dissolved in D2O to identify slowly exchanging amide protons in the protein. In the case of the N-TIMP-2-N-MMP-3 complex, experiments carried out were two-dimensional 15N/1H HSQC, two-dimensional 13C/15N HMQC, three-dimensional 15N/15C/1H HNCA and CBCA(CO)NH (25–27).

The three-dimensional spectra were recorded over about 65 h with acquisition times in the indirect dimensions (F2, F3) of 9.4–15.5 ms for 15N, 6–11.3 ms for 13C, and 18.7–24.6 ms for 1H, as appropriate, and in the real time domain of 48–128 ms. Water suppression in the experiments was achieved by using the pulsed-field-gradient-based WATERGATE method (28).

Two- and three-dimensional NMR data were processed essentially as described previously (10, 14, 29) using the NMRPipe software package on Silicon Graphics workstations (30). All the spectra were analyzed on-screen using the program EXASI (31).

**Structural Calculations**—Structurally significant intra- and inter-residue NOEs were identified in three-dimensional 15N/1H NOEY-HSQC, three-dimensional 13C/15N HMQC-NOEY, and two-dimensional NOEY spectra of N-TIMP-2 recorded with mixing times of 125 ms. The relationship between NOE intensity and interproton distance was calibrated using NOEs corresponding to known distances in regular beta-sheet and alpha-helical regions of N-TIMP-2, and on this basis the NOEs were converted to upper distance constraints using the program CALIBA (32) with the maximum upper distance limit set to 6 Å. Where appropriate, standard distance corrections were applied to constraints involving methyl and aromatic ring protons (33, 34). In addition, where spectral overlap prevented reliable determination of volumes for NOE cross-peaks the distance constraints were set to the upper limit of 6 Å.

The ratios of diagoonal to cross-peak volumes in HNHA spectrum allowed reliable JNNH,NH coupling constants (± 1 Hz) to be determined for 100 residues of N-TIMP-2. In addition, the relative intensities of the HN to HJ cross-peaks in the HNHB spectrum allowed JNNH,NH coupling constants to be estimated as either large (–5 ± 0.5 Hz) or small (–1 ± 0.5 Hz). This coupling constant data together with intraresidue and sequential NOEs were used as input for the program HABAS (32), which produced 89, 89, 89, and 42 chi torsion angle constraints.

The high resolution solution structure of N-TIMP-2 was calculated with the program DYANA (35), which uses simulated annealing combined with restrained molecular dynamics annealing. The Overhauser effect data, were used to determine stereospecific assignments and chi angles for more residues. At each stage in the calculation cycle new generations of N-TIMP-2 structures were calculated from 100 random starting coordinates, with the additional information obtained now included. In the final stages of the N-TIMP-2 structure refinement, additional distance constraints were included in the calculations corresponding to NMR determined hydrogen bonds between backbone amide and carbonyl groups. Hydrogen bonds were included only for residues whose amide proton was detectable at least 1 h in D2O and where the distance between the hydrogen bond acceptor and donor atoms was less than 2.5 Å and the NH–O bond angle greater than 135°. For each hydrogen bond identified in N-TIMP-2, appropriate lower and upper distance limits were used to constrain NH to O to 1.8–2.3 Å and N to O to 2.4–3.3 Å.

Results and Discussion

High Resolution Solution Structure of N-TIMP-2—The solution structure of N-TIMP-2 was determined using a total of 3413 NMR-derived structural constraints (26.8/residue), including 3132 NOE-based upper distance limits (672 intraresidue, 784 sequential (i, i + 1), 602 medium-range (i, i ± 4), and 1074 long range (i, i ± 5)), 56 hydrogen bonding constraints corresponding to 14 protein backbone hydrogen bonds and 220 torsion angle constraints (89 phi, 89 psi, and 42 chi). In addition, the calculations included a further 18 distance constraints for the three disulfide bonds (Cys1–Cys72, Cys3–Cys101, and Cys13–Cys126) previously mapped in N-TIMP-2 and TIMP-1 (10, 36).

Specific stereochemical assignment information was incorporated for three glycine alpha-protons, 37 beta, 12 gamma, 3 delta, and 6 epsilon-methylene groups and 5 gamma- and 3 methyl groups.

After the final round of DYANA calculations, 49 satisfactorily converged N-TIMP-2 structures were obtained from 100 random starting conformations. The converged structures contain no distance constraint or van der Waals violations greater than 0.5 Å and no dihedral angle violations greater than 5°. The sum of the violations for upper distance limits, lower distance limits, van der Waals contacts, and torsion angle constraints were 64.9 ± 1.5 Å, 0.8 ± 0.2 Å, 34.6 ± 1.1 Å, and 40.7 ± 4.9°, respectively. Similarly, maximum violations for the converged structures were 0.42 ± 0.04 Å, 0.19 ± 0.05 Å, 0.35 ± 0.04 Å, and 3.8 ± 0.6°, respectively. The solution structure of N-TIMP-2 is determined to very high precision, which is reflected in very low root mean squared deviation values for both backbone and all heavy atom coordinates of 0.38 ± 0.06 and 0.74 ± 0.08 Å, respectively, for the family of 49 converged structures.

The residue by residue distribution of the NOEs identified for N-TIMP-2 is shown in Fig. 1. The observed NOEs are distributed relatively evenly throughout the protein, with only residues near the N (Cys1–Ser2) and C termini (His120–Tyr122) and Gly125–Glu127) and in three short stretches corresponding to Ser6–Thr10, Thr19–Thr23, and Arg163–Arg169. The most striking feature of the protein remains the closed, five-stranded beta-barrel, which is formed from beta-strands A (Val17–Asp41), B (Asn38–Phe53), C (Phe62–Ala66), D (Tyr84–Glu91), and E (Lys95–Ile98). The
**Characterization of the MMP Catalytic Domain Binding Site on N-TIMP-2**—The binding site for the catalytic domain of stromelysin-1 (N-MMP-3) on N-TIMP-2 was mapped by analyzing the changes in chemical shifts of backbone HN, N, and Cα resonances of N-TIMP-2 that occur on formation of the N-TIMP-2-N-MMP-3 complex. The chemical shifts of signals from NMR active nuclei depend on their environment, hence this approach identifies residues in N-TIMP-2 that undergo an environmental change on MMP binding, either due to close proximity to bound N-MMP-3 or as a consequence of a binding-induced conformational change in the protein. Sequence-specific $^{15}$N, $^{13}$C, and $^3$H backbone resonance assignments were obtained for 87% of the residues in $^{13}$C/$^{15}$N-labeled N-TIMP-2 bound to unlabeled N-MMP-3 by analysis of spectra from three-dimensional CBCA(CO)NH and HNCA experiments. No signals could be identified from the backbone amide groups of Cys$^1$–Ser$^4$, Asn$^{14}$, Ile$^{35}$, Ile$^{40}$, Ser$^{68}$–Ala$^{70}$, Cys$^{72}$, Ser$^{75}$, Thr$^{112}$, Thr$^{113}$, His$^{120}$, and Arg$^{121}$. The inability to detect these resonances for N-TIMP-2 in the complex probably arises because they are significantly exchange broadened compared with signals from other backbone groups in the protein. Interestingly, many of these signals are also not detected in free N-TIMP-2, in particular the two stretches around the C1-C72 disulfide bond (Cys$^1$–Ser$^4$ and Ser$^{68}$–Cys$^{72}$). We previously proposed that these signals could be broadened due to intermediate exchange between different local conformations (10, 14).

To identify N-TIMP-2 signals perturbed by N-MMP-3 binding, the backbone assignments for bound N-TIMP-2 were compared with those determined for the free protein and the chemical shift change noted for each signal. The shifts in backbone amide $^{15}$N and α carbon $^{13}$C signals were then scaled to take some account for the difference in spectral dispersion compared with backbone amide $^1$H signals giving a more equal weighting to each data set ($^{15}$N range 130–107 = 23 ppm; $^{13}$C range 67–44 = 23 ppm; $^1$H range 10.1–6.9 = 3.2 ppm; giving a correction factor of 0.14 for both $^{15}$N and $^{13}$C signals). Correction in this manner allows an average change in backbone chemical shift to be calculated for each residue in N-TIMP-2, results of which are shown in Fig. 4. Examination of the chemical shift differences clearly identifies 8 residues in N-TIMP-2 whose backbone signals undergo large chemical shift changes on complex formation, specifically Ser$^{32}$, Tyr$^{36}$, Ile$^{40}$, Lys$^{41}$, Ala$^{70}$, Val$^{71}$, Gly$^{73}$, and Cys$^{101}$. These residues fall into three distinct regions of the protein: (i) the end of strand A, the AB loop, and the beginning of strand B (Glu$^{88}$–Lys$^{41}$); (ii) Ala$^{70}$–Gly$^{73}$ in the long loop between strands C and D, which is disulfide-bonded to Cys$^1$ through Cys$^{72}$; and (iii) in the loop between strands E and F (His$^{97}$–Ile$^{104}$), which is also linked to the N terminus by the Cys$^3$–Cys$^{101}$ disulfide bond. Val$^{71}$ showed the largest chemical shift changes, more than twice as large as those of any other residue, whereas signals from about 70% of the residues in N-TIMP-2 showed no appreciable chemical shift changes on complex formation.

![Fig. 1](image_url)

**High Resolution Structure of TIMP-2**

distance constraints obtained for residues in helix-1 and -2, in particular, from the $^{13}$C/$^2$H HMBC-NOESY spectra. Apart from the orientation of helix-2, the low resolution structure reported previously for N-TIMP-2 has proved to be essentially correct and has already been very successful in guiding and interpreting site-directed mutagenesis work (38).

The two N-terminal residues (Cys$^1$–Ser$^2$) and a few others (Glu$^{61}$, Ala$^{90}$, Asp$^{102}$) are significantly less ordered. Many side chain resonances could be identified from the backbone amide groups of Cys$^1$–Ser$^4$, Asn$^{14}$, Ile$^{35}$, Ile$^{40}$, Ser$^{68}$–Ala$^{70}$, Cys$^{72}$, Ser$^{75}$, Thr$^{112}$, Thr$^{113}$, His$^{120}$, and Arg$^{121}$. The inability to detect these resonances for N-TIMP-2 in the complex probably arises because they are significantly exchange broadened compared with signals from other backbone groups in the protein. Interestingly, many of these signals are also not detected in free N-TIMP-2, in particular the two stretches around the C1-C72 disulfide bond (Cys$^1$–Ser$^4$ and Ser$^{68}$–Cys$^{72}$). We previously proposed that these signals could be broadened due to intermediate exchange between different local conformations (10, 14).

To identify N-TIMP-2 signals perturbed by N-MMP-3 binding, the backbone assignments for bound N-TIMP-2 were compared with those determined for the free protein and the chemical shift change noted for each signal. The shifts in backbone amide $^{15}$N and α carbon $^{13}$C signals were then scaled to take some account for the difference in spectral dispersion compared with backbone amide $^1$H signals giving a more equal weighting to each data set ($^{15}$N range 130–107 = 23 ppm; $^{13}$C range 67–44 = 23 ppm; $^1$H range 10.1–6.9 = 3.2 ppm; giving a correction factor of 0.14 for both $^{15}$N and $^{13}$C signals). Correction in this manner allows an average change in backbone chemical shift to be calculated for each residue in N-TIMP-2, results of which are shown in Fig. 4. Examination of the chemical shift differences clearly identifies 8 residues in N-TIMP-2 whose backbone signals undergo large chemical shift changes on complex formation, specifically Ser$^{32}$, Tyr$^{36}$, Ile$^{40}$, Lys$^{41}$, Ala$^{70}$, Val$^{71}$, Gly$^{73}$, and Cys$^{101}$. These residues fall into three distinct regions of the protein: (i) the end of strand A, the AB loop, and the beginning of strand B (Glu$^{88}$–Lys$^{41}$); (ii) Ala$^{70}$–Gly$^{73}$ in the long loop between strands C and D, which is disulfide-bonded to Cys$^1$ through Cys$^{72}$; and (iii) in the loop between strands E and F (His$^{97}$–Ile$^{104}$), which is also linked to the N terminus by the Cys$^3$–Cys$^{101}$ disulfide bond. Val$^{71}$ showed the largest chemical shift changes, more than twice as large as those of any other residue, whereas signals from about 70% of the residues in N-TIMP-2 showed no appreciable chemical shift changes on complex formation.

Fig. 5 shows a space-filled view of N-TIMP-2 in which residues are color-coded according to the changes in average backbone chemical shift observed on N-MMP-3 binding (red > 0.20 ppm, yellow > 0.05 ppm, blue < 0.05 ppm, and white unknown). This clearly shows that the MMP binding site is formed from the region around the Cys$^1$–Cys$^{72}$ and Cys$^3$–Cys$^{101}$ disulfide bonds, which connect loops CD and EF to the N

**Fig. 1** Histogram showing the distribution and classification of NOE distance constraints for N-TIMP-2. White columns represent short range (i, i + 1); light gray columns represent medium range (i, i ≤ 4); and black columns represent long range (i, i ≥ 5) distance constraints.
terminus, and by the prominent β-hairpin involving residues Glu28–Lys41 that links strands A and B (Figs. 2C and 5). Although no chemical shift data were obtained for Cys1–Ser4, the central position of these residues in the NMR-mapped MMP binding site clearly suggests that they play a key role in TIMP-MMP interactions.

Further evidence for the location of the MMP binding site on N-TIMP-2 comes from analysis of chemical shift changes for
methyl groups in N-TIMP-2. Sequence-specific $^1$H and $^{13}$C resonance assignments for methyl groups in bound N-TIMP-2 were made from three-dimensional $^{13}$C/$^1$H HMQC-NQESY spectra of the $^{15}$N/$^{13}$C-labeled N-TIMP-2-unlabeled N-MMP-3 complex, using the $\alpha$ and $\beta$ carbon assignments obtained from CBCA(CO)NH and HNCA spectra as starting points. This approach allowed resonance assignments to be made for all methyl groups in the protein apart from those of Ile$^{35}$, Ile$^{40}$, Ala$^{66}$, and Val$^{71}$. No significant chemical shift changes occurred for any assigned methyl signals on N-MMP-3 binding, except for Ala$^{70}$. These results are entirely consistent with the chemical shift changes seen for the protein backbone resonances of N-TIMP-2. $^1$H signals from a pair of methyl groups were each shifted by over 0.5 ppm on complex formation, but unfortunately showed no NOEs to $\alpha$ or $\beta$ protons and so could not be assigned. These highly shifted methyl resonances must belong to either Ile$^{35}$, Ile$^{40}$, or Val$^{71}$; all of these residues are also implicated in MMP binding by the changes seen in the chemical shifts for their backbone signals.

In an earlier report (14), we described a preliminary mapping of the N-MMP-3 binding site on N-TIMP-2 using a nearest peak-based approach in which lower limits for chemical shift changes in backbone amide signals on complex formation were determined by noting the $^{15}$N/$^1$H cross-peaks in HMQC spectra of the complex that were nearest to an assigned peak in a comparable HMQC spectrum of free N-TIMP-2. Now that real values (rather than lower limit values) for the chemical shift changes of the backbone nuclei have been determined (Fig. 4), the success of the earlier “nearest-peak” approach can be assessed. In fact, the nearest-peak approach was found to be highly successful in identifying the overall location of the N-MMP-3 binding site on N-TIMP-2, but the availability of actual chemical shift changes on complex formation allows one to focus more clearly on those residues involved in MMP binding as their true degree of change relative to the other residues is not attenuated by the inherent underestimates of chemical shift changes determined by the nearest-peak approach for the most perturbed residues.

Conformational Heterogeneity in the MMP Binding Site on N-TIMP-2—The inability to detect backbone amide resonances from Cys$^{1}$-Ser$^{4}$ and Ser$^{68}$-Cys$^{72}$ in free N-TIMP-2 indicates that these signals are either significantly broadened compared with other backbone amide signals from the protein or there is intermediate or fast exchange with water protons catalyzed by the local environment (14, 15). Localized signal broadening from exchange processes could result if the protein in these regions exists in different conformational states in intermediate exchange on the NMR chemical shift time scale. Although these two regions form a large part of the MMP binding site, the formation of a very tight and stable N-TIMP-2-N-MMP-3 complex does not result in the backbone amide signals for residues in this region of N-TIMP-2 becoming detectable in HSQC, CBCA(CO)NH, and HNCA spectra of the complex, except for Val$^{71}$. This implies that complex formation had little effect on the processes responsible for the backbone amide signal line broadening of residues Cys$^{1}$-Ser$^{4}$ and Ser$^{68}$-Cys$^{72}$. The binding of N-MMP-3 to N-TIMP-2 almost certainly prevents these backbone amides from being in contact with bulk water, which would argue against rapid exchange via exchange with the water. Thus, the backbone amide line broadening for residues Cys$^{1}$-Ser$^{4}$ and Ser$^{68}$-Cys$^{72}$ in N-MMP-3-bound N-TIMP-2 would appear to arise from interconversion between multiple local conformations of the protein. The model of the TIMP-1-N-MMP-3 complex determined by x-ray diffraction (13) does not suggest a clear mechanism for this conformational heterogeneity. Indeed, the x-ray model shows many of the affected backbone amide protons in well defined conformations participating in hydrogen bonds with backbone carbonyl oxygen atoms of N-MMP-3.

Another interesting observation for bound N-TIMP-2 is the detection of two sets of signals from the side chain methyl groups of Ile$^{43}$ and of a pair of substantially shifted methyl resonances arising from either Ile$^{40}$, Ile$^{35}$, or Val$^{71}$. No other signals appeared to be doubled, and the ratio of major:minor forms (80:20) is the same for both sets of signals and did not change with time or between different preparations of the complex. This suggests that the methyl signal doubling arises from the presence of two conformational states of bound N-TIMP-2 in this local region, which are in slow exchange on the NMR time scale. Interestingly, the residues involved are again implicated in N-MMP-3 binding; however, it seems likely that this conformational heterogeneity in N-TIMP-2 is distinct from that affecting Cys$^{1}$-Ser$^{4}$ and Ser$^{68}$-Cys$^{72}$ discussed previously.

Comparisons between the N-MMP-3 Binding Sites on TIMP-1 and TIMP-2—The N-MMP binding site identified by NMR on N-TIMP-2 is entirely consistent with that seen in the crystal structure of the related TIMP-1-N-MMP-3 complex (13), in which the N-MMP-3 binding site on TIMP-1 consists of the N terminus (Cys$^{1}$-Val$^{14}$), CD loop (Ala$^{65}$-Cys$^{72}$), AB loop (Val$^{29}$, Thr$^{33}$-Tyr$^{35}$), and EF loop (Thr$^{38}$-Cys$^{49}$). The first two sites make three-quarters of all intermolecular contacts, whereas the last two make direct but weaker side chain-mediated hydrophobic interactions. The corresponding residues in TIMP-2 are Cys$^{1}$-Ser$^{4}$, Pro$^{67}$-Cys$^{72}$, the AB loop region around Val$^{29}$-Lys$^{41}$ and Leu$^{100}$-Cys$^{101}$, respectively, exactly those residues identified as participating in N-MMP-3 binding by this NMR study. A significant structural difference between TIMP-1 and -2 is the insertion of 7 residues in the AB loop of TIMP-2, which allows it to make more extensive contacts on binding to N-MMP-3. In the case of TIMP-1, Thr$^{38}$ and Leu$^{44}$ make hydrophobic contacts with N-MMP-3 and are homologous to Ile$^{40}$ and Lys$^{41}$ in TIMP-2, both of which show substantial chemical shift changes for backbone signals on complex formation. On the other side of the AB $\beta$-hairpin, Val$^{29}$, which makes hydrophobic interactions with N-MMP-3 in the crystal structure, is conserved in both TIMPs and together with Glu$^{28}$ also shows
significant chemical shift changes for backbone resonances on binding of N-MMP-3. The additional 7 residues present in the AB loop of N-TIMP-2 are inserted between these two sites and substantial chemical shift changes are observed for signals from several of these residues on complex formation, namely Ser$^{31}$, Gly$^{32}$, Asp$^{34}$, Tyr$^{36}$, and Gly$^{37}$. Tyr$^{36}$ is located at the end of the AB loop in N-TIMP-2 and together with Ile$^{35}$ (for which no backbone chemical shift data could be obtained) are strong candidates for making additional contacts with N-MMP-3. The chemical shift changes seen on binding for signals from Ser$^{31}$

![Chemical shift changes for the backbone nuclei of N-TIMP-2 on complex formation with N-MMP-3. Values shown are an average of HN, N, and C$\alpha$ chemical shift changes scaled by the relative differences in spectral widths (0.14 for $^{15}$N and $^{13}$C). The chemical shift change for proline residues was determined solely from C$\alpha$ measurement as are residues 14, 40, 70, 75, 112, 113, 120, and 121. No resonance assignments could be made for residues 1–4, 35, 39, 67–69, and 72; these residues are marked with an asterisk on the histogram.](image1)

![Space-filled model (no hydrogen atoms) of N-TIMP-2 showing the MMP binding site. Residues with backbone nuclei experiencing a substantial environmental change on binding (average chemical shift change > 0.2 ppm) are shown in red, those experiencing a small change are shown in yellow (average chemical shift change > 0.05 ppm), and those experiencing no change are shown in blue. Residues for which no assignments could be made are shown in white. Residues on the reverse side of N-TIMP-2 (not shown) showed no changes in chemical shifts. This figure was prepared using the N-TIMP-2 structure closest to the mean and produced using MOLMOL (39).](image2)
and Gly\(^{32}\) may also indicate additional interactions with N-MMP-3. However, it is interesting to note that these two residues form part of a β-bulge in strand A, which could facilitate movement of the AB loop in N-TIMP-2 to optimize interactions with the MMP catalytic domain.

A comparison of the β-barrel in the high resolution solution structure of free N-TIMP-2 with that of TIMP-1 in the crystal structure of the complex (13) reveals that there is no major conformational change in the core of the TIMPs on binding to N-MMP-3, which is reflected in an root mean squared deviation of 1.6 Å for backbone atoms of homologous residues in the β-barrels. This finding is consistent with our NMR data for the complex, as no significant chemical shift changes were detected for nuclei distant from the N-MMP-3 binding site on complex formation, indicating that there is no overall conformational change in N-TIMP-2 on binding to N-MMP-3. The only major differences in the topologies of the two TIMPs are the extension of the AB β-hairpin in N-TIMP-2 (discussed above) and a short C-terminal β₄ helix present in TIMP-1 but not found in N-TIMP-2. It is possible that this helix is absent from TIMP-2, or destabilized by removal of the C-terminal domain, and therefore only present in the full-length protein.

Comparisons of the conformations of exposed loops in N-TIMP-2 and TIMP-1 highlight several interesting differences between the local conformations of the two proteins, particularly in the TIMP/MMP interaction site. Superposition of the two TIMP structures using the coordinates for the backbone atoms of the β-barrel (Fig. 6) places the N-terminal residues of both inhibitors in very similar positions, which would allow the N-terminal amide of N-TIMP-2 to interact with the zinc in the catalytic site of N-MMP-3 in the complex, as observed for TIMP-1 (13). However, the conformation of the CD loop (Pro\(^{67}\)–Cys\(^{72}\)) is very different in bound TIMP-1 and free N-TIMP-2. Thus, with free N-TIMP-2 superimposed on the β-barrel of TIMP-1 in the complex, residues in the CD loop of N-TIMP-2 do not occupy the active site cleft of N-MMP-3 at positions P2 and P3 as seen for TIMP-1, but lie to one side, pointing away from the enzyme. Consequently, if TIMP-2 is to bind N-MMP-3 in a similar manner to that for TIMP-1, then this region (Pro\(^{67}\)–Cys\(^{72}\)) must undergo a substantial conformational change on N-MMP-3 binding, to place the backbone and side chains in the correct orientation to form TIMP-1-like hydrogen bonds and hydrophobic interactions with the enzyme. The largest displacement of the backbone atoms between the free and bound forms of TIMP in this region is over 9 Å. In addition to the CD loop, the AB β-hairpin of N-TIMP-2 must also change its conformation, probably by a reorientation with respect to the rest of the protein, on binding of N-MMP-3, as when N-TIMP-2 is superimposed on TIMP-1 in the complex, the extended AB β-hairpin of free N-TIMP-2 penetrates the catalytic domain making numerous van der Waals clashes (Fig. 6). This extended β-hairpin is probably somewhat less coiled in the complex, thereby changing its orientation with respect to the β-barrel and allowing favorable interactions with N-MMP-3. This change in conformation could occur about the Ser\(^{31}\)Gly\(^{32}\) β-bulge, as suggested earlier. The increased length of the TIMP-2 AB hairpin allows specific interactions by residues at the end of this region (i.e. Ile\(^{65}\) and Tyr\(^{66}\)) with the MMPs that are not possible for the TIMPs with shorter AB loops, for example TIMP-1. It is tempting to speculate that these additional interactions could be important for the specificity differences seen between the TIMPs for the membrane-bound MMPs, where TIMP-2 shows much faster binding than TIMP-1 (8).

Acknowledgments—Most of the spectra were recorded at the Medical Research Council Biomedical NMR Center, National Institute for Medical Research, London. We thank Prof G. Murphy and Dr. A. J. P. Docherty for valuable discussion and advice and Dr. W. Bode for supplying the coordinates of the TIMP-1-N-MMP-3 crystal structure (13).

REFERENCES

1. Woessner, J. F. (1991) FASEB J. 5, 2145–2154
2. Docherty, A. J. P., O’Connell, J., Crabbe, T., Angel, S. & Murphy, G. (1992) Trends Biotechnol. 10, 200–207
3. Docherty, A. J. P., Lyons, E., Smith, B. J., Wright, E. M., Stephens, P. E., Harris, T. J. R., Murphy, G. & Reynolds, J. J. (1985) Nature 318, 66–69
4. Boone, T. C., Johnson, M. J., DeClerck, Y. A. & Langley, K. E. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2600–2604
5. Apte, S. S., Mette, M.-G. & Olsen, B. R. (1994) Genomics 19, 86–90
6. Greene, K., Wang, M., Liu, Y. E., Raymond, I. A., Rosen, C. & Shi Y. E. (1996) J. Biol. Chem. 271, 30375–30380
7. Murphy, G. & Willenbrock, F. (1995) Methods Enzymol. 248, 496–510
8. Will, H., Atkinson, S. J., Bulter, G. S., Smith, B. & Murphy, G. (1996) J. Biol. Chem. 271, 17119–17123
9. Murphy, G., Houbrechts, A., Cockett, M. I., Williamson, R. A., O’Shea, M. & Docherty, A. J. P. (1993) Biochemistry 32, 8097–8102
10. Williamson, A. R., Martorell, G., Carr, M. D., Murphy, G., Docherty, A. J. P., Freedman, R. B. & Feeny, J. (1994) Biochemistry 33, 11745–11759
11. Murzin, A. G. (1993) EMBO J. 12, 861–867
12. Williamson, A. R., Natalia, D., Gee, C. K., Murphy, G., Carr, M. D. & Freedman R. B. (1996) Eur. J. Biochem 241, 476–483
13. Gomis-Ruth, F.-X., Maskos, K.-E., Betz, M., Huber, R., Suzuki, K., Yoshiida, N., Nagase, H., Brew, K., Bourne, G. P., Bartunik, H. & Bode, W. (1997) Nature 389, 77–80
14. Williamson, A. R., Carr, M. D., Frenkel, T. A., Feeny, J. & Freedman, R. B. (1997) Biochemistry 36, 13882–13889
15. Neuhau, D., Nakaseko, Y., Schwabe, J. W. R. & Klug, A. (1992) J. Mol. Biol. 228, 637–651
16. Marcy, A. I., Eiberger, L. L., Harrison, R., Chan, H. K., Hutchison, N. I., Hagemann, W. K., Cameron, P. M., Boulton, D. A. & Hermes, J. D. (1991) Biochemistry 30, 6476–6483
17. States, D. J., Haberkorn, R. A., Ruben, D. J. (1982) Magn. Reson. 48, 286–292
18. Marion, D., Kay, L. E., Sparks, S. W., Torchin, D. A. & Bax, A. (1989) Am. Chem. Soc. 111, 1515–1517
19. Ikura, M., Kay, L. E., Tschudin, R. & Bax, A. (1990) Magn. Reson. 36, 204–209
High Resolution Structure of TIMP-2

31. Bartels, C., Xia, T. H., Billeter, M., Guntert, P. & Wuthrich, K. (1995) *Biomol. NMR* 6, 277–293
32. Guntert, P., Braun, W. & Wuthrich, K. (1991) *Mol. Biol.* 217, 517–530
33. Wuthrich, K. (1986) *NMR of Protein and Nucleic Acids*, John Wiley & Sons, Inc., New York
34. Wuthrich, K., Billeter, M. & Braun, W. (1983) *Mol. Biol.* 169, 949–961
35. Guntert, P., Mumenthaler, C. & Wuthrich, K. (1997) *Mol. Biol.* 273, 283–298
36. Williamson, R. A., Marston, F. A. O., Angal, S., Koklitis, P., Panico, M., Morris, H. R., Carne, A. F., Smith, B. J., Harris, T. J. R. & Freedman, R. B. (1996) *Biochem. J.* 208, 267–274
37. Hyberts, S. G., Goldberg, M. S., Havel, T. F. & Wagner, G. (1992) *Protein Sci.* 1, 736–751
38. Huang, W., Meng, Q., Suzuki, K., Nagase, H. & Brew, K. (1997) *J. Biol. Chem.* 272, 23086–23091
39. Koradi, R., Billeter, M. & Wuthrich, K. (1996) *J. Mol. Graphics* 14, 51–55