Tyrosine 36 Plays a Critical Role in the Interaction of the AB Loop of Tissue Inhibitor of Metalloproteinases-2 with Matrix Metalloproteinase-14*

The tissue inhibitor of metalloproteinases-2 (TIMP-2) is potentially an important inhibitor of all known matrix metalloproteinases (MMPs). However, it has been shown to undergo specific interactions with both MMP-2 (gelatinase A) and MMP-14 (MT1-MMP), and it has been proposed that these three proteins function as a cell surface-based activation cascade for matrix metalloproteinases and as a focus of proteolytic activity. In this study, we have carried out mutagenesis and kinetic analyses to examine the unique interactions between the AB loop of TIMP-2 and MMP-14. The results demonstrate that the major binding contribution of the AB loop is due solely to residue Tyr-36 at the tip of the hairpin. From this work, we propose that TIMP-2 may be engineered to abrogate MMP-14 binding, whereas its binding properties for other MMPs, including MMP-2, are maintained. Mutants of TIMP-2 with more directed specificity may be of use in gene therapeutic approaches to human disease.

The role of the tissue inhibitors of metalloproteinases (TIMPs)1–4 in the regulation of matrix metalloproteinases (MMPs) and hence extracellular matrix protein turnover has now been well documented (reviewed in Refs. 1 and 2). One important focus of research has been the relationship of the structure of TIMPs to their function as MMP inhibitors, studies that have been significantly promoted by the determination of two crystal structures for MMP-TIMP complexes (3, 4) and NMR studies mapping the MMP binding site on N-TIMP-2 (5–7). Domain and site-directed mutagenesis studies on the TIMPs have highlighted both the basic similarities in their binding mechanisms to different MMPs and further unique binding contribution of the AB hairpin is specific to MMP-14 based studies. The work presented here clearly shows that the binding contribution of the AB hairpin is specific to MMP-14 and that the binding contribution is almost entirely due to the side chain of Tyr-36.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Residue substitutions were introduced into the human N-TIMP-2 (Δ128–194 TIMP-2) AB loop region by the overlap extension method (14) using the pET23d-N-TIMP-2 construct as a template (15). The ΔΔ34-140 deletion mutant was similarly generated, but the two halves of the gene were ligated together using T4 ligase before the second round of polymerase chain reaction amplification. Mutated N-TIMP-2 DNA was ligated into pET23d (Novagen), and the sequence was verified by dideoxy chain termination sequencing. The N-TIMP-2 DNA sequence used in this study codes for Thr at position 21. Ala is more commonly found in this position, but the A21T substitution had no effect on the inhibitory activity of the N-TIMP-2 molecule (8) and is therefore referred to as the wild-type protein for the purposes of this study.

**Protein Production**—N-TIMP-2 and site-directed mutants were prepared from inclusion bodies after expression in E. coli BL21 (DE3) pLysS as described previously (12, 15). Full-length TIMP-1 and MMP-14 were prepared from inclusion bodies after expression in E. coli after activation of Pro-MMP-2 (16) and a transmembrane deletion mutant of MMP-14 was expressed and refolded as described by Butler et al. (12).

**Equilibrium Denaturation Studies**—Guanidine hydrochloride dena-

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* This work was supported by the Biotechnology and Biological Sciences Research Council (United Kingdom), the Arthritis Research Campaign (United Kingdom), and the Wellcome Trust (United Kingdom). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

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‡ The abbreviations used are: TIMP, tissue inhibitor of metalloproteinases; N-TIMP-2, N-terminal domain (residues 1–127) of human TIMP-2; GdmCl, guanidine hydrochloride; MMP, matrix metalloproteinase.

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2 M. O’Shea and G. Murphy, unpublished data.
N-TIMP-2 concentration of 10 μM in the indirect dimensions of 9.4 ms (15N) and 18.8 ms (1H) and the three-dimensional total correlation spectroscopy and nuclear Overhauser assignments of wild-type N-TIMP-2 (7, 20). The mixing times for nuclear single quantum coherence experiments with reference to the binding site.

The MMP-14 catalytic domain is shown as a Van der Waals surface (blue), Ile-35 (green), and Asn-38 (orange) shown as solid spheres. The disulfide bonds of N-TIMP-2 are shown in yellow. This figure was produced using MOLMOL (32).

NMR Studies—The 15N-labeled I35G+Y36G mutant was produced as described previously (5). NMR experiments were collected from a 1.2 mM sample prepared in 25 mM sodium phosphate buffer (pH 6.0) containing 100 mM NaCl and 16% D2O on a Varian UnityPlus 600 MHz spectrometer. All data were collected in phase-sensitive mode (19) at a temperature of 35°C. Nearly complete backbone assignments (1H, 15N, 1H) for I35G+Y36G were made from two-dimensional 15N/1H heteronuclear single quantum coherence experiments with reference to the known assignments of wild-type N-TIMP-2 (7, 20). The mixing times for the three-dimensional total correlation spectroscopy and nuclear Overhauser enhancement spectroscopy-heteronuclear single quantum coherence experiments were 50 and 125 ms, respectively, and the spectra were recorded for 70 h with acquisition times in the indirect dimensions of 9.4 ms (15N) and 18.8 ms (1H) and 128 ms in the real-time domain (1H). Water suppression in the NMR experiments was achieved using the pulsed-field gradient-based WATERGATE method (21). The NMR data were processed using NMRPipe (22) and analyzed using the XEASY program (23).

Kinetic Analyses—The concentrations of the stock MMP solutions used in the kinetic studies were determined by titration using standardized preparations of TIMP-1 or TIMP-2 (8). For N-TIMP-2 mutants, titrations were carried out against several enzymes to ensure that an accurate estimate of the protein concentration was obtained. All fluorescence-based kinetic experiments were performed in 50 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl2, and 0.05% Brij 35, pH 7.5, using a PerkinElmer Life Sciences LS-50B spectrofluorimeter with a thermostatically controlled cuvette holder set to either 25°C or 37°C. MMP activity was assessed by measuring the rate of hydrolysis of 1 μM quenched fluorescent substrate Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH2 for MMP-3 or Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 for the other MMPs (24).

The association rate constants (k on) for the formation of TIMP-MMP complexes were determined by following the rate of inhibition of MMP activity by N-TIMP-2 in a continuous fluorometric enzyme activity assay. The association rate constant was estimated from the progress curves using published equations and the Grafit program (25). The apparent Kd (K app) for the inhibition of MMP-2 or MMP-14 by N-TIMP-2 was measured essentially as described previously (12). The MMP was incubated overnight with increasing amounts of N-TIMP-2 at the final assay volume to reach binding equilibrium. 1 μM (final concentration) of the quenched fluorescent substrate was added, and the rate of substrate hydrolysis was measured immediately. The K app was determined from the steady-state rates plotted against TIMP concentration. These data were fitted to the tight binding Kd equation shown below to obtain an estimate of K app:

\[ v_s = \frac{(v_o - 2E_o) [(K_{app} + I - E_o)^2 + 4K_{app}E_o]}{3 - (K_{app} + I - E_o) } \]  

(Eq. 1)

where \( v_s \) is the rate in the absence of inhibitor, \( E_o \) is the total enzyme concentration, and \( I \) is the total inhibitor concentration (26). Where possible, \( K_{app} \) was measured at several enzyme concentrations below the apparent \( K_d \) to ensure that a true value was obtained. The time required to reach binding equilibrium was estimated by incubating the MMPs and N-TIMP-2s together for increasing periods of time to obtain linear (rather than curved) plots of substrate cleavage versus time, which is indicative of equilibrium having been reached. This time

**FIG. 1.** Crystal structure of the TIMP-2-MMP-14 complex (4) showing the positions of the mutated residues in the AB loop binding site. The MMP-14 catalytic domain is shown as a Van der Waals surface (blue), with calcium ions in gray. The N-TIMP-2 molecule is shown as a ribbon diagram, with the side chains of the mutated residues Asp-34 (yellow), Ile-35 (green), Tyr-36 (magenta), and Asn-38 (orange) shown as solid spheres. The disulfide bonds of N-TIMP-2 are shown in yellow. This figure was produced using MOLMOL (32).

**FIG. 2.** GdmCl denaturation curve analysis for mammalian cell-expressed wild-type N-TIMP-2 (●), E. coli-expressed and refolded wild-type N-TIMP-2 (□), ΔD34-I40 mutant (○), and I35G+Y36G mutant (▲). Protein denaturation was followed by a change in \( \lambda_{max} \) of emission with excitation at 280 nm.

**FIG. 3.** Backbone chemical shift change for the I35G+Y36G mutant compared with wild-type N-TIMP-2. Chemical shift values are shown as a weighted average of 15N, 1HN, and 1H resonances (\( \Delta^{15}N/8^{1H} + \Delta^{1H} + \Delta^{1H}/3 \)) to allow an equal contribution from each. Data were not included for residues 35 and 36 due to the change in side chain to Gly, and no resonance assignments could be made for Cys-1, Ser-2, Cys-3, Asp-93, Thr-113, His-120, and Arg-121. Backbone chemical shift values for wild-type N-TIMP-2 can be found in the BioMagResBank (BMRB) NMR data base (accession number 4214).
A method of comparing the structure of mutant proteins with Heteronuclear NMR provides a sensitive and informative fingerprint of the protein that is dependent on the precise environment of the backbone nuclei and is therefore precise enough to provide sufficient 15N-labeled material for heteronuclear NMR analysis. Backbone resonance assignments were obtained for 95% of the protein and compared with those for wild-type N-TIMP-2 (5, 7, 20). Fig. 3 shows the cumulative backbone chemical shift change for the mutant and reveals that the overall structure of I35G+Y36G was essentially unchanged from that of the wild-type, except in the AB β-hairpin region (residues Ser-31 to Lys-41), where large differences in chemical shift were seen. The largest chemical shift changes occurred at the end of the hairpin (i.e. nearest the site of the mutations), with the perturbation becoming less as the two strands approach the stabilizing influence of the β-barrel. These data suggest a local conformational change (unfolding) to the tip of the β-hairpin and confirm that the rest of the protein structure is unaffected by the change at this site.

**Kinetic Analysis of N-TIMP-2 Mutants**—Each of the mutant forms of N-TIMP-2 was assessed for its rate of association (k dissociation) and final equilibrium constant (K dissociation) with MMP-2 and MMP-14 (Table I). We had previously found that mutation of Tyr-36 to Gly or Trp significantly affected binding to MMP-14, with the dissociation rate constant for MMP-14 binding being reduced about 36-fold as compared with 180-fold for the Gly mutation and 120-fold for the Trp mutation. A K dissociation value of about 20-fold greater than wild-type N-TIMP-2 was obtained for the Y36F mutant and MMP-14, but the binding constant for MMP-2 was unchanged. This is comparable to the data obtained previously for Y36G and Y36W, where the K dissociation values for MMP-14 were reduced 100- and 15-fold, respectively, and MMP-2 binding was unchanged. Ile-35 was found to be less important than Tyr-36, with mutation to Gly having no significant effect on the k dissociation or final binding equilibrium (K dissociation) for the interaction of mutants of the AB loop of N-TIMP-2 (5, 7, 20). The K dissociation value for either proteinase, although the K dissociation for MMP-14 increased 13-fold (Table I). When coupled with the Y36G mutation in the double mutant I35G+Y36G, no further change to the Y36G modification of MMP interactions was seen. On the basis of the TIMP-2/MMP-14 structure, Asp-34 and Asn-38 were also postulated to be involved in the interactions made by the AB loop and are comparable to Asp-34 and Asn-38 in TIMP-4 (Fig. 4). Mutation of Asn-38 in TIMP-2 to Asp (i.e. comparable to TIMP-4) resulted in no large-scale changes in association constants for binding to either MMP-14 or MMP-2 but did result in a 2-3-fold increase in K dissociation for MMP-2, and a substantial 50-fold decrease in K dissociation for MMP-14 was seen. Analysis of the binding of mutant D34A+N38A confirmed that these residues are of little importance for the association (k dissociation) or final binding equilibrium (K dissociation) for MMP-2.

The k dissociation value for MMP-14 binding was reduced 180-fold, but the K dissociation value was not changed. Finally, we examined the effect of truncation of the TIMP-2 AB loop by deletion of residues
TIMP-2/MMP-14 Interactions

TIMP-2 (residues V17-F53)

A

V V I R A K A S E K E V D S G

F M K O K T E X I R K I P N Y

B

TIMP-4 (residues L17-F52)

A

L V I R A K I S E K E V S P A D P

F M K O K T E X I R K I P N Y

B

TIMP-3 (residues 117-F47)

A

I V I R A K V K K L V K E G

Y M K O K T X Y L T G F P M

B

TIMP-1 (residues 117-Y46)

A

L V I R A K F P E V N Q T

Y M K M K T E Y R Q Y L T T

B

TIMP-2 (ΔD34-I40 mutant)

A

V V V I R A K A S E K E V D S G

F M K O K T E X I R K I P N Y

B

Fig. 4. β-strand alignments for the AB hairpins of TIMPs 1–4 (in order of size) as determined by the cross-strand hydrogen bonding pattern predicted from the available NMR and x-ray diffraction data and based on the known properties of the OB protein fold. Strong H–H nuclear Overhauser effects seen in the NMR structure analysis of N-TIMP-2 are shown by double-headed arrows and identify residues adjacent to one another in the β-strands. The hydrogen bonding pattern is shown by single-headed arrows from NH to O. Residues with side chains internalized into the hydrophobic core of N-TIMP-2 are boxed. Residues conserved within the sequences for human TIMPs are shown in black (other residues are shown in gray). The glycosylated Asn residue of TIMP-1 is labeled cho. Residues within the AB hairpin mutated in this study are underlined. The predicted strand alignment for the ΔD34-I40 deletion mutant is also shown for comparison.

Having previously identified that the Tyr-36 residue at the tip of the AB loop in TIMP-1 (Fig. 4) apparently underwent specific interactions with surface features of the catalytic domain of MMP-14 (12), we wished to characterize the AB loop binding to MMP-14 in more depth and to compare features of this structure in TIMP-2 with those in TIMP-4. Alignments of the β-strands in the AB hairpins of TIMPs 1–4 (Fig. 4) were made using the available NMR and x-ray diffraction data for TIMP-1 (3, 27) and TIMP-2 (4, 7, 28) and based on the known properties of the OB protein fold (19, 29). In free TIMP-2, the strand alignment for the β-hairpin is very well defined in both the NMR (7) and crystal (28) structures and ends in a type I β-turn with a G1-β bulge (30, 31). In the crystal structure of the TIMP-2-MMP-14 complex (4), the strand alignment is less clear, and there is some evidence of strand realignment in the middle section (Glu-26 to Asp-30) that may occur as part of the large conformation change seen for this region on binding to MMP-14 (28). The structural data for TIMP-1 show no significant difference in strand alignment between the free (27) and MMP-3-bound protein (3), and the predicted hydrogen bonding pattern suggests that this hairpin ends in a 4-residue turn (i.e. 4 residues in the loop positions; Ref. 30). No structural data are currently available for either TIMP-3 or TIMP-4, but their predicted strand alignments and hydrogen bonding pattern suggest that both these hairpins may end in type I β-turns (i.e. 2 residues in the loop positions). The predicted β-strand alignment of the ΔD34-I40 mutant of N-TIMP-2 is also shown in Fig. 4, and the hydrogen bonding pattern suggests that this hairpin will end in a longer 5-residue loop.

NMR-based structural analysis of the I35G+Y36G mutant clearly showed that the glycine substitutions had no effect on the overall folding of the protein, except at the tip of the AB hairpin (Fig. 3). Although this region is highly solvent-exposed and does not interact with the rest of the protein structure (7), it was found to make a significant contribution to the conformational stability of the molecule (Fig. 2). The change in structure at the tip of the AB loop in the I35G+Y36G mutant resulted in a denaturation midpoint shift of 0.39 M GdmCl, whereas the removal of the entire tip region (ΔD34-I40) resulted in a larger shift in stability of 0.68 M GdmCl. The lower stability of the deletion mutant may be due in part to the new non-native turn between strands A and B, which is unlikely to be as energetically stable as the well-ordered turn found in the wild-type protein. The findings from the structural studies on I35G+Y36G and ΔD34-I40 serve to highlight the structural independence of the AB hairpin and suggest that quite large changes to the sequence can be made in this region without perturbing the structure of the rest of the protein molecule.

The kinetic studies of the binding of N-TIMP-2 mutants to MMP-14 (Table I) clearly showed that the side chain of Tyr-36 is the most important feature of the AB loop in terms of both initial association and final binding. Complete deletion of the tip of the AB hairpin (ΔD34-I40) of N-TIMP-2 did not significantly modify these parameters, suggesting that there is no net binding contribution from any other residues in this region apart from Tyr-36. This finding further supports our previous suggestion that although the position of the extended AB hairpin will necessitate its close contact with a proteinase bound at the inhibitory site of TIMP-2 (see Fig. 1), this interaction need not contribute to the overall binding affinity in all cases and could, in some cases, conceivably weaken the overall binding interaction by making unfavorable contacts with the proteinase (6). The crystallographic data for the TIMP-2-MMP-14 complex have shown that the side chain of Tyr-36 is positioned on the surface of the MMP-14 catalytic domain in a cavity formed by the MT loop, the side chains of Asp-212 and Phe-180, and the S loop, which allows the formation of a hydrogen bond between the tyrosine hydroxyl group and the carboxylate oxygen of Asp-212 as well as with a number of Van der Waals contacts (4).

N-TIMP-4 associates with MMP-14 at a 20-fold slower rate than N-TIMP-2,3 which may be due to the lack of a residue

3 V. Knäuper and G. Murphy, unpublished data.
comparable to Tyr-36 in the AB β-turn (see Fig. 4). However, N-TIMP-4 has been shown to have a similar $K_{a}^{app}$ value for MMP-14 binding as N-TIMP-2 (830 pM), suggesting that the lack of the binding contribution from Tyr-36 is compensated for by other interactions elsewhere. The charged and polar residues Asp-34 and Asn-38 were considered as potentially important residues for TIMP-2/MMP-14 association because they occupy the same positions as Asp-34 and Asp-37 in TIMP-4. Mutation of both residues to Ala did markedly reduce the rate of initial binding to MMP-14 ($k_{on}$ was decreased by 180-fold) but had little effect on the final $K_{a}^{app}$ value. Interestingly, the substitution of Asn-38 to Asp in TIMP-2 (making it comparable for binding to MMP-14. The side chain of Asn-38 is involved in making a hydrogen bond to its own backbone (amide O to HN) and in making weaker electrostatic interactions across the hairpin with the side chain of Asp-34 and to the proteinase with the side chain of Asn-208 and the backbone HN of Ile-209. Substitution of Asn-38 with the negatively charged Asp may cause some structural rearrangement at this site, allowing stronger interactions to form between the tip of the AB hairpin and the catalytic domain. It is interesting to speculate that Asp-37 in N-TIMP-4 may help compensate for the lack of a residue equivalent to Tyr-36, allowing the overall binding constant for MMP-14 to be similar to that measured for N-TIMP-2.

The precise biological significance of the unique interactions between the AB loop of TIMP-2 and MMP-14 can only be speculated upon, but they may play an important role in the stabilization of the TIMP-2/MMP-14 complex in which the C-terminal region of TIMP-2 is free to bind to the hemopexin-like domain of MMP-2. This would represent the basis of a cell model and in vivo studies using appropriate mutants of full-length TIMP-2 will be carried out to determine the role of the AB loop in MMP-14-mediated activation of MMP-2 in association with events such as angiogenesis and tumorigenesis.

Acknowledgments—We thank Tom Frenkietel (National Institute for Medical Research, London, United Kingdom) for expert help and advice with NMR data collection and Meng-Huee Lee for stimulating discussions.

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J. Biol. Chem. 2001, 276:32966-32970.
doi: 10.1074/jbc.M101843200 originally published online June 4, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101843200

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