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Solution Structure of Inhibitor-Free Human Metalloelastase (MMP-12) Indicates an Internal Conformational Adjustment

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Abstract

Macrophage metalloelastase or MMP-12 appears to exacerbate atherosclerosis, emphysema, aortic aneurysm, rheumatoid arthritis, and inflammatory bowel disease. An inactivating E219A mutation, validated by crystallography and NMR spectra, prevents autolysis of MMP-12 and allowed us to determine its NMR structure without an inhibitor. The structural ensemble of the catalytic domain without inhibitor is based on 2813 NOEs and has an average RMSD to the mean of 0.25 Å for the backbone and 0.61 Å for all heavy atoms for residues Trp109 - Gly263. Compared to crystal structures of MMP-12, helix B (hB) at the active site is unexpectedly more deeply recessed under the beta-sheet. This opens a pocket between hB and β -strand sIV in the active site cleft. Both hB and an internal cavity are shifted toward β -strands sI and sIII and hA on the back side of the protease. About 25 internal NOE contacts distinguish the inhibitor-free solution structure and indicate hB's greater depth and proximity to the sheet and hA. Line broadening and multiplicity of amide proton NMR peaks from hB are consistent with hB undergoing slow conformational exchange among subtly different environments. Inhibitor binding-induced perturbations of NMR spectra of MMP-1 and -3 map to similar locations across MMP-12 and encompassing its internal conformational adjustments. Evolutionary trace analysis suggests a functionally important network of residues that encompasses most of the locations that adjust in conformation, including 18 residues with NOE contacts unique to inhibitor-free MMP-12. The conformational change, sequence analysis, and inhibitor perturbations of NMR spectra agree in the network they identify between structural scaffold and active site of MMPs.

Introduction

Matrix metalloproteinase-12 (MMP-12) is also known as metalloelastase or macrophage elastase and is secreted by macrophages at sites of inflammation ^{1; 2; 3}. Smoking-induced emphysema in mice requires MMP-12 ⁴, apparently because it releases tumor necrosis factor- α (TNF- α) from macrophages to trigger inflammatory processes ⁵. MMP-12 promotes atherosclerosis in mice and rabbits ², increasing the size of the lesions and destabilizing them ^{3; 6}. Its presence correlates with tissue damage at sites of aortic aneurysms ^{7; 8}, ulcerative colitis ⁹, inflammatory arthritis ¹⁰ and rheumatoid arthritis ¹¹. MMP-12 is an anti-target in cancer therapy, where it should be left active ¹². Nonetheless, specific inhibition of MMP-12 has been proposed as a strategy to stabilize atherosclerotic plaques and forestall heart attack

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and stroke^{6; 13}. MMP-12 also appears to be a target for treatment of emphysema in smokers⁴. Consequently, inhibitors of MMP-12 are under development^{14; 15; 16; 17}.

Tissue invasion by macrophages requires MMP-12¹⁸, which can hydrolyze several components of the extracellular matrix (ECM) and basement membrane^{19; 20}. Macrophages from MMP-12^{-/-} mice retain less than 5% of the elastolytic activity of wild-type macrophages¹⁸. MMP-12 is distinctively high in activity upon elastin^{20; 21}, α 1-antitrypsin (α 1-AT, serpin inhibitor of neutrophil elastase)²⁰, and pro-TNF- α ¹⁹ that are important in inflammatory diseases of arteries, lungs, joints and skin. MMP-12 localizes and binds to proteolytically damaged elastin fibrils at sites of abdominal aortic aneurysm⁷. MMP-12 generated fragments of elastin attract monocytes in emphysema, increasing destruction of the lung²². Insufficient α 1-AT to inhibit the serine protease neutrophil elastase leaves it free to damage inflamed tissue²³. MMP-12 inactivation of α 1-AT²⁰ promotes inflammation²⁴. MMP-9 also regulates neutrophil elastase by inactivating α 1-AT in inflammation of skin²⁵. Neutrophil elastase inactivation of TIMP-1 may reciprocally result in higher MMP activities^{26; 27}.

MMP-12 needs only its catalytic domain for its high activity upon elastin fibrils. This is evident from mature MMP-12 retaining only its catalytic domain after autolytic removal of its C-terminal domain, both *in vivo* and *in vitro*^{18; 21; 28}. By contrast, the gelatinases, MMPs-2 and -9, require their fibronectin-like inserts in order to hydrolyze elastin²⁹. They hydrolyze tropoelastin at different set of sites than MMP-12³⁰. Collagenases and stromelysins are nearest kin to MMP-12, yet fail to digest insoluble elastin. MMP-12's S₁' pocket is a long and open tube extending to a surface distant from the active site cleft³¹. The similarity of their S₁' pockets places MMP-12 with MMP-13 and -8 (collagenases) and MMP-3 (stromelysin-1) in a group^{32; 33}. However, the activity of MMP-1 (collagenase 1) upon α 1-AT appears to be hundreds-of-fold lower than that of MMP-12^{20; 34}. The catalytic domain of MMP-12 shares sequence identity of 58.5% with MMP-1 and 61% with MMP-3. Despite the differences in activities, the backbone conformations of inhibitor complexes of MMP-12 and other MMPs are almost identical except for variability in the III-IV loop, V-B loop, and S₁' specificity loop between helices B and C^{31; 32; 35; 36}. MMP-12 coordinates differ from other MMP catalytic domains by backbone RMSDs of 0.7 to 0.8 Å³¹. Given this high degree of structural similarity, it is challenging to discern what structural nuances, residues, or other properties of the MMP-12 catalytic domain confer its high activity on α 1-AT and uniquely high activity on elastin. Such substrates have modest affinities for MMP-12 (Palmier et al., unpublished). A ligand-free structure of inactivated MMP-12 in solution will be useful for NMR studies of its distinctive substrate interactions.

Inhibitor-free structures of MMPs, in comparison with structures of complexes with inhibitors, can aid rational drug design by suggesting conformational adjustments linked to inhibitor binding. The solution structure of inhibitor-free MMP-1 is available³⁷. Crystal structures without a small molecule inhibitor bound are available for MMP-1³⁸, MMP-3³⁹, MMP-8⁴⁰, and MMP-12⁴⁰. However, the active sites of these have been occupied in the crystals used to obtain each of these four structures. The active site of the MMP-1 structure with PDB code 1CGE is occupied by the N-terminus of another MMP-1 molecule³⁸. The active site of MMP-3 (1CQR) is occupied by the C-terminus of another molecule of MMP-3³⁹. A weak hydroxamate inhibitor present when the MMP-8 and -12 were crystallized was subsequently washed out in order to obtain their inhibitor-free structures (2OY4 and 2OXU, respectively)⁴⁰. These crystal structures of MMP-1, -3, -8, and -12 are compared with crystal structures of four or five inhibitor complexes of each MMP in terms of backbone RMSD in Figure S1 in Supplementary Data. Among MMP-1, -8, and -12, the "inhibitor-free" structures agree within 0.3 Å of the inhibitor complexes through most of the helices and β -strands. MMP-3 structures with and without inhibitor agree to within 0.5 Å through most of the secondary structure. The larger variability in the loops is intrinsic to MMPs, regardless of what is in the active site, and

consistent with dynamic processes in their loops³⁵. Large variability in MMP crystal structures at the C-terminal end of the S₁' specificity loops distal from the active site, e.g. residues 245–250 in MMP-12 numbering or 223–230 in MMP-3 numbering (Figure S1 in Supplementary Data), can be interpreted in light of mobility underlying the loop variability³⁵. Comparison of structures of inhibited and free state MMP-1 and -3 suggests that inhibitors slightly displace residues they contact around the active site in the S₁' specificity loop and the calcium-binding loop 37; 38; 39; 41. Structural changes of MMP-12 residues Pro238 to Lys241 in the S₁' specificity loop (Figure S1 of Supplementary Data) may likewise be attributable to their contacts with inhibitor. NNGH inhibition of MMP-12 has little effect on the crystal structure (Figure S1 of Supplementary Data)³⁵, but induces large chemical shift changes in NMR spectra (Figure 1a). (This compares the NNGH inhibitor complex of the F171D mutant 35 with the free state of the E219A mutant 42.) These perturbations are largest around the active site at Thr239 to Lys241, β -strand IV, and the V-B loop. Smaller perturbations occur across MMP-12 (Figure 1).

The limited availability of MMP structures with a vacant active site is a consequence of autolysis (self proteolysis) intrinsic to several MMPs³⁷. MMP-12's high rate of autolysis 31, at sites such as Leu250 32, is severe within a few hours of concentration, rendering NMR of active MMP-12 infeasible 43. An inactivating mutation in the active site is a way to overcome this. Replacement of the conserved general base Glu by either Gln or Ala in the active sites of MMP-1, -2, and -3 renders them inactive, unchanged in susceptibility to activation by another protease, and unchanged in binding to the TIMP-1 protein that inhibits them 44; 45; 46. The E→Q and E→A substitutions of MMP-7 diminish its k_{cat}/K_m by 600-fold and 1900-fold, respectively⁴⁷. The crystallographic coordinates of inhibitor-bound complexes of MMP-3 with and without the Glu to Gln substitution lack local or global structural consequences of the lesion (RMS difference of 0.7 ± 0.1 Å) and lack effect on the binding mode of the inhibitor⁴⁶. This study recommended mutating the active site Glu of MMPs in cases of autolysis 46. This advice was followed in that the E219A mutation was exploited for the first crystal structure of MMP-12 at 1.1 Å resolution bound to batimastat 31 and for solution NMR of inhibitor-free MMP-12 42. Improved purification and rapid acquisitions with a cryogenic probe helped overcome short sample life times in solution for complete assignments of NMR spectra of MMP-12 42. The crystal structure of MMP-12(E219A)³¹ (PDB code 1JK3) is compared in Figure 2 with five other crystal structures of MMP-12 having wild type sequence in the active site. The backbone RMSD of the MMP-12(E219A) crystal structure (1JK3) from the other crystal structures averages 0.7 ± 0.1 Å and less than 0.3 Å in most of the secondary structure. (The deviations in the exposed loops again are commonplace when comparing MMP structures 31; 35; 36.) The lack of local or global structural impact of the E219A substitution on the crystal structure of MMP-12 is evident in Figure 3a where the E219A-substituted structure 31 (green) is superposed with crystal structures without this mutation 32; 40 (red and yellow).

As a step in addressing the question of sources of MMP-12's unique and pathophysiologically important specificities, we solved the NMR structure of MMP-12 to high-resolution in absence of inhibitor. The solution structure of inhibitor-free MMP-12 reveals an intriguing internal conformational difference from high-resolution crystal structures. Helix B (hB) at the active site is recessed more deeply under the β -sheet, deepening a pocket at the floor of the active site cleft. In this novel position, hB makes more contacts with hA and β -strands sI and sIII distant from the active site (s for strand and h for helix). Structural adjustments near these contacts accommodate the deeper positioning of hB. Consequently, inhibitor-induced chemical shift perturbations occur in residues around MMP-12's internal conformational adjustment and active site, not only in MMP-12 but also in MMP-1 and MMP-3. Bioinformatics ranking of the importance of MMP residues identifies an internal network of side chains from core to active site that is consistent with both the conformational change and chemical shift perturbations.

Results and Discussion

The crystal structure of E219A-substituted MMP-12 with an inhibitor bound ³¹ (1JK3; green in Figure 3a) can scarcely be distinguished from crystal structures having wild-type sequence in hB (red or yellow in Figure 3a). To verify the minimal structural effect of the E219A mutation in solution, we compared the TROSY spectra of wild-type, short-lived MMP-12 and stable MMP-12(E219A). The wild-type sample retains an intact NMR spectrum within the first hour after buffer change and concentration. The stabilizing E219A mutation causes essentially no chemical shift perturbations of most peaks (Figure 1a). The E219A lesion causes modest chemical shift perturbations $\Delta\omega_{\text{HN}}$ of 0.2 to 0.29 ppm for seven residues (Figure 1a). Most of these residues are immediate neighbors of the E219A mutation (His183, Ala184, His222). Others are a little more distant as ligands of the structural zinc above the sheet (Gly169, His183, His196) or in the S₁' specificity loop (Thr239). These modest perturbations are an order of magnitude smaller than the much larger ones from inhibitors (Figure 1a). Such an inhibitor (NNGH) binding results in no recognizable structural adjustment of the backbone (Figure S1 of Supplementary Data). Consequently, it is quite unlikely that the E219A mutation results in conformational change beyond the change of the side chain and its immediate environment.

NMR structure of inhibitor-free MMP-12

The NMR structure of inhibitor-free MMP-12(E219A) is determined primarily by 2445 interresidue NOEs (Table 1), an average of more than 15 per residue. The 20 accepted NMR structures of inhibitor-free MMP-12(E219A) average an RMSD to the mean structure of 0.25 $\pm$ 0.07 Å for backbone atoms and 0.61 $\pm$ 0.05 Å for all heavy atoms (Figure 1b). PROCHECK-NMR assessment of stereochemical qualities ⁴⁸ suggests equivalent resolutions ranging from 1.8 Å for dihedral angles G-factor to 2.7 Å for the less-determined χ^2 side chain torsion angles. See the Table 1 for these and other indices of structural quality. MMP-12 (E219A) without inhibitor has the well-known fold with a sheet of four parallel β -strands, one strand anti-parallel, three helices, methionine turn near the histidines coordinating zinc in the active site, two zinc binding sites, and three calcium binding sites ³¹; ³²; ³⁶. The sequence locations of the strands and helices are listed in Table 1.

A few details of the fold not discussed in the literature are given here. N-terminal residues Pro104 – Pro107 jut out into solution and are disordered. Helix A (hA) begins with an N' $\rightarrow$ N4 capping box⁴⁹ in which Asn126 is the Ncap position, Asp129 is N3, and Met125 packs internally with Val130. hB packs against hA and forms the floor of the active site cleft. hB ends with a Schellman C-capping box 49 with Gly225 in the Ccap position. The C'' $\rightarrow$ C3/G' Gly box 49 is indicated by backbone torsion angles of Ccap, C' and C'' positions 50; packing of Leu226 (C'') with Gly221 (C3); strong $d_{\alpha\text{N}}$ NOE between Gly225 (C') and Leu226 (C''); and $d_{\alpha\text{N}}$ NOE between His222 and Leu226. Pro238 – Lys141 contribute to the rim around the S' subsite, a cavity that tunnels through to a remote surface (Figure 4a). hC has an N' $\rightarrow$ N4 capping box⁴⁹ with Ser251 (Ncap) and Asp254 (N3) protected from solvent exchange due to the H-bonding of the reciprocal contacts of their side chains with their backbone amide groups. The N-capping in hC includes the internal packing of Leu250 with Ile255. Since hydrogen exchange protection continues only to Gly257, hC may fray through the C-terminal end at Gly263.

Comparing backbone NMR chemical shifts of inhibitor-free ⁴² and inhibited MMP-12 ³⁵; ⁴³ reveals sizable chemical shift perturbations around the active site and smaller perturbations remote from the active site. The largest perturbations encompass sIV, the V-B loop, hB, and Met236 – Tyr240 of the B-C loop (S₁' specificity loop) (Figure 1b, gold). Significant spectral perturbations by an inhibitor with $\Delta\omega_{\text{HN}} > 0.3$ ppm also occur at the termini, the zinc- and calcium-binding III-IV loop, the IV-V loop, and sV (Figure 1b, orange). Modest perturbations by inhibitor with $0.1 \text{ ppm} < \Delta\omega_{\text{HN}} < 0.3 \text{ ppm}$ also occur at or near sI, hA, sII, sIII, and hC

(Figure 1b, violet). The inhibitor-induced changes in the NMR spectrum herald a conformational adjustment inside and across MMP-12.

Internal conformational adjustment involving helix B

Comparison of the NMR structure of inhibitor-free MMP-12 with crystal structures of MMP-12 is intriguing. The three crystal structures of MMP-12 used for comparison in Figure 2 have high resolution ($< 1.4 \text{ \AA}$) and vary in mutations and ligand bound. sIV, sV, hC, the calcium-binding I-A loop, the calcium- and zinc-binding III-IV loop, and the V-B loop of this NMR structure superpose with the representative crystal structures (Figure 2, colored symbols) nearly as well as the crystal structures superpose with each other (Figure 2, black symbols). Where the backbone C α RMSD difference of the solution structure from crystal structures (colored symbols in Figure 2) exceeds the variability among crystal structures (black symbols in Figure 2), the structural difference of the inhibitor-free NMR structure is experimentally significant. The differences in the NMR structure in the II–III and IV–V loops and in other loops coincide with the consistently high variability of the loops in solution structures of MMPs³⁵. Clear displacements of well-defined sI, sIII, and hB in the active site are evident in this ligand-free solution structure (Figure 2). hA is displaced more modestly. The changes are independent of mutation or ligand present in the crystal structure used for reference. (Inhibitors were present at time of crystallization of MMP-12 for all X-ray structures to date.) Since the E219A mutation has little structural consequence, effects of ligand binding and / or crystallization are the best hypotheses to explain the conformational adjustment seen in solution without inhibitor.

We examined the conformational adjustments among the well-packed secondary structure elements more closely. sI, hA, sIII, and hB pack closer together in the inhibitor-free solution structure than in the crystal structures (Figure 3a). This closer packing contrasts the tendency of NMR structures to be slightly expanded compared to crystal structures⁵¹. The closer packing is significant beyond the 0.6–0.7 $\text{\AA}$ backbone RMSD between the inhibitor-free NMR ensemble and X-ray structures (1JK3³¹, 1JIZ³², and 1RMZ³⁵), when excluding the displaced segments and less defined loops, i.e. when comparing residues 119–124, 137–149, 160–165, 174–185, 194–208, and 248–262 (Figure 2). We compared distances within the free state in solution (blue in Figure 3a) and within the crystal structures 1JK3³¹ and 1JIZ³² (green and gold in Figure 3a). In the inhibitor-free solution structure, the backbone of surface-exposed Arg110 - Tyr113 averages 1.6 $\text{\AA}$ closer to the C-terminal end of hB at Leu224. The backbone of sI residues Ile114 - Arg117 average 2.1 $\text{\AA}$ closer to the backbone of hB at Ile220. One end of the backbone of sIII from Asp158 through Leu160 averages 1.7 $\text{\AA}$ closer to hB (Ser223 C α) in the free state than in these inhibited crystal structures.

We investigated why the NMR refinement of the inhibitor-free state displaced N-terminus, sI, sIII, hA, hB, and some loop segments from crystal structures of MMP-12. We compared contacts within MMP-12 between crystal structures and solution structure without inhibitor. The vast majority of the NOE contacts without inhibitor agree with the coordinates of MMP-12 crystal structures. However, 25 NOE contacts distinguish the free state in solution from crystal structures. hB makes distinctive contacts with sIII such as Leu212 to Phe163, Ala216 to Val161, and Ala219 to Val161 (Figure 3b). Leu214 contacts Asp198 of sV. Ile220 of hB contacts Tyr116 of sI. Arg117 of sI contacts Asp158 just prior to sIII. Gly257, Tyr262 and Gly263 of hC make distinctive contacts with N-terminal Arg101 and Lys111, respectively. Tyr262 and Gly263 make novel internal contacts respectively with Trp141 and Leu147 of the A-II loop. The unique internal NOE contacts include several from hB (among Leu212 to His218) to hA (among Asp129 to Val140) (Figure 1B). Where hA and hB meet, the side chains of Phe138 and Ile220 draw closer for tight packing as do Ala137 and Val217. The slight adjustments of these and neighboring side chains in the interface accommodates the closer approach of hA and hB in solution without inhibitor. This follows known principles of inter-

helical adjustments now observed here ⁵²: (i) Neighboring helices can undergo rigid-body translations of up to 1.8 Å relative to each other. (ii) This is facilitated by small interfacial side chain adjustments. (iii) Long-range conformational effects can accompany these adjustments. (iv) Ligand-induced changes to active site clefts can be related to inter-helical adjustments ⁵².

The adjustment of contacts within MMP-12 can be considered in light of the general hypothesis of conformational substates in proteins ^{53; 54; 55}, which can couple to functional sites ^{56; 57; 58}. In MMP-12 without inhibitor and either wild type or E219A sequence, the backbone amide NMR peaks of the full length of hB at the active site exhibit a multiplicity of two or more overlapping peaks spread or broadened in the ¹H dimension (see Figure S2 in Supplementary Data). This suggests at least two substates of the environment of hB. These states might interconvert more slowly than the ¹H spectral shift differences of several Hz. In MMP-12 bound to the inhibitor NNGH, splitting of backbone amide NMR peaks is present from residues 217 – 240 ³⁵, including much of hB. In the X-ray structure of MMP-12 bound to NNGH, the carbonyl groups of Glu219 and Ile220 have two conformations and the nearby B-C loop that forms the S₁' specificity pocket has several side chains disordered ³⁵. Clearly, conformational substates generalize from free states of wild type and E219A variants to one inhibitor complex. However, multiplicity or broadening of amide peaks from hB was not observed in MMP-12 bound to Wyeth's proprietary inhibitor (M. Markus, pers. communication) ⁴³. Analogous NMR spectral behavior was reported at the active site of MMP-1. In free MMP-1, eight residues of hB and seven residues of the following B-C loop manifest doubling of their amide NMR peaks that disappears upon inhibitor binding ⁵⁹. The authors attributed this to a slow but unclear conformational change. The conformational change observed in MMP-12 suggests a new hypothesis for the conformational equilibrium. Perhaps helix B slowly samples different depths.

Pockets and cavities in the inhibitor-free solution structure

A consequence of hB's more recessed location under the β-sheet is that the active site cleft is subtly more open and deeper in the inhibitor-free solution structure. The channel across the active site between hB and the wall formed by His228 and Pro238-Thr239 (running vertically over the zinc ion and deep pocket in Figure 4) is slightly wider in the inhibitor-free NMR structure (Figure 4a) than in the crystal structures. The depth between backbone of hB and Met236 – Thr240 is almost 1 Å greater in the solution structure without inhibitor. This NMR structure shows a narrow pocket opening underneath Ala182 and His183 of sIV and reaches toward the backbone of His196 of sV (middle arrow in Figure 4a). The smaller side chain introduced by the E219A mutation probably helps widen the opening to this narrow pocket, the one structural artifact resulting from this mutation. The narrow channel coincides with E219A's modest chemical shift perturbations of His183 and His196 (Figure 1a). The pocket between hB and sIV continues to Gly179 (upward in Figure 4a) where it is more shallow and approaches the S₁' tunnel. This pocket between hB and His183 to Gly179 is minimal in most crystal structures of MMP-12 and absent from the "closed", active state after inhibitor has been washed out ⁴⁰ (Figure 4b; 2OXU). The pocket underneath His183 to Gly179 is however present when the inhibitor batimastat is bound (Figure 4c; 1JK3).

The S₁' specificity pocket (right arrow in Figure 4) in solution without inhibitor is intermediate in size among MMP-12 structures. Dimensions reported by the CASTp server ⁶⁰ and visual inspection suggest the relative sizes of the active site mouth to the S₁' cavity are: crystal structure with NNGH inhibitor bound 35 (1RMZ) > crystal structure with acetohydroxamate bound 35 (1Y93) > inhibitor-free solution structure (2POJ) ≥ crystal structure with a hydroxamate inhibitor (1JIZ) > crystal structure with batimastat bound (1JK3) crystal structure with acetohydroxamate washed out 40 (2OXU).

A cavity lies buried in the core between the β -sheet, hA, and hB in the high-resolution structures of MMP-12 and other MMP catalytic domains (left arrow in each panel of Figure 4). This cavity is larger in the inhibitor-free NMR structure (Figure 4a) and an X-ray structure with a hydroxamate inhibitor (1JIZ, not shown) than in other X-ray structures (Figure 4b,c). In the NMR structure without inhibitor, this buried cavity is shifted with hB further back and away from the active site cleft.

Inhibitor effects on NMR spectra and conformational adjustment

Without inhibitor, the deeper location of hB within the core places it closer to sIII and sV (Figure 3a). The unique NOE contacts hB has with Val161 and Phe163 of sIII and Asp198 of sV (Figure 3b) in absence of inhibitor coincide with inhibitors' perturbations of the NMR chemical shifts of these residues (Figure 1). Likewise, other NOEs unique to the free state to Lys111 and Leu148 just prior to sI and sII and to Ser251 and Gly257 of hC (Figure 3b) correlate with chemical shift changes at these sites when inhibitor binds (Figure 3a). High completeness of the side chain assignments of the complex with Wyeth's proprietary inhibitor⁴³ allows comparison with inhibitor-free MMP-12(E219A)⁴². Since spectral perturbations of the E219A mutation on the backbone are an order of magnitude lower than perturbations by Wyeth inhibitor, the mutation presumably makes similarly smaller perturbations of side chains. That is, the spectral differences can be attributed primarily to the inhibitor. The ¹³C side chain assignments with and without this inhibitor (excluding the mutated Glu) differ by an average of 2.2 ppm. The large differences throughout hB are consistent with its change in depth within MMP-12. This inhibitor's perturbation of side chain ¹³C chemical shifts averages 0.7 ppm for hA and 0.25 ppm for hC, consistent with the progressively more modest conformational adjustment of these two helices (Figures 2 and 3). Among the side chains encapsulating the aforementioned internal cavity that shifts (Figure 4) are Phe138, Ile159, Val161, Ala195, and Phe197. The conformation of these side chains adjusts with the displacement of the internal cavity. Whereas the backbone chemical shifts of these five residues are not significantly perturbed by the inhibitor, their side chains in direct contact with the internal cavity are clearly perturbed. The largest side chain ¹³C chemical shift perturbations by Wyeth's inhibitor are 1.6, 3.5, 1.0, and 1.5 ppm for Phe138 (hA), Ile159 (sIII), Val161 (sIII), and Phe197 (sV), respectively. Since an inhibitor's chemical shift perturbations extend across MMP-12 and qualitatively correlate with the structural differences from crystal structures, this has an important implication. It suggests that binding of inhibitor may at least partly account for the conformational difference of inhibitor-free MMP-12 in solution compared to crystal structures.

Inspection of published NMR assignments of MMP-1 and MMP-3 indicate inhibitor binding-induced chemical shift perturbations across both MMPs at similar sites as in MMP-12. Moreover, the propagation of the perturbations across these MMPs is unequivocally dependent on the inhibitor, as no mutation was involved in those studies. A hydroxamate inhibitor⁶¹ perturbs the backbone chemical shifts of free, active MMP-3⁶² at sites similar to MMP-12. A hydroxamate and sulfonamide-substituted inhibitor perturbs the chemical shifts of free, active MMP-1^{37; 41; 59} at sites similar to MMP-12 and -3. The inhibitor perturbations of chemical shifts of MMP-1 are just as large as for MMP-12 (compare Figure 1a with Figure S3 of Supplementary Data). MMP-1 has more affected residues in the N-terminal 40% than does MMP-12. Like MMP-12, the inhibitors' biggest perturbations of NMR spectra of MMP-1 or MMP-3 occur around the active site in sIV, hB, and the B-C loop or S₁' specificity loop (Figure S3 of Supplementary Data). Like MMP-12, more modest perturbations of NMR peaks of MMP-1 or MMP-3 by inhibitors lie in all of the other β -strands and α -helices, as well as in other loops. This pattern of inhibitor binding-induced chemical shift perturbations shared among MMP-1, MMP-3, and MMP-12 may conceivably be explained by the internal conformational adjustment now evident within MMP-12.

Internal network identified by sequence analysis

Evolutionary trace analysis (ET) predicts a protein family's functional surfaces from the systematic variation of sequence positions that distinguish branches of the phylogenetic tree. These residue positions that can *differ* between subfamilies of a protein superfamily may help tune specificities^{63; 64; 65; 66}. Internal networks of residues of functional communication have also been recognized using either ET^{67; 68} or statistical coupling analysis^{69; 70}. Real-valued ET combines ET with sequence entropy. This hybrid method is an enhanced way to rank to the importance of residues to a protein and its family⁷¹. The residues that real-valued ET ranks as the 10% of most importance are colored green in Figure 5. The residues it ranks in the second and third deciles in importance are colored yellow and red, respectively (Figure 5). These top 30% of residues form a contiguous network encompassing principal residues of the core, active site, and the most important of the divalent cation binding sites (Figure 5b). The most important 10% of the residues tend to occupy central locations at the zinc binding sites and at the confluence of the three helices (Figure 5b). Adjoining these are residues ranked in the second decile in importance (yellow in Figure 5) that are particularly enriched in the S-shaped III-IV loop that binds calcium and zinc ions. The third decile in importance (red) are dispersed to the flanks of the more highly ranked residues. The third decile includes Thr215, Pro238, and Tyr240 ringing the key S₁' specificity pocket. Thr215 distinguishes human MMP-12³¹ and lies at the N-terminal end of hB. In yellow in Figure 5 above Thr215 are Leu181 and Ala182 that are also key components of the primed side of the active site cleft. Other important residues around the active site are not ranked in the top 30%.

The similarity between the most important 30% of residues (Figure 5a) and residues perturbed in chemical shift by inhibitor binding (Figure 1b) is striking. In both groups, similar sites appear in helices A, B, and C, strands II – V, and the long III–IV and B–C (S₁' specificity) loops. This correspondence suggests that the network of most important side chains extending through the core (Figure 5b) transmits subtle structural adjustments through MMP catalytic domains. This network may account for NMR chemical shift perturbations in the MMP catalytic domain far from the active site when inhibitors bind at the active site (Figure 1 and Figure S3 in Supplementary Data). The most important buried residues encompass the internal conformational adjustment. 18 of the residues ranked among the most important 30% are involved in the unique NOE contacts in the interior that distinguish the solution structure of inhibitor-free MMP-12. These residues are Ala137, Phe138, Trp141, Leu147, Val161, Phe163, Asp198, Leu212–Ala216, His218, Glu219Ala, Met236, Gly257, Tyr262, and Gly263. These 18 residues participate in the conformational adjustment. Thus, quantitative sequence analysis, inhibitor-induced chemical shift perturbations, and the internal structural adjustment provide mutually consistent identification of a network connecting the structural core and scaffold with the active site of MMP catalytic domains. This network may identify key structural features indirectly supporting MMP function and interfacial contacts with substrates and inhibitors in the active site.

Conclusions

The E219A mutation of MMP-12 that stabilizes the free state is quite unlikely to account for the overall conformational adjustment of active site helix hB relative to hA and the β -sheet. However, it appears to help open a narrow, localized pocket (Figure 4a, middle arrow). The loops of MMP catalytic domains have inherent structural variability and dynamics³⁵. The NMR structure of MMP-12 without inhibitor presents a new example where key secondary structural elements also have adjusted in relative position. Internal helix B in this solution structure is subtly but measurably deeper than in crystal structures (from crystals formed with inhibitors present). Accompanying the greater depth of hB in the free state in solution are small inward displacements of sI and sIII toward hB, displacement of the internal cavity with hB, adjustments of coordinates and NMR peaks positions of core side chains, and modest deepening

and widening of the active site cleft. The adjustments within MMP-12 are reminiscent of the phenomenon of rigid body movements among helices via subtle adjustments of their side chains in contact^{52; 72}. The inhibitor-dependent chemical shift perturbations of wild-type MMP-1 and MMP-3 (Figure S3 of Supplementary Data) at very similar sites as in MMP-12 raise the question of whether MMP-1 and -3 also undergo similar subtle internal structural adjustments in solution upon inhibitor binding. Quantitative sequence analysis, NMR chemical shift perturbations from inhibitors, and the internal structural adjustment agree in the patterns of linkage that they identify in MMPs between the structural scaffold and active site.

Materials and Methods

MMP-12 Samples

The wild-type MMP-12 and MMP-12(E219A) constructs contain the catalytic domain from Phe100 through Gly263. ¹⁵N and ¹⁵N, ¹³C labelled proteins were expressed from a pGEMEX expression plasmid (Promega) in an *E. coli* BL21(DE3) RIL host using ¹⁵NH₄Cl and ¹³C-glucose-based M9 medium supplemented with Celtone (Spectral Stable Isotopes) at 20% (v/v). The expressed protein is found in inclusion bodies. The protein was extracted in 8 M urea buffer and purified initially through a gel filtration column. The protein was then refolded as described⁷³. It was prepared typically at 0.5 mM in 20 mM imidazole (pH 6.6), 10 mM CaCl₂, and 20 μM ZnCl₂ for NMR at 26 °C using a 600 MHz cryogenic probe⁴¹. Early samples contained 1 mM octyl glucoside for solubility, but had life times of only 5 days. (Octyl glucoside's reputed stability of only around a week may be related.) Enhanced purity of preparations allowed omission of the detergent, boosting sample life times to as much as two months.

NOEs and Structural Refinement

Virtually complete NMR peak assignments of MMP-12 (E219A) without inhibitor are available⁴². Distance restraints were derived from ¹⁵N-separated NOESY and ¹³C-separated NOESY spectra collected on both aliphatic and aromatic regions, each with 100 ms mixing times. Approximately 1200 NOEs were assigned manually in order to launch structure determination. Crystallographic distances of the five metal ions to coordinating ligands³¹ were used as distance restraints to the metal ions during structure calculations. Structures were calculated in torsion angle space using CYANA 2.1^{42; 43} using as initial coordinates the crystal structure 1JK3³¹. The iterative approach of CYANA automatically assigns initially ambiguous NOE-based distance restraints, refining it to a progressively less ambiguous list. This resulted in the final list of 2813 NOE-based restraints, 2445 of which are between residues (Table 1). From the final 100 structures computed, 50 structures with lowest target function were minimized with restraints. The 20 of these with lowest energy were accepted. See Table 1 for statistics on restraint violations and parameters of structural quality.

Hybrid Evolutionary Trace Analysis

The real-valued Evolutionary trace analysis was performed at the online web server⁷⁴: http://mammoth.bcm.tmc.edu/report_maker/. The multiple sequence alignment used 299 sequences averaging 158 residues long. Most of the sequences are from vertebrates. Small percentages of the sequences are from arthropods, plants, viruses, and prokaryotes. The alignment is 166 positions long.

Figures 1b and 3a were plotted with MolMol⁷⁵. Figures 3b, 4, and 5 were plotted with PyMol (DeLano Scientific, <http://www.pymol.org>).

Protein Data Bank accession code

Coordinates and restraints for the ensemble of 20 NMR structures of MMP-12(E219A) without inhibitor are deposited in the RCSB Protein Data Bank under accession code 2POJ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ET	evolutionary trace analysis
hA – hC	helices A – C
sI - sV	β -strands I–V
MMP	matrix metalloproteinase
NOE	nuclear Overhauser effect

(a)

(b)

Figure 1.

(a) NMR chemical shift perturbations of MMP-12 by inhibitors or E219A mutation at the active site. The radial chemical shift changes are defined as: $\Delta\omega_{HN} = [(\Delta\omega_H)^2 + (\Delta\omega_N/5)^2]^{1/2}$ relative to the peak positions in inhibitor-free MMP-12(E219A) 42. Red circles mark differences from MMP-12(F171D) bound to NNGH 35. Blue triangles mark differences from MMP-12 bound to Wyeth's proprietary inhibitor 43. Black squares mark differences of the short-lived, wild-type spectrum from that of E219A-substituted MMP-12. Helices are marked by cylinders and β -strands by arrows. (b) The backbone of the ensemble of 20 accepted structures (PDB code 2POJ) is plotted in stereo. It is colored gold where inhibitor causes $\Delta\omega_{HN} > 1.0$ ppm, orange-red where $1.0 \text{ ppm} > \Delta\omega_{HN} > 0.3$ ppm, and violet where $0.3 \text{ ppm} > \Delta\omega_{HN} > 0.1$ ppm. Calcium ions are colored orange and zinc gray.

Figure 2.

Comparison of coordinates of MMP-12 X-ray and NMR structures having the E219A mutation (1JK3 and 2POJ, respectively) with X-ray structures lacking this inactivating point mutation. Black squares indicate the average C α RMSD of the crystal structure of MMP-12(E219A) with batimastat bound (1JK3) 31 to crystal structures with wild-type (1JIZ 32, 1ROS 17) or F171D mutant sequence (1RMZ 35, 2OXU 40, 2HU6 80). The inhibitor-free solution structure with the E219A mutation (2POJ) is compared to crystal structures of active MMP-12 with F171D mutation (blue triangles, 2OXU), NNGH-inhibited F171D variant (red triangles, 1RMZ), and batimastat-inhibited E219A variant (green squares, 1JK3).

Figure 3.

(a) NMR structure of MMP-12 free of inhibitor (blue; 2POJ) is superposed with crystal structures of MMP-12 with inhibitor washed away (red; 2OXU)40 or a hydroxamate inhibitor bound (either E219A-substituted 1JK3 31 in green or 1JIZ 32 in gold), as shown in stereo. The inhibitor CGS27023A from 1JIZ is drawn with ball and sticks. Lines point out closer approaches of hB with sI, sIII or hA in the solution structure without inhibitor. For clarity, the S-shaped III-IV loop and N-terminal six or seven residues are clipped from view. Zinc and calcium ions in the solution structure are gray and orange, respectively. (b) Dotted blue lines indicate 25 NOEs that distinguish the ligand-free solution structure from X-ray structures using crystallization with inhibitors. Side chain and backbone groups involved in the contacts unique to this NMR structure are plotted with sticks.

Figure 4.

Comparison of MMP-12's active site surfaces and internal pocket in (a) the inhibitor-free solution structure (model 1 of 2POJ), (b) structure from crystals formed with acetohydroxamate inhibitor subsequently washed out to allow activity (2OXU)⁴⁰, and (c) crystal structure with batimistat bound but not shown (1JK3)³¹. A viewing slab 12 Å thick clips off near and far surfaces to reveal internal cavities. Surface-accessible hydrogen atoms are colored light gray, carbon green, nitrogen blue, and oxygen red. A sphere marks the zinc ion in the active site. Its histidine ligands are drawn with sticks. The arrow at right in each panel points to the S₁' specificity pocket. The arrow at left in each panel points to the buried cavity. The middle arrow in (a) points to a pocket unique to the solution structure of inhibitor-free MMP-12(E219A).

Figure 5. Residues were ranked in importance to structure and function of MMP-12 and other MMPs using the real-valued Evolutionary trace method 71; ⁷⁴, and marked on the solution structure of MMP-12. The first, second, and third most important deciles are colored green, yellow, and red, respectively. Panel (a) plots the backbone. Panel (b) also plots the side chain heavy atoms with sticks and dots for the top three deciles of residues. Zinc and calcium ions are gray and orange, respectively.

Table 1

Structural statistics of MMP-12 ensemble without inhibitor.

Average RMSD to the mean for MMP-12 residues 109–263		
Backbone	0.25 +/- 0.07 Å	
Heavy	0.61 +/- 0.05 Å	
Number of experimental restraints		
Intraresidue NOEs	368	
Sequential NOEs	1075	
Medium range NOEs	605	
Long range NOEs	765	
Total NOEs	2813	
Dihedral angle restraints (derived using TALOS 76)	220	
RMSD from idealized covalent geometry		
Bond lengths (Å)	0.001	
Bond Angles (deg)	0.2	
Restraint violations per structure		
NOE violations > 0.5 Å	0	
Dihedral violations > 5 deg	0	
Statistics of Overall Structural Quality		
Close contacts (Procheck)	2 (S189 O – I191 H; A167 Hα– A173 Hα)	
	Mean score	Z-score
Procheck G-factor (phi / psi only)	–0.84	–2.99
Procheck G-factor (all dihedrals)	–1.04	–6.15
Verify3D ⁷⁷	0.46 ± 0.01	0.00
ProsaII (–ve) ⁷⁸	0.58 ± 0.02	–0.29
MolProbity ⁷⁹ clash score	67.62 ± 3.94	–10.08
Ramachandran analysis (residues 109–263, from PROCHECK-NMR ⁴⁸)		
Residues in most favored region [%]	79.5	
Residues in additionally allowed regions [%]	19.4	
Residues in generously allowed regions [%]	1.1	
Residues in disallowed regions [%]	0.0	
Locations of β-strands:	Y113 - I118, K148 - I152, I159 - A164, A182 - A184, A195 - D198	
Locations of helices:	N126 - S142, L212 - L224, S251 - G263	