Enzyme Mechanisms

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Snapshots of the Reaction Mechanism of Matrix Metalloproteinases**

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Matrix metalloproteinases (MMPs) are a family of proteins involved in tissue remodeling and cell signaling.[1–3] As such, they are validated drug targets.[4,5] MMPs are endopeptidases with zinc at the active site of a catalytic domain.[6]

The enzymology of peptidases has been a popular area of research since the investigation of carboxypeptidase A (CPA)[7–11] and thermolysin (TLN)[12–15] in the 1980s. Both proteins are rich in charged and ionizable groups, such as Glu, Arg, and His, in the active site. A role for these residues in catalysis was proposed. In contrast, MMPs are simple proteins with only one Glu residue in the active site besides the three His residues coordinated to the catalytic zinc ion. In the most widely accepted mechanism, which is similar to that proposed for CPA and TLN, this Glu residue assists in the nucleophilic attack at the relevant peptide carbonyl group of the substrate by a zinc-coordinated water molecule.[16] The interest in MMPs has grown again because of the need for inhibitors that are as specific as possible for each of the 23 human proteins.[17–22]

We have now solved a series of X-ray crystal structures under a variety of conditions in an attempt to obtain models of the various steps of the reaction mechanism of MMPs. The protein tends to digest itself, so crystallization is always carried out in the presence of an inhibitor. By experimenting with various crystals under crystal-washing conditions we could solve the structure of the uninhibited form of MMP-12 at a resolution of 1.2 Å (Figure 1a; see also the Supporting Information) and that of MMP-8 at a resolution of 1.7 Å (see the Supporting Information). These are the first X-ray crystal structures of the uninhibited, active form of MMP. In MMP-12, the active site contains three water molecules coordinated to zinc, one of which is hydrogen bonded to Glu219, in an almost regular octahedral geometry. In the case of MMP-8, only the water molecule that is hydrogen bonded to Glu219 has full occupancy, whereas the other two water molecules have occupancies of only about 20%. Despite the lower resolution of the MMP-8 structure, the electron densities of the latter water molecules are clearly appreciable (see the Supporting Information). If these two water molecules are neglected, the resulting geometry is a flattened tetrahedron, with ample room for the binding of an exogenous ligand.

We then attempted to soak the 204–209 peptide fragment of type I/III of the alpha-1 collagen chain, *ProGlnGlyIle-AlaGly*, which is known to be cleaved at the *Gly–Ile* bond, into the active MMP-12 and MMP-8 crystals. No peptide-bound form could be identified, as a result of rapid hydrolysis. Even with the mutation Glu219Ala, which had been reported to lower the enzymatic activity of MMP-7 by three orders of magnitude,[23] the substrate underwent hydrolysis. On the other hand, well-resolved X-ray crystallographic structures that show a hydrolysis product inside the active site could be obtained for both MMP-12 (resolution: 1.1 Å; Figure 1d) and MMP-8 (resolution: 1.5 Å; see the Supporting Information), and for the former even a structure with both hydrolysis products was obtained (resolution: 1.8 Å; Figure 1c). We then modeled the substrate in the form of a tetrahedral *gem*-diol intermediate (in analogy to the structure of a pseudosubstrate described in the literature[24]) by taking the uninhibited form of MMP-12 and that with two peptides in the active site as the starting points (Figure 1b). Figure 1a–d show a plausible series of events in the catalytic cycle of MMPs, as discussed below. A four-snapshot movie of the steps illustrated in Figure 1a–d can be downloaded as Supporting Information for this article.

The cycle starts with the uninhibited MMP-12 enzyme (Figure 1a). Figure 1b shows that one of the two oxygen atoms of the *gem*-diol that results from substrate binding

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

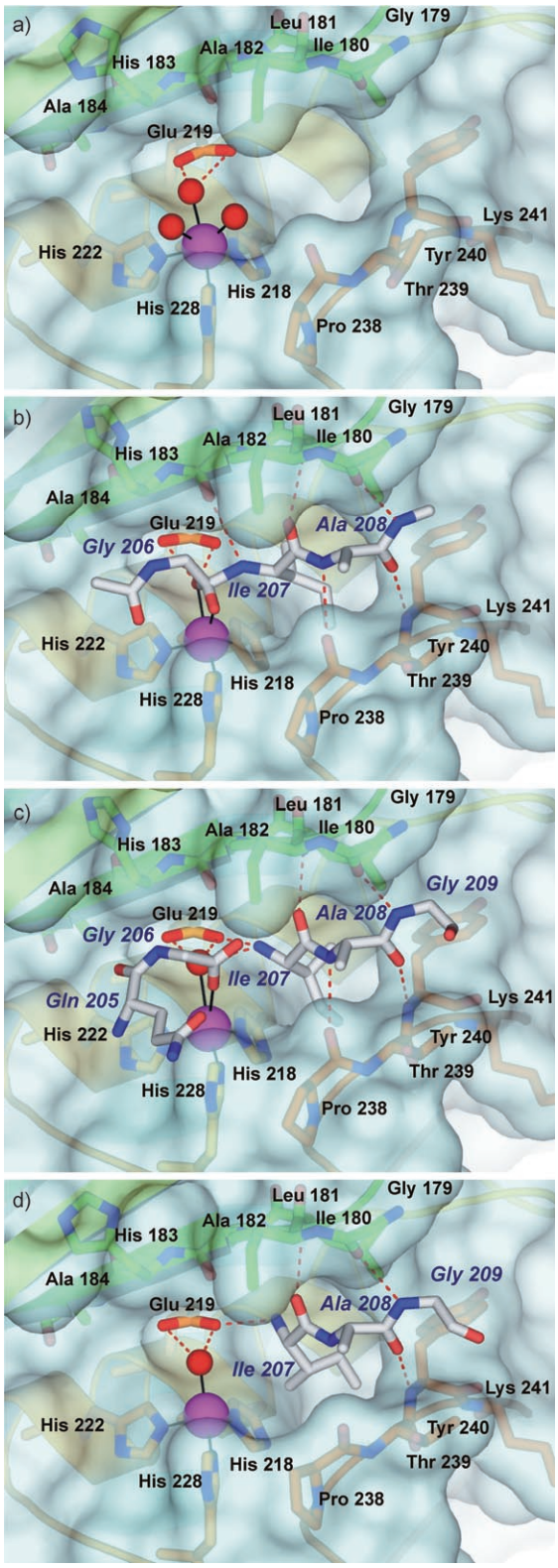

Figure 1. a) Active, uninhibited form of MMP-12. b) A plausible *gem*-diol intermediate[24] of MMP-12 modeled by using the structures of the uninhibited (a) and two-peptide (c) forms as templates. c) Two-peptide intermediate observed upon soaking the active uninhibited MMP-12 crystals with the collagen fragment *ProGlnGlyIleAlaGly*. d) *IleAlaGly* adduct of MMP-12.

indeed sits in the position previously occupied by the catalytically relevant water molecule and is also hydrogen bonded to Glu 219, whereas the other occupies a coordination site between the positions of the other two more external water molecules in Figure 1 a. The peptide NH group of the scissile bond is hydrogen bonded to the carbonyl oxygen atom of Ala 182, and the side chain of *Ile 207* partially enters the S_1' cavity, whereas the rest of the body of the peptide is stabilized by four hydrogen-bonding interactions with the backbone of the protein. The latter interactions involve the carbonyl oxygen atom of *Ile 207* and the nitrogen atom of Leu 181, the nitrogen atom of *Ala 208* and the carbonyl oxygen atom of Pro 238, the carbonyl oxygen atom of *Ala 208* and the nitrogen atom of Tyr 240, and the nitrogen atom of *Gly 209* and the carbonyl oxygen atom of Gly 179. As the modeling did not provide a convincing conformation for the Pro and Gln residues of the peptide, they are omitted from Figure 1 b.

After peptide-bond cleavage, both hydrolysis fragments, *ProGlnGly* and *IleAlaGly*, sit in the active-site cavity, as shown in Figure 1 c. The peptide *ProGlnGly*, for which a clear electron density is observed only for the whole *Gly 206* residue and part of *Gln 205*, is apparently not involved in any significant stabilizing interaction with the protein backbone. The carboxylate end of *Gly 206* binds the zinc ion in an *anti*-monodentate fashion. A water molecule is also semicoordinated to zinc (Zn–O distance: 2.8 Å) and hydrogen bonded to the catalytically relevant Glu 219 residue; it acts as a "spacer" between Glu 219 and the coordinated carboxylate end of *Gly 206*. The zinc ion is thus five-coordinated in this intermediate. The semicoordinated water molecule occupies a position similar to that of the catalytically relevant water molecule observed in the free uninhibited enzyme. The full *IleAlaGly* fragment can be detected clearly. Its orientation in the active site is such that the side chain of *Ile 207* partially enters the S_1' cavity, whereas the rest of the body of the peptide is stabilized by four hydrogen-bonding interactions with the backbone of the protein. The hydrogen-bonding distances are 2.91 Å between the carbonyl oxygen atom of *Ile 207* and the nitrogen atom of Leu 181, 3.16 Å between the nitrogen atom of *Ala 208* and the carbonyl oxygen atom of Pro 238, 2.81 Å between the carbonyl oxygen atom of *Ala 208* and the nitrogen atom of Tyr240, and 2.71 Å between the nitrogen atom of *Gly 209* and the carbonyl oxygen atom of Gly 179. The N-terminal nitrogen atom of the *IleAlaGly* fragment is involved in two strong hydrogen-bonding interactions with the water molecule coordinated to the zinc atom (2.69 Å) and with one oxygen atom of Glu 219 (2.24 Å), whereas there are no hydrogen-bonding interactions of this nitrogen atom with the protein backbone. It is difficult to ascertain whether the N-terminal nitrogen atom is part of a neutral NH_2 group or a positive NH_3^+ group. Overall, the active-site crevice is slightly more open than in the active uninhibited enzyme, in contrast to what was observed for thermolysin and astacin.[25,26]

The *ProGlnGly* fragment, which is coordinated to zinc through the carboxylate group of the C-terminal *Gly 206* residue, is the first to leave the cavity, whereas the *IleAlaGly* fragment remains in the cavity (Figure 1 d; see also the Supporting Information). The zinc ion appears in this case to

be tetracoordinated, with a more strongly bound water molecule (2.28 Å), which is in turn hydrogen-bonded to Glu219. The interaction between this water molecule and Glu219 is still very strong; they are separated by a distance of 2.60 Å. This water molecule occupies roughly the same position as in the free uninhibited enzyme, but the other two coordinated water molecules are not present, so that the zinc ion has a flattened tetrahedral coordination sphere. Given the high resolution of the structure, the existence of other immobilized water molecules in the zinc coordination sphere can be safely ruled out (see the Supporting Information). The bond lengths around the zinc ion (see the Supporting Information) are also consistent with a lower coordination number of the zinc center. The amino terminus of the *IleAlaGly* fragment moves away from the zinc center and loses the strong hydrogen-bonding interaction with the zinc-coordinated water molecule, whereas the interaction with one of the oxygen atoms of Glu219 (2.74 Å) is retained. The side chain of *Ile207* alters its position and increases its interactions with the S_1' cavity. The rest of the body of the peptide is stabilized by three of the four hydrogen-bonding interactions with the backbone of the protein that were observed when two peptides occupied the active site (carbonyl oxygen atom of *Ile207* with the nitrogen atom of Leu181, 2.77 Å; carbonyl oxygen atom of *Ala208* with the nitrogen atom of Tyr240, 2.74 Å; nitrogen atom of *Gly209* with the carbonyl oxygen atom of Gly179, 3.03 Å). However, the interaction between the nitrogen atom of *Ala208* and the carbonyl oxygen atom of Pro238 no longer exists, and this carbonyl bond is itself reoriented by almost 90° away from the center of the active crevice, as observed in several inhibitor adducts with hydrophobic groups in the S_1' cavity.[27] Overall, the active-site crevice is more open with respect to the previous snapshot (Figure 1c). The observation of a similar adduct with MMP-8[*] (see the Supporting Information) and in a self-interacting form of MMP-12[28] confirms the generality of our findings.

Based on the details of the structures shown in Figure 1a–d and in the corresponding movie, a consistent sequence of events emerges:

1) The uninhibited enzyme has a coordinated water molecule whose position is determined by a strong hydrogen bond with Glu219. The position of this water molecule suggests that the Zn-H_2O-Glu219 moiety has lost at least one proton, as has already been proposed for CPA and TLN. During catalysis the water hydrogen atom in the hydrogen bond to the carboxylate group of Glu219 may actually move to Glu219 as a proton; the coordinated water molecule is thus transformed into a hydroxide ion and its nucleophilicity increased.[29] The active-site crevice is in a "closed" form; that is, it is somewhat narrower than when observed in the presence of the hydrolysis products or inhibitors.
2) The incoming substrate binds zinc with the carbonyl group of *Gly206* (replacing the additional labile water molecules when present), and establishes a number of stabilizing interactions with the protein through its C-terminal section (*IleAlaGly*). It is thought that the N-terminal section may not be involved in relevant interactions with the protein on the basis of the bent conformation observed for the Gln side chain in the product (Figure 1c). The strongly coordinated water molecule (hydroxide ion) performs a nucleophilic attack on the *Gly206* carbonyl group to give the *gem*-diol intermediate modeled in Figure 1b.
3) After the peptide bond has been broken, both peptide fragments remain bound to the protein initially. However, the *ProGlnGly* fragment is only held in place by monodentate coordination of the *Gly206* carboxylate group to the zinc center, whereas the *IleAlaGly* fragment is strongly bound to the protein (Figure 1c). An incoming water molecule loosely binds zinc and separates the bound carboxylate group from the Glu219 residue. The formation of this five-coordinate intermediate facilitates the detachment of the *ProGlnGly* fragment through an associative ligand-exchange mechanism.
4) The remaining protein-bound fragment *IleAlaGly* (Figure 1d) undergoes a relatively small but significant rearrangement in the active-site cavity. The driving force for this rearrangement could rest in the possibly strained pose of this fragment in the two-peptide adduct as a result of several interactions that still exist between the two fragments; these interactions are mediated by the zinc-coordinated water molecule and the Glu219 residue. After the release of the *ProGlnGly* fragment, the increased repulsion between the positively charged zinc ion and the incipient NH_3^+ moiety cause the Ile side chain to enter deeper into the S_1' cavity and optimize its hydrophobic interactions with the cavity. The change in the position of the Ile side chain occurs at the expense of the interaction between the nitrogen atom of *Ala208* and the carbonyl oxygen atom of Pro238; this interaction is lost as a result of the opening up of the cavity. The release of the *IleAlaGly* fragment would from this point follow the same pathway as that of any S_1'-directed inhibitor.

This model may also provide some clues on the pH dependence of the catalytic activity of MMPs. The k_{cat}/K_M profile is bell-shaped, as it is for CPA and TLN, with pK_a values of 4.3 and 9.6. In the absence of any other ionizable groups in the active-site cavity, the simplest interpretation would be that the two pK_a values apply to the successive deprotonations of the coordinated water–Glu219 system. However, mutations in which Glu219 is replaced by a nonionizable residue decrease but do not abolish activity, and, more importantly, the bell-shaped activity profile is maintained.[23] For example, the Glu219Ala mutation in MMP-7 reduces the activity to 0.1%, shifts the first pK_a value to 5.4 (an increase of 1.1 pH units), and does not change the second pK_a value appreciably.[23] In this mutant, the only ionizable residue is the coordinated water molecule, which itself can not account for two pK_a values. One possibility is that one of the

[*] In the MMP-8 adduct the orientation of the *IleAlaGly* fragment is more similar to that observed in the two-peptide adduct of MMP-12, and two water molecules are coordinated to zinc; these water molecules occupy similar positions to those of the two *gem*-diol oxygen atoms in the modeled structure in Figure 1b.

two pK_a values is product-linked. Our mechanism might suggest that the high pK_a value of 9.6 corresponds to the deprotonation of the *IleAlaGly* product. A neutral NH_2 amino terminus could stabilize the *IleAlaGly* adduct, thereby preventing the clearance of the active site for the next enzymatic reaction.

Experimental Section

The catalytic domains of MMP-12 (Gly106–Gly263, F171D mutant) and MMP-8 (Asn85–Gly242) were expressed and purified as reported previously.[30] Crystal growing, data collection, and structure refinement are described in the Supporting Information. The following structures have been deposited to the PDB: Uninhibited MMP-12; the adduct of MMP-12 with two peptides; the adduct of MMP-12 with one peptide; uninhibited MMP-8; the adduct of MMP-8 with one peptide. The PDB file of the model of the MMP-12–*gem*-diol adduct is available as Supporting Information.

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