Role of the S_1' Subsite Glutamine 215 in Activity and Specificity of Stromelysin-3 by Site-Directed Mutagenesis†

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ABSTRACT: The influence of Gln215 in stromelysin-3 (MMP-11), a residue located in the S_1' subsite, was determined by producing three single mutants of this position. As compared to wild-type stromelysin-3, the kinetic parameters K_M and k_{cat} for the degradation of the fluorogenic substrate Dns-Pro-Leu-Ala-Leu-Trp-Ala-Arg-NH_2 (Dns-Leu) by these mutants indicated that the Gln/Leu substitution led to a 4-fold decrease in catalytic efficiency, whereas the mutations Gln/Tyr and Gln/Arg increased this parameter by a factor 10. The cleavage of α1-protease inhibitor (α1-PI), a natural substrate of stromelysin-3, by these mutants was also determined. Their relative activities for the degradation of α1-PI correspond to those observed with the synthetic substrate Dns-Leu. The catalytic efficiency of wild-type stromelysin-3 and its mutants to cleave the P_1' analogue of Dns-Leu, containing the unusual amino acid Cys(OMeBn) (Dns-Cys(OMeBn)), was also determined. The values of the specificity factor, calculated as the ratio $(k_{cat}/K_M)^{Dns-Cys(OMeBn)}/(k_{cat}/K_M)^{Dns-Leu}$, were observed to vary from 26 for the wild-type stromelysin-3 to 120 for the Gln/Leu mutant and 25 for the Gln/Arg mutant. The Gln/Tyr mutant did not cleave the substrate when its P_1' position is substituted by the unusual amino acid Cys(OMeBn). Altogether these observations established that both the catalytic activity and the specificity of stromelysin-3 are dependent on the nature of the residue in position 215. Finally, the cleavage efficiency of the Dns substrates by three representative matrixins, namely, MMP-14 (215 = Leu), MMP-1 (215 = Arg), and MMP-7 (215 = Tyr), was determined. Interestingly, the trends observed for these enzymes were similar to those established for the three mutants of stromelysin-3, pointing out the influence of position 215 toward the selectivity in this family of enzymes.

Matrix metalloproteinases (MMPs), also known as matrixins, form a group of structurally related extracellular zinc endoproteinases collectively able to degrade all components of the extracellular matrix (*1*). MMPs are believed to be mediators of both normal and pathological tissue remodeling processes. Their overexpression has been observed and linked to a variety of diseases, including cancer, arthritis, multiple sclerosis, and arteriosclerosis (*2*−*4*).

Among the 15 members belonging to the human MMP multigene family (*5*), stromelysin-3 (MMP-11), which was first described in fibroblastic cells of invasive breast carcinoma (*6*), displays a number of unusual features: mature form of MMP-11 appears unable to degrade any major extracellular matrix component; MMP-11 is secreted under active form (*7*), whereas most of the other secreted MMPs must be activated extracellularly (*8*); finally, while most MMPs possess in their S_1' pocket a conserved leucine in position 215, this residue is replaced by glutamine in MMP-11. These findings suggest that MMP-11 may have a unique role in tissue remodeling processes, including those associated with tumor progression, and may represent a particular target regarding the development of MMP inhibitors. In this respect, it is worth noting that recent studies on the role of MMP-11 in cancer development led to the conclusion that MMP-11 may act as a cancer cell survival factor and could be involved in tumor formation more than in tumor growth (*9*).

As part of our efforts to develop highly specific inhibitors of this particular MMP, we initiated studies dealing with the specificity of this enzyme to cleave different fluorogenic synthetic substrates. Recently, we reported that MMP-11 and MMP-14 prefer to cleave synthetic substrates containing in their P_1' position unusual residues rather than natural ones

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[1] Abbreviations: Dns, dansyl; Cys(OMeBn), S-*para*-methoxybenzylcysteine; α1-PI, α1-protease inhibitor; TIMP, tissue inhibitor of metalloproteases. The sequence numbering used in the text for all MMPs and MMP-11 mutants corresponds to that of MMP-11.

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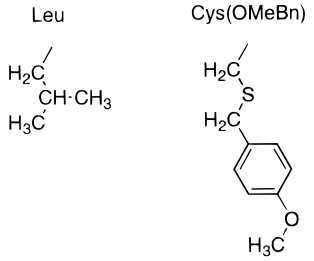
Scheme 1



(*10*). These observations have pointed out the existence in this family of proteases of a very peculiar S_1' subsite able to accommodate very long aryl-alkyl side chains, like that of Cys(OMeBn) (Scheme 1).

The aim of the present study was to evaluate the role played by the glutamine residue in position 215, a residue located in the S_1' subsite of MMP-11, in the activity and specificity of this enzyme. To this end, three mutants of MMP-11 were produced by site directed mutagenesis. The mutations introduced in position 215 correspond to a leucine, the residue mostly observed in MMPs, an arginine and a tyrosine, the residues respectively found in position 215 in MMP-1 and MMP-7. We determined the kinetic parameters K_M and k_{cat} of these three mutants toward two fluorogenic synthetic substrates of general formula Dns-Pro-Leu-Ala-Xaa-Trp-Ala-Arg-NH_2 in which the position Xaa is occupied either by leucine or the unusual residue Cys(OMeBn). This study was completed by determining the catalytic efficiency of MMP-14, MMP-1, and MMP-7 to cleave the two aforementioned fluorogenic substrates.

EXPERIMENTAL SECTION

Enzyme Production. Construction of murine MMP-11 mutants was achieved according to the following procedure: the cDNA containing the full-length coding region of mMMP-11 was cloned into the pBluescript SK+ vector and modified by site-directed mutagenesis. The Gln215 residue preceding the catalytic zinc-binding site was substituted by either Leu215 (primer 5′-ttcatgagccgccactAAcagcaggtcagttccc-3′) or Arg215 (primer 5′-ttcatgagccgccactCTcagcaggtcagttccc-3′) or Tyr215 (primer 5′-ttcatgagccgccacAtAcagcaggtcagttccc-3′). The catalytic domain of these constructs were amplified by polymerase chain reaction (PCR) as previously described. The PCR products were digested with NdeI and XhoI and ligated into the modified pET-3b vector to obtain pET-3b mMMP-11:Q215L, pET-3b mMMP-11:Q215Y, and pET-3b mMMP-11:Q215R, respectively. All recombinant plasmids were sequenced and were found to be 100% identical with the expected sequence. Enzyme production and purification (MMP-11, MT1, and MMP-7) were achieved as previously described (*11*).

Recombinant full-length collagenase-1 was expressed in NSO myeloma cells and purified as described before (*12*). Pro-MMP-1 was treated with trypsin (10 μg/mL) for 25 min at 25 °C, followed by bovine pancreatic trypsin inhibitor (100 μg/mL).

Chemistry. The synthesis of the fluorogenic substrates Dns-Pro-Leu-Ala-Cys(OMeBn)-Trp-Ala-Arg-NH_2 (Dns-Cys-(OMeBn)), Dns-Pro-Leu-Ala-Leu-Trp-Ala-Arg-NH_2 (Dns-Leu), Dns-Pro-Leu-Ala-OH, Leu-Trp-Ala-Arg-NH_2, and Cys(OMeBn)-Trp-Ala-Arg-NH_2 were synthesized as previously described (*10*).

Kinetic Studies. Enzyme assays were performed in 50 mM Tris/HCl buffer, pH = 6.8, 10 mM $CaCl_2$ at 25 °C, in a 10 × 10 × 45 mm quartz fluorescence cell. Substrate solutions were prepared in dimethyl sulfoxide (DMSO) and their concentrations were determined spectrophotometrically by measuring their optical densities ($\epsilon_{340\ nm}$= 4300 $M^{-1}cm^{-1}$). The profile of activity as a function of the pH was determined between pH = 5.5 and 8.5 for MMP-11 and its mutants according to a previously reported study (*13*). No significant shift in the pH optimum of the activity was observed for the mutants as compared to the wild-type enzyme. Enzyme concentrations were determined from optical density using their respective extinction coefficient calculated by the methods of Gill and von Hippel (*14*). These estimations were confirmed by titration of solutions of MMP-11 and its mutants by TIMP2. The two methods gave similar results within the experimental errors.

The quantities of DMSO added in the reaction cell did not exceed 0.5% of the final volume. The solubility of compounds Dns-Cys(OMeBn)and Dns-Leu in the buffer solution was checked spectrophotometrically and exceeded 180 μM, as proved by the linearity of the optical density as function of the concentration.

Progress curves were monitored through Trp fluorescence (λ_{ex} = 280 nm, λ_{em} = 356 nm; slit width 15 nm/15 or 15 nm/20 nm), on a Perkin-Elmer LS50B luminescence spectrometer, for substrate concentrations ranging from 1 to 50 μM. To take into account the "inner filter effect", calibration curves simulating the degradation of the substrates S_i into their corresponding products P_i and P_i' were carried out (P_i =Dns-Pro-Leu-Ala-OH; P_i'=Leu-Trp-Ala-Arg-NH_2, P_i'=Cys-(OMeBn)-Trp-Ala-Arg-NH_2 for the substrates Dns-Leu and Dns-Cys(OMeBn) respectively). Within the range of the initial concentrations S_{i_0} used, we measured the fluorescence of samples containing increasing amount of the two products P_i and P_i' (with $P_i = P_i'$) whereas the concentration S_i was decreased so as to maintain $S_{i_0} = S_i + 0.5 \times (P_i + P_i')$ constant. Thus for a given initial substrate concentration, considering that the enzyme does not contribute to the inner filter effect, the slope of fluorescence increase under steady-state condition can be directly converted into the rate of products appearance. In any case, less than 10% of the substrate was converted into product.

The kinetic parameters K_M and k_{cat} were estimated according to the direct linear plot method (*15*−*17*). This methods allow to determine confidence intervals on the k_{cat} and k_{cat}/K_M (*18*). When the K_M value was greater than 100 μM, the k_{cat}/K_M ratio was determined from first-order full-time reaction curves recorded at $S \ll K_M$ (S = 0.2 or 1 μM), fitted with the integrated Michaelis−Menten equation (*19*).

Evaluation of α*1-PI Cleavage*. α1-PI (3 μg) was incubated at 22 °C with 80 ng of either MMP-11, MMP-11:Q215L, MMP-11:Q215R, or MMP-11:Q215Y in 50 mM Tris/HCl pH = 6.8, 10 mM $CaCl_2$, 200 mM NaCl, in a total volume of 20 μL. The cleavage of α1-PI was quenched after 5, 15, and 25 min by addition of 5 μL of 100 mM 1,10-orthophenantroline. The cleavage products were analyzed by 7.5% SDS-PAGE and visualized by Coomassie Blue staining.

Table 1: Kinetic Parameters for the Hydrolysis of Synthetic Substrates Dns-Pro-Leu-Ala-Xaa-Trp-Ala-Arg-NH_2, (where Xaa is Leu or Cys(OMeBn)) by MMP-11 and its S_1' Mutants[a]

	$P_{1'}$ = Leu					$P_{1'}$ = Cys(OMeBn)					
	E 10^{-9} M	*S*-range 10^{-6}M	K_M (10^{-6}M)	k_{cat} (10^{-3} s^{-1})	k_{cat}/K_M ($M^{-1}.s^{-1}$)	*E* 10^{-9} M	*S*-range 10^{-6}M	K_M (10^{-6}M)	k_{cat} (10^{-3} s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)	*R*
MMP-11	72	1−37	21	12 (9−19)	570 (360−600)	18	0.5−17	5.2	78 (61−92)	15000 (12 00019 500)	26
MMP-11:Q215L	110	7.6−45.4	88	13 (9−21)	150 (130−160)	14	0.3−20.3	4.5	83 (71−95)	18000 (14 600−21 000)	120
MMP-11:Q215R	16	1−13.4	3	16 (14−17)	5300 (4600−6000)	3.7	3.4−13.5	6.4	650 (580−760)	135000 (125 000−156 000)	25
MMP-11:Q215Y	14	1−15.5	5.7	38 (33−45)	7000 (6100−9700)					NC[b]	≈0

[a] The corresponding specificity factor $R = (k_{cat}/K_M)^{Dns-Cys(OMeBn)}/(k_{cat}/K_M)^{Dns-Leu}$ is indicated. The confidence limits ($r = 0.95$) on the k_{cat} and the k_{cat}/K_M values are indicated into brackets. The enzyme concentration (*E*) and the range of substrate (*S*) used to determine the kinetic parameters are indicated. [b] NC = Not Cleaved.

RESULTS

The analysis of the kinetic parameters K_M and k_{cat} for the degradation of the fluorogenic substrate Dns-Pro-Leu-Ala-Leu-Trp-Ala-Arg-NH_2 by wild-type MMP-11 and the three mutants of position 215 (MMP-11:Q215L, MMP-11:Q215R, MMP-11:Q215Y; Table 1) indicates that the Gln/Leu substitution (MMP-11:Q215L) led to a 4-fold decrease in catalytic efficiency (k_{cat}/K_M). This mutation only affected the K_M value and had no consequence on k_{cat}. In the case of MMP-11:Q215R, the replacement of Gln215 by an arginine led to an increase in catalytic efficiency by a factor about 9. This change results from a lower K_M value, whereas k_{cat} is almost unaffected. In the presence of a tyrosine in position 215 of the S_1' subsite (MMP-11:Q215Y), a similar 10 fold increase in catalytic efficiency was observed, corresponding to an optimization of both K_M and k_{cat}.

We evaluated the relative activities of MMP-11 and its S_1' mutants to cleave α1-PI, a natural substrate of MMP-11. This enzyme cleaves α1-PI between Ala350 and Met351 within the reactive-site loop (*20*). The results reported in Figure 1 indicate that both MMP-11:Q215R and MMP-11:Q215Y mutants process more rapidly α1-PI than the wild-type enzyme. A reduced processing of α1-PI was observed for MMP-11:Q215L mutant as compared to the wild-type enzyme.

The results obtained for the cleavage of the substrate containing the unusual residue Cys(OMeBn) in P_1' position (Dns-Cys(OMeBn); Table 1), reveal that, in this case, the Gln/Leu substitution (MMP-11:Q215L) had no significant effect on the kinetic parameters. In contrast, the replacement of Gln215 by a tyrosine (MMP-11:Q215Y) led to an inactive enzyme toward this substrate, whereas the presence of an arginine in position 215 (MMP-11:Q215R) yielded a mutant displaying a 7-fold higher activity than the wild-type enzyme. This higher catalytic efficiency for the cleavage of Dns-Cys-(OMeBn) is due to an increase in k_{cat} by a factor about 10.

The kinetic parameters K_M and k_{cat} for the degradation of the two fluorogenic substrates Dns-Leu and Dns-Cys-(OMeBn), by MMP-11, MMP-14, MMP-1, and MMP-7 are reported in Table 2. The catalytic efficiency (k_{cat}/K_M) of these four MMPs to degrade the Dns-Leu substrate varies from 570 $M^{-1}s^{-1}$ for MMP-11 to 25000 $M^{-1}s^{-1}$ for MMP-14. In each case, the catalytic efficiency is the result of a particular combination of K_M and k_{cat}. These results disclose the ability

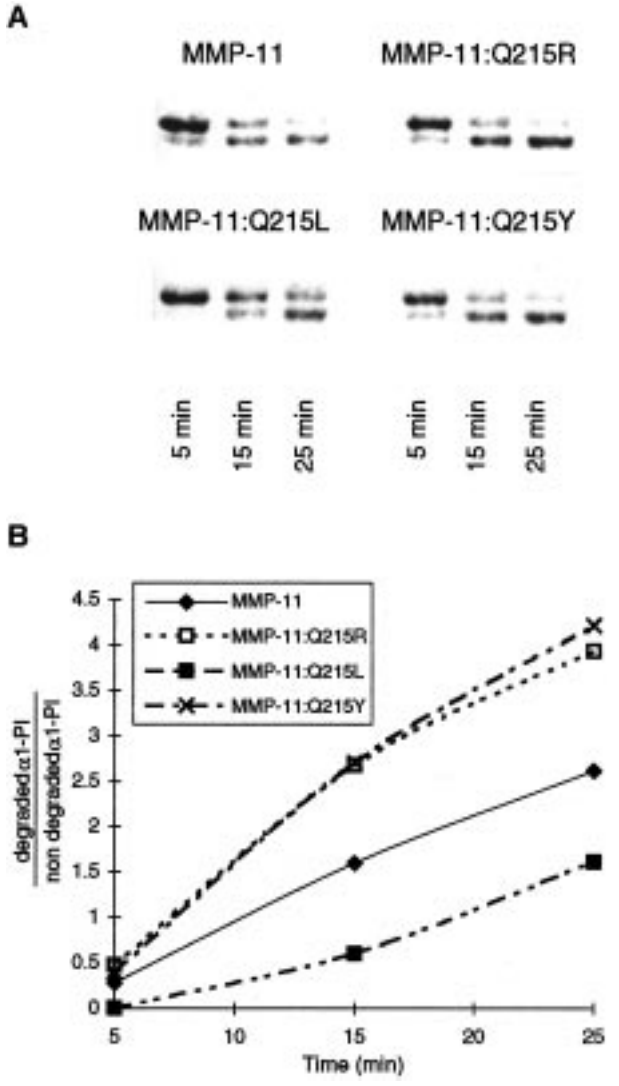


FIGURE 1: Rate of cleavage of α1-PI by MMP-11, MMP-11:Q215L, MMP-11:Q215R, and MMP-11:Q215Y. A: SDS-PAGE analysis. B: Ratio of the intensity of the band corresponding to cleaved α1-PI on that of uncleaved α1-PI as function of the incubation time.

of each enzyme to interact with and process this particular substrate, but the dispersion of the catalytic efficiencies shows that the specific amino acid composition of the active site of the different MMPs controls the cleavage of this substrate. However, the comparison of the results obtained

Table 2: Kinetic Parameters for the Hydrolysis of Synthetic Substrates Dns-Pro-Leu-Ala-Xaa-Trp-Ala-Arg-NH_2, (where Xaa is Leu or Cys(OMeBn)) by MMP-11, MMP-14, MMP-1, and MMP-7[a]

	$P_{1'}$ = Leu					$P_{1'}$ = Cys(OMeBn)					
	E 10^{-9} M	*S*-range 10^{-6}M	K_M (10^{-6}M)	k_{cat} (10^{-3} s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	*E* 10^{-9} M	*S*-range 10^{-6}M	K_M (10^{-6}M)	k_{cat} (10^{-3} s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	*R*
MMP-11	72	1−37	21	12 (9−19)	570 (360−600)	18	0.5−17	5.2	78 (61−92)	15000 (12 000−19 500)	26
MMP-14	7	5−45.8	27	670 (620−730)	25000 (22 600−26 100)	0.2	1.7−15.2	8	5600 (4600−8100)	700000 (583 000−903 000)	28
MMP-1	12	3.4−49.8	145	417 (200−1850)	7300 ± 1600[b]	10	3−25	17	150 (116−209)	8800 (7100−11 200)	1.2
MMP-7	3.7	7.8−45.8	114	3480 (2710−4870)	23 000 ± 2100[b]	18	4−56	20	90 (76−117)	5000 (4000−6900)	0.2

[a] The corresponding specificity factor $R = (k_{cat}/K_M)^{Dns-Cys(OMeBn)}/(k_{cat}/K_M)^{Dns-Leu}$ is indicated. The confidence limits ($r = 0.95$) on the k_{cat} and the k_{cat}/K_M values are indicated into brackets. The enzyme concentration (*E*) and the range of substrate (*S*) used to determine the kinetic parameters are indicated. [b] Determined using first-order full-time reaction curves (±SD).

for the cleavage of the two substrates, differing only by the nature of the residue occupying their P_1' position, makes it possible to probe the S_1' subsite specificity of each MMP in the reported series. The ratio $(k_{cat}/K_M)^{Dns-Cys(OMeBn)}/(k_{cat}/K_M)^{Dns-Leu}$, hereafter called *R* specificity factor, provides a quantitative indication of the ability of the S_1' subsite of the four MMPs to accommodate the long unusual side chain. These *R* factors (Table 2) vary over a factor 100. For MMP-14 and MMP-11, the values of the *R* factor (about 25) indicate that the S_1' subsite of these enzymes accommodates very well a Cys(OMeBn) residue. The S_1' subsite of MMP-1 accepts the Cys(OMeBn) residue as well but, in this case, the presence of this side chain in P_1' position does not lead to an improvement in cleavage efficiency as compared to the Dns-Leu substrate. In the case of MMP-7, the *R* factor of 0.2 reveals a clear preference of the S_1' subsite of this enzyme to interact with leucine than with the unusual Cys-(OMeBn) side chain.

The analysis of the *R* factor calculated for the three MMP-11 mutants reveals that the replacement of the glutamine by a leucine entails a variation of the *R* factor from 26 for wild-type MMP-11 to 120 for MMP-11:Q215L (Table 1). The *R* factor calculated in the case of MMP-11:Q215R shows that the substitution of the glutamine in position 215 for an arginine has only little consequence on the specificity factor ($R = 25$), whereas in MMP-11:Q215Y, due to the absence of cleavage of the Dns-Cys(OMeBn) substrate, the *R* factor is 0.

DISCUSSION

Comparison of the 3D-structure of the catalytic domain of several MMPs under free form or in complexes with active site competitive inhibitors led to a precise definition of their S_1' subsites (*21*−*27*). Among the residues that delineate the S_1' pocket, the residue in position 215 has been proposed to play an important role in the enzyme specificity and possibly enzyme activity. Among the 15 human MMPs known at present, the majority contains a leucine in position 215. In contrast, MMP-7, MMP-1, and MMP-11 possess, respectively, a tyrosine, an arginine, and a glutamine in this position.

The role of Gln215 in MMP-11 activity was investigated through the cleavage of synthetic and natural (α1-PI) substrates of MMP-11. The present results demonstrate that this residue plays a role in the enzyme activity. Previous site-directed mutagenesis studies of MMP-7 and MMP-3 demonstrated the influence of position 215 in the activity of these enzymes (*28*). In MMP-7, the substitution Tyr/Leu was observed to decrease the enzyme activity by a factor 9. In MMP-3, the Leu/Tyr mutation (in a double mutant MMP-3:L215Y/V216A) led to 10-fold increase in enzyme activity toward a synthetic substrate bearing a leucine in P_1' position.

We investigated the role of residue in position 215 in MMP-11 specificity, as the shape of the S_1' pocket is clearly dependent on the side chain in this position and its orientation. By comparison of two substrates, differing only by the residue in P_1' position, our observations establish that Gln215 in MMP-11 also participates in the enzyme selectivity. Thus, the *R* factor increases from 26 for wild-type MMP-11 to 120 when the glutamine is replaced by leucine. In contrast, the Gln/Tyr mutation in position 215 yields an enzyme unable to cleave the substrate containing a Cys(OMeBn) residue in P_1' position.

The S_1' pocket in the enzyme family may have contrasting aspects. MMP-8 and MMP-3, two MMPs possessing a leucine in position 215, display a S_1' subsite that corresponds to a very large pocket, with a tunnel shape that passes all the way through the catalytic domain of the enzyme. In MMP-7 and MMP-1, the presence of either a tyrosine or an arginine in position 215, instead of a leucine, clearly affects the shape of the S_1' subsite, leading to a reduction in size of this cavity. On the basis of these observations, the S_1' pocket of MMP-11 has been proposed to belong to the second category, with a S_1' pocket of reduced size. These arguments might suggest that in this second category of MMPs, the cleavage of synthetic substrates would be impered by the presence of a long side chain in P_1' position. Our previous report (*10*), as well as the present data, do not support this hypothesis since MMP-11 exhibits a preference to cleave synthetic substrates containing a long unusual aryl-alkyl side chain in P_1' position. In the case of MMPs possessing a leucine in position 215, as recently shown in the crystal structure of MMP-14/TIMP-2 complex, the S_1' subsite should accommodate long bulky side chains (*29*). This proposal was supported by the observation that MMP-14 cleaves the substrate with a Cys(OMeBn) in P_1' position much better than the substrate with a leucine. The above remarks suggest that, in MMP-11, the Gln215 side chain may move to open

the S_1' pocket, thereby increasing its size. Such motion would depend on the P_1' side chain of the substrate. This view is supported by recent structural studies of MMP-1 and MMP-7, two MMPs exhibiting a "small size" S_1' pocket. Indeed, 3D-structures of these two MMPs, complexed with inhibitors possessing either a leucine or a long aryl-alkyl side chain (bi-phenyl-propyl or phenyl-propyl side chain) in P_1' position, have revealed the existence of a conformational transition leading to the formation of a large S_1' when the inhibitor contained a long side chain in P_1' position (*30*). In this "open form", the S_1' pocket of MMP-1 and MMP-7 has a size similar to that observed in MMPs harboring a leucine in position 215.

This conformational shift also involves a fragment, hereafter, called the S_1' loop that joins Tyr241 to the third helix of the catalytic domain of MMPs (*30*). Importantly, this S_1' loop appears somewhat flexible as suggested by the B-factors in the available X-ray structures of the catalytic domain of MMPs. The overall consideration suggests that MMP-1 and MMP-7 could adapt their S_1' subsite to accommodate the Cys(OMeBn) side chain. This interpretation is in agreement with the ability of MMP-1 and MMP-7 to cleave synthetic substrates containing very long side chain in P_1' position. However, the specificity factor (*R*) of MMP-1 and MMP-7 is much lower than that of MMP-14. This difference might correspond to the energetic cost associated with the conformational shift occurring in MMP-1 and MMP-7 when they interact with the unusual substrate. In this respect, the higher specificity factor of MMP-11, as compared to MMP-1 and MMP-7, suggests a lower energetic cost for the conformational transition. In agreement with the above interpretation, the specificity factor of MMP-11: Q215L is increased as compared to wild-type MMP-11 and the Gln/Leu substitution in MMP-11 should enlarge the S_1' subsite, thereby suppressing the need for a conformational shift.

The hypothesis of a lower energetic cost associated with a S_1' conformational transition in MMP-11 is also consistent with recent observations showing that the substitution Leu/Cys(OMeBn) in P_1' position of phosphinic inhibitors increases the affinity of the inhibitors for MMP-11 but decreases their potency toward MMP-1 and MMP-7 (*31*). This conformational transition in the S_1' pocket of MMP-11 may depend on the nature of the side chain in position 215. The values of the *R* factor reported in Table 1 reveal that the Gln/Arg mutation provides an enzyme that is still able to process the unusual substrate, whereas MMP-11:Q215Y is unable to cleave this substrate. These results, as compared to the *R* factor reported for MMP-1 and MMP-7, suggest the existence of differences between the S_1' pocket of these enzymes. The S_1' loop of MMPs, as mentioned above, is certainly one of the important components that controls the S_1' selectivity in these enzymes. It should be noted that the length and the sequence of this loop display important variations in the MMP family. The resolution of the 3-D structure of the catalytic domain of MMP-11 would give very important insights regarding the S_1' subsite and the S_1' loop in this MMP and validate our suggestions. This knowledge would certainly help in developing either highly selective synthetic substrates or inhibitors of this particular MMP, a goal not yet fully achieved.

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