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Expression and high yield production of the catalytic domain of matrix metalloproteinase 12 and of an active mutant with increased solubility☆

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Dedicated to Professor Renato Ugo on the occasion of his 65th birthday

Abstract

In the general frame of a project aiming at screening candidate drugs against several different matrix metalloproteinases (MMP) to find a rationale for selectivity, the catalytic domain of MMP-12 (metalloelastase) has been expressed in *E. coli* strain BL21D3 and its production optimized to about 30 mg/dm^3. The chosen construct spans residues 106–267 of the whole MMP-12 and contains two additional methionines at positions 104–105. This is at variance with the previously published constructs which span residues 99–279 [J. Mol. Biol. 312 (2001) 743] and 100–262 [J. Mol. Biol. 312 (2001) 731], respectively. The protein, expressed in inclusion bodies, is solubilized in high urea concentration and properly refolded, as judged from ^{1}H to ^{15}N HSQC, by stepwise urea dilution in the presence of the mild inhibitor acetohydroxamic acid. The latter can be easily dialysed out when needed for activity or inhibition studies. The solubility of this catalytic domain construct of MMP-12 is around 250–300 μM. To increase solubility, a mutant (F171D) has been designed that should not alter the activity and should not interfere with the contacts between the catalytic domain and either the pro-domain or the C-terminal domains that precede and follow it in the full-length protein. The F171D mutant was produced and indeed resulted fully active and three times more soluble than the WT, greatly facilitating its use in NMR screening experiments. Comparison of X-ray data for the present [Angew. Chem. Int. Ed. 42 (2003) 2673] and previous [J. Mol. Biol. 312 (2001) 731; J. Mol. Biol. 312 (2001) 743] constructs of the catalytic domain of MMP-12 suggested that elimination of Met 105 and of the stretch 264–267 should lead to an even more soluble and stable construct. The latter was produced and showed to have the desired properties.

Keywords: Metalloelastase; Mutant; NMR; Drug adducts

1. Introduction

The endopeptidases matrix metalloproteinases (MMP) are currently receiving much attention as potential drug targets, because of their involvement in the degradation of the extracellular matrix and, more generally, in the turnover and remodeling of

☆ This is a tribute to Renato Ugo, a pioneer in the whole spectrum of chemical sciences, an outstanding manager, and a friend of the Florentine School of Inorganic Chemistry.

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connective tissues [1,2]. Some MMPs are membrane proteins that stick out of the cell walls, several others are soluble proteins excreted by cells. There are 23 proteins [3–5] recognized as MMPs in the human genome, six membrane proteins and 16 soluble proteins including gelatinases, collagenases and stromelysins, and many of them are implicated in diseases such as rheumatoides arthritis, carcinomas, and metastasis of melanomas and menigiomas. In all these diseases MMPs are found to be hyperactive, either because overexpressed or upregulated [6–9] and this is the reason why selective inhibitors of one or another MMP involved in a particular disease are actively sought for. Many molecules have been shown to have high affinity for MMPs (dissociation constants in the nanomolar region) [10–13] but, so far, none has shown the necessary selectivity for any MMP to survive until the end of the drug development pipeline.

Drug discovery strategies, either based on irrational or rational drug design, benefit from the knowledge of the high resolution three-dimensional structure of the target protein, as the interaction with any potential drug needs structural validation to accompany all phases of development before it can be considered a lead of industrial interest. In the early development phases NMR may play an important role, both as a high-throughput screening tool and as a tool to rapidly assess the structural features of the adduct between the target and the candidate drug. In the case of MMPs, where the challenge is now more on selectivity than on potency, a rational drug design approach seems more promising. This requires a detailed structural knowledge not only of the MMP selected as target but ideally also of the other MMPs, in such a way as to test, first in silico and then in vitro, the synthetic strategies aimed at increasing selectivity. Structural characterization of the target as well as of its potential drug adducts in solution through NMR, however, requires relatively high concentrated samples. For this, good solubility of the targets is needed.

MMPs are generally constituted by four domains with well-defined function [9]: a pre-domain (or signal sequence) of about 18–20 aa that leads the protein in the right cell compartment; a pro-domain of about 80 aa with a inhibitory function which is then cleaved to yield the active protein; a catalytic domain of about 150 aa containing a catalytic zinc ion, a structural zinc ion and 1–3 structural calcium ions; and a C-terminal domain of about 200 aa possibly implicated in substrate recognition. The catalytic domain has obviously focused the attention of researchers. The 3D structures of the catalytic domains of MMP-1 [14], MMP-2, MMP-3 [15], MMP-7 [16], MMP-8 [17], MMP-9 [18], MMP-12 [19,20], MMP-13 [14] and MMP-14 [21] are now available, as well as the structures of the whole MMP-1 [22] and MMP-2 [23]. The catalytic domains of some MMPs such as MMP-1, MMP-3 and MMP-13 are soluble in the millimolar range, and NMR studies are also available for them [2,24,25].

In a project aimed at using NMR to develop possible strategies to increase selectivity by screening the same candidate drugs against several MMPs, we have cloned and expressed-or initiated the expression of the catalytic domain of MMPs 1, 3, 7, 10, 12, 13, 15 and 28. We describe here the development of a high yield expression system for MMP-12, and the design and high yield expression of two different mutants: a single point mutant which maintains the activity and enhances the solubility of MMP-12 by more than a factor 3 from 0.3 to 1 mM, and a double mutant which, in addition to the F171D substitution, presents a deletion of four amino acids at the C-terminal region. This double mutant increases the protein solubility up to 2 mM.

Because of their high solubility these twoMMP-12 mutants are much more amenable than the wild-type protein to high resolution NMR studies.

2. Experimental

The cDNA encoding the sequence Gly 106–Asn 267, encompassing the catalytic domain of human MMP-12 was generated by a polymerase chain reaction (PCR) from an IMAGE consortium clone (ID 196612) using two synthetic oligonucleotides as primers. The cDNA obtained was cloned into the pET21 vector (Novagen) using *Nde*I and *Bam*HI as restriction enzymes. Two extra methionines at positions 104–105 were present in the final expression product.

The first step of protein purification was performed with a size exclusion chromatography (Pharmacia, HiLoad Superdex 75 16/60) in 6 M urea and 50 mM sodium acetate at pH 5.0. Afterwards the protein was loaded with the same buffer on the cation exchange column Mono-S (Pharmacia). The elution was performed using a linear gradient of NaCl up to 0.5 M.

The protein was refolded by using a multi-step dialysis against solutions containing 50 mM Tris pH 7.2; 10 mM $CaCl_2$; 0.1 mM $ZnCl_2$; 0.3 M NaCl; 2 mM NaN_3. During these steps the concentration of urea was gradually decreased from 4 to 2 M and then completely removed from the buffer.

2.1. Sample preparation and NMR spectroscopy

The refolded protein was exchanged, by dialysis, with a buffer with 10 mM Tris pH 7.2; 10 mM $CaCl_2$; 0.1 mM $ZnCl_2$; 0.3 M NaCl; 2 mM NaN_3; 200 mM acetohydroxamic acid and then was concentrated using an Amicon-stirred cell, fitted with a YM10 membrane in nitrogen atmosphere at 4 °C. To the final solution 10% of D_2O was added.

CD spectra in the UV region were obtained with a Jasco 500 spectropolarimeter at 22 °C.

NMR spectra were recorded at 298 K on a DRX Bruker 500 MHz spectrometer equipped with a TXI cryo-probe. Amide NH resonances were detected through ^1H–^{15}N HSQC experiments [26,27] implemented with the sensitivity enhancement scheme [28,29].

3. Results and discussion

3.1. Expression and purification of MMP-12

The human MMP-12 active catalytic domain (163 aa) was chosen, corresponding to the stretch Gly 106–Asn 267 and containing two additional methionines at 104–105. After several expression trials, the vector pET 21, one of a family of vectors that contains the strong phage T7 promoter, turned out to behave optimally. The *E. coli* strain BL21D3 transfected with the above vector was grown in rich medium at 37 °C and the protein expression was induced during the exponential growth phase with 0.5 mM of IPTG. Cells were harvested 5 h after induction.

As for all recombinant MMPs reported so far [14,30–32], MMP-12 is expressed as inclusion bodies. After cell lysis, inclusion bodies were collected and solubilized in 8 M urea. There are two possible strategies for protein purification at this stage. One involves purification under denaturing conditions and then refolding, while the other involves refolding first. In the latter case, however, the protein is exposed for a longer time to possible self-hydrolysis, and therefore purification under denaturing conditions was chosen. Size exclusion chromatography was used first to remove lipids, DNA and membrane fragments possibly present in the inclusion bodies. The eluate was then brought to a 6 M urea concentration at pH 5.0, passed on a cationic Mono-S column and eluted with a 0–0.5 M NaCl gradient. After optimization of this procedure the yield of purified protein was approximately 30 mg/dm^3.

The optimized refolding procedure consisted of a multi-step dilution of the denaturant, keeping the protein concentration below 150 μg/cm^3 to minimize intermolecular interactions which could cause misfolding. Zinc and calcium ions were added to the solution in concentrations of 0.1 and 10 mM, respectively. Precipitation and unfolding occurred when the refolding was carried out in the presence of only 0.5 mM of calcium ion. Refolding started below 2 M urea, as judged from circular dichroism. Starting from this point 200 mM acetohydroxamic acid, a mild inhibitor of MMPs with dissociation constant around 20 mM, was added to minimize the risk of auto-digestion of the refolded protein. This procedure recovers about 70% of expressed active protein (see below).

Protein expression was also optimized in minimal medium containing 1.25 g/l of $(^{15}NH_4)_2SO_4$ in order to produce ^{15}N-enriched protein for routinely use ^1H–^{15}N HSQC NMR spectra as a diagnostic tool. The yield of protein grown in minimal medium turned out to be above 60% of that obtained in rich medium. Typically, about 22 mg of enriched protein could be obtained from a 1 dm^3 culture.

The progress of the optimization in the production of MMP-12 can be followed by comparing the three ^1H–^{15}N HSQC spectra shown in Fig. 1a and b. Fig. 1a shows a typical ^1H–^{15}N HSQC spectrum of the initial WT protein samples obtained without the addition of acetohydroxamic acid during the refolding procedure, while Fig. 1b shows the ^1H–^{15}N HSQC spectrum obtained on WT protein sample containing 200 mM of acetohydroxamic acid. Although the overall appearance of the spectra is similar, in the first sample a small number of signal are present and with a smaller signal spreading. This is not only related to the lower concentration of the WT protein sample but indicates the presence of a fraction of either non properly

folded protein or of misfolded fragments coming from auto-digestion. Mass spectrometry (not shown) confirmed the presence of protein fragments in the first preparations. So the presence of the inhibitor seems to be important either to prevent the auto-digestion or to favor the folding.

3.2. Design and production of an active MMP-12 mutant of improved solubility

The catalytic domain of MMP-12 obtained as described above could be concentrated safely up to around 250–300 μM, above which partial protein precipitation occurred. This behavior could be in part due to the lack of either the pro-domain or the C-terminal domain or both. Indeed, both domains show meaningful contacts with the catalytic domain and their absence could expose to the solvent relatively hydrophobic regions of the catalytic domain, as judged from the structure of the full MMP-1. One obvious route to increase solubility would be to mutate one or more surface residues in the hydrophobic contact regions. The long-term goal of this project is that of testing the catalytic domains of many if not all MMPs against candidate drugs to look for strategies to increase selectivity. Therefore, it was decided to look for surface regions that were sufficiently far from both the catalytic site and the interdomain contact regions to ensure minimal interference with all plausible drug targets. A careful analysis of the surface properties

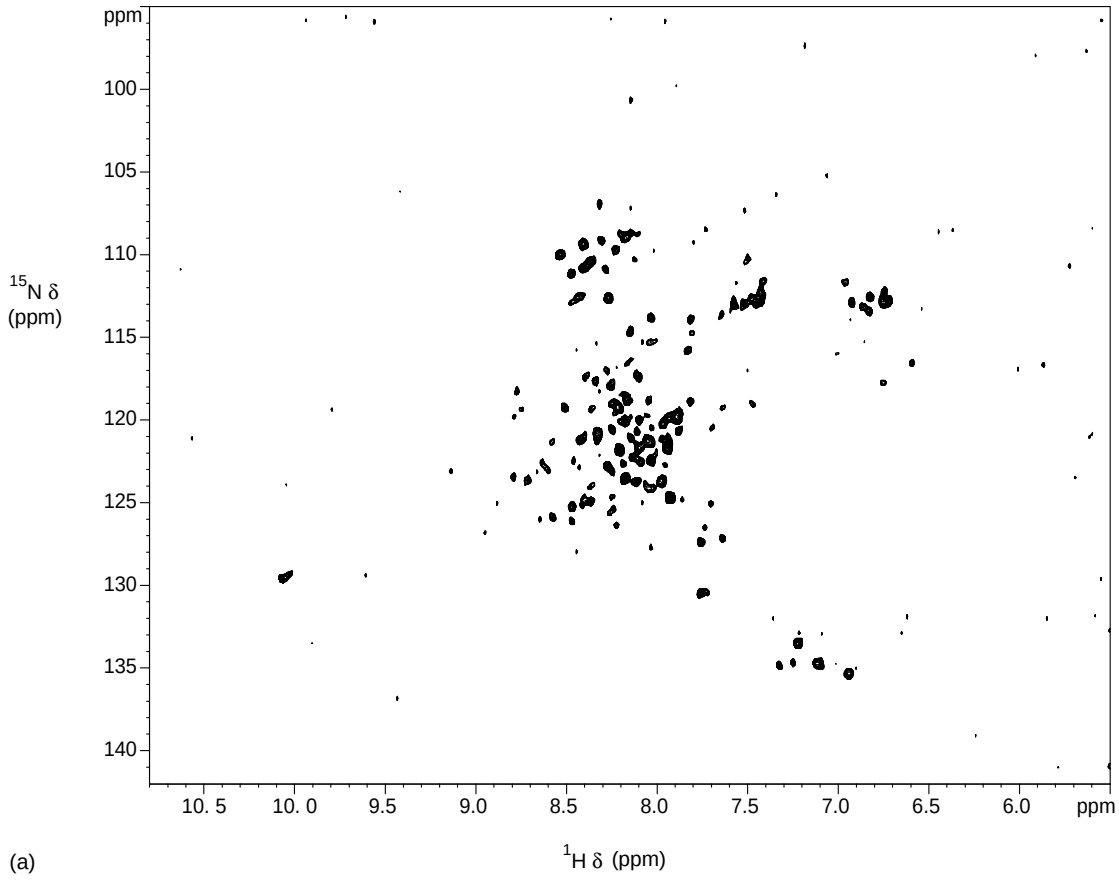

Fig. 1. (a) 2D ^{1}H–^{15}N HSQC spectrum of 0.2 mM ^{15}N-enriched WT MMP-12 without acetohydroxamic acid at pH 7.2, 25 °C. (b) 2D ^{1}H–^{15}N HSQC spectrum of 0.3 mM ^{15}N-enriched WT MMP-12 with 200 mM of acetohydroxamic acid added to the NMR sample at pH 7.2, 25 °C. (c) 2D ^{1}H–^{15}N HSQC spectrum of 0.5 mM ^{15}N-enriched F171D MMP-12 (106–267 construct) refolded with 200 mM of acetohydroxamic acid at pH 7.2, 25 °C.

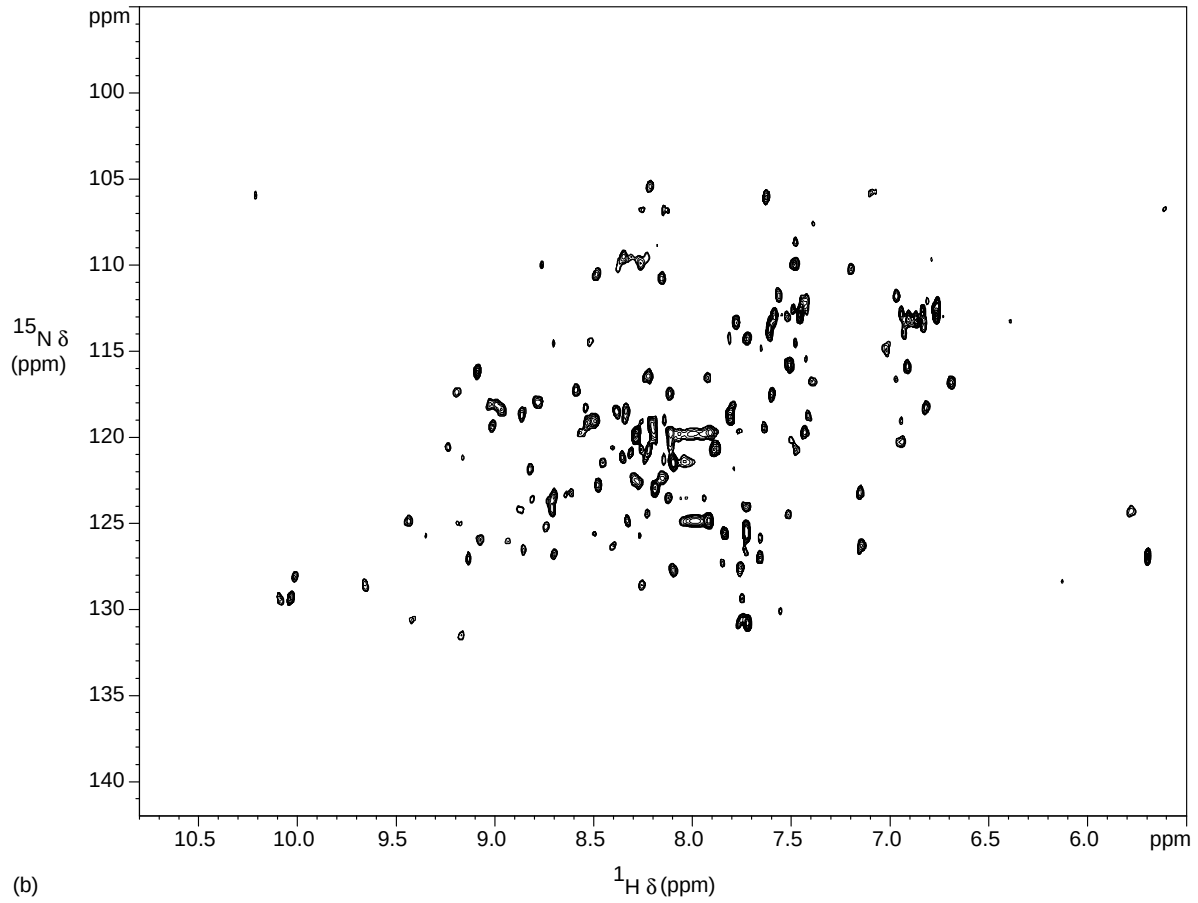

Fig. 1. (*Continued*).

of the catalytic domain of MMP-12 as it appears from the X-ray structure after excluding the active site and interdomain contact regions suggested the presence of hydrophobic residues as a possible place to perform mutagenesis. Among the above residues, Phe 171 was the single residue contributing most to the hydrophobicity of the area, and the possibility of mutating it with a hydrophilic residue was considered. The selection was performed with the program PROSA [33,34], by mutating Phe 171 with several different hydrophilic residues and selecting the one that gave the lowest energy derivative. The Asp 171 mutant resulted to have even lower energy than the WT protein, and was therefore selected.

The F171D mutant was produced using the quick-change site-directed mutagenesis kit (Quiagen), and the expression and purification of the protein and of its ^{15}N-enriched version completed using the same procedure described above. The final yield was as good as the best yield obtained during optimization of the WT production. The activity of the mutant was assayed by fluorimetry using the fluorogenic peptide substrate Mca-PLGLDpa-AR-NH_2 [35]. k_{cat}/K_m values of $2 \times 10^5\,M^{-1}\,s^{-1}$ at 37 °C were obtained, which compare well with the literature value for the WT of $6 \times 10^4\,M^{-1}\,s^{-1}$ at 25 °C [35]. The activity of the mutant was also compared directly with that of the WT by a parallel assay consisting of incubating equal amounts of the two proteins, in the absence of the hydroxamic inhibitor, with the α-1 proteinase inhibitor (α_1-PI) [36,37], and then monitoring the intensities of the bands relative to the peptide fragments

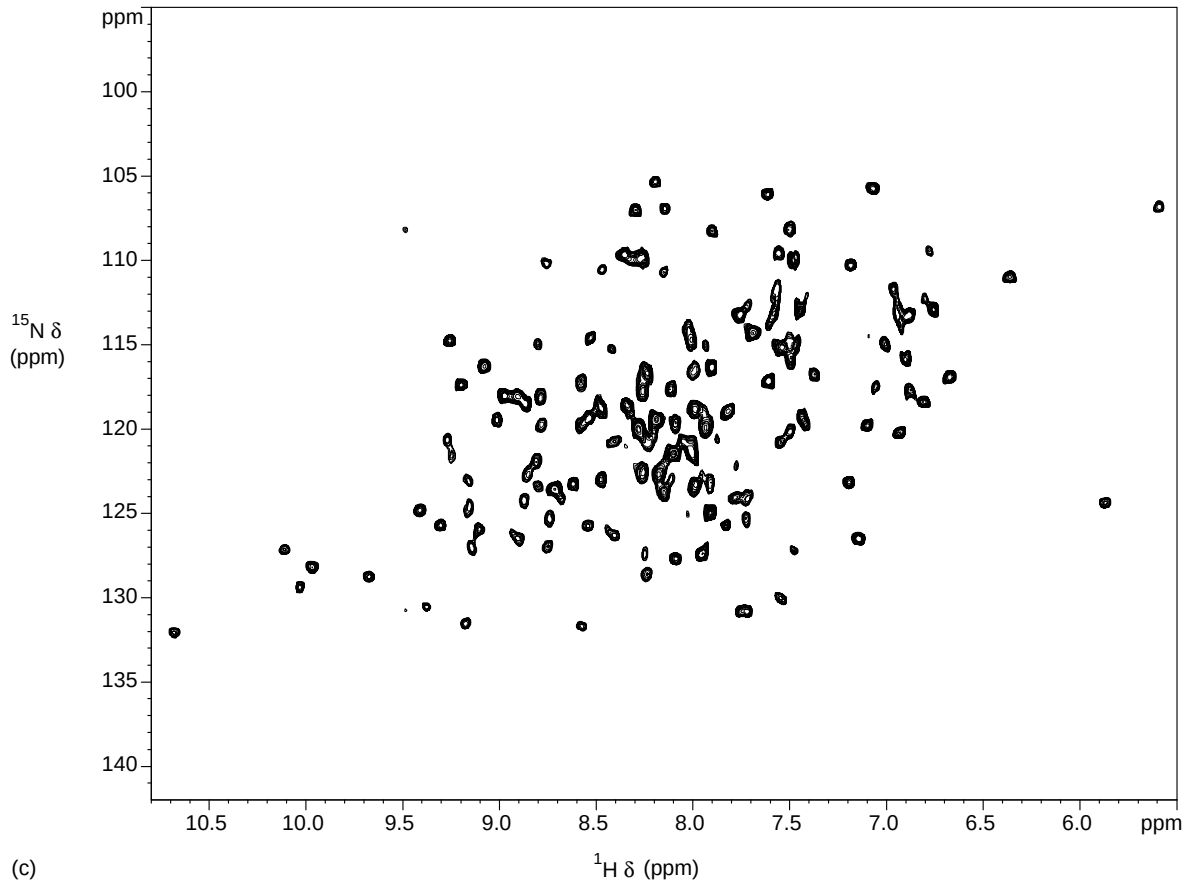

Fig. 1. (*Continued*).

in a SDS PAGE. The activity of the mutant resulted to be equal to that of the WT within the sensitivity of the method. The F171D mutant was easily concentrated up to 1 mM, and the solution did not show the formation of protein precipitate on standing at room temperature for 15 days.

We recently succeeded in obtaining the X-ray structure of the present construct of the catalytic domain of MMP-12 [38]. The construct contained the expected complement of two Zn^{2+} and three Ca^{2+} ions. Surprisingly, the molecules are arranged in chains where the N-terminal residue of one molecule are hosted into the catalytic domain of another molecule, mimicking a possible enzyme-product complex. This is probably due to the presence of the extra residues Met 104–Met 105. While this finding may provide new insight into the catalytic action [38], it also shows that Met 104 may have a determinant role in decreasing the solubility of the construct. Furthermore, recent X-ray structures of longer constructs show that residues 100–105 [19] and 264–279 [20] may be disordered in solution and this may complicate further analysis. To further optimize the catalytic domain for activity and NMR studies we thus planned a shorter construct, lacking both Met 104 and residues 264–267. A SMART [39,40] analysis suggests that this construct should still be representative of the full catalytic domain. Therefore the original 104–267 construct was further engineered into a Met-106-263 construct, still containing the F171D mutation, and expressed in the same vector. A schematic representation of the latter construct is shown in Fig. 2.

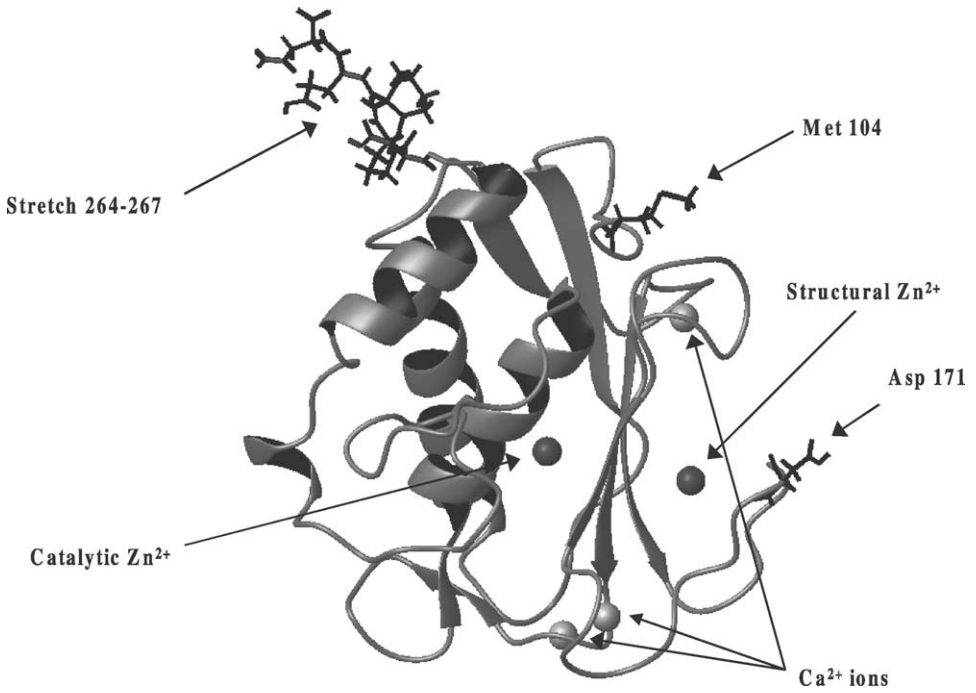

Fig. 2. X-ray structure of MMP-12 F171D [38], where the mutation and the N- and C-terminal deletions described in the text are highlighted.

The expression yield of the ^{15}N-enriched new construct was the same as the original construct. The protein was purified and refolded using the same protocol described above. It experiences a solubility almost double than that of the first construct as it can be easily concentrated up to 2 mM. The ^{1}H–^{15}N HSQC NMR spectra showed a very good spreading of the cross-peaks in both dimensions indicating a completely folded protein.

4. Conclusions

Concluding, a mutant of the catalytic domain of MMP-12 has been prepared that has all the requisites of activity and solubility to be used in a multiple NMR screening project together with other soluble MMPs to search for candidate drugs with improved selectivity. A second MMP-12 mutant has been devised which displays even higher solubility. This paper shows that, in addition to the optimization of protocols for protein expression, the careful optimization of the domain construct is an advisable strategy for the obtainment of soluble and stable proteins for structural studies, catalytic activity studies and drug screening.

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