

Research article

Open Access

Nonnatural amino acid incorporation into the methionine 214 position of the metzincin *Pseudomonas aeruginosa* alkaline protease

Paula Walasek and John F Honek*

Address: Department of Chemistry, University of Waterloo, 200 University Avenue, Waterloo, Ontario, Canada N2L 3G1

Email: Paula Walasek - pwalasek@scimail.uwaterloo.ca; John F Honek* - [jhoneyk@uwaterloo.ca](mailto:jhonek@uwaterloo.ca)

* Corresponding author

Published: 12 October 2005

Received: 11 August 2005

BMC Biochemistry 2005, 6:21 doi:10.1186/1471-2091-6-21

Accepted: 12 October 2005

This article is available from: <http://www.biomedcentral.com/1471-2091/6/21>

© 2005 Walasek and Honek; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: The alkaline protease from *Pseudomonas aeruginosa* (AprA) is a member of the metzincin superfamily of metalloendoproteases. A key feature of these proteases is a conserved methionine-containing 1,4-tight β turn at the base of the active site zinc binding region.

Results: To explore the invariant methionine position in this class of protease, incorporation of a nonnatural fluorinated methionine, L-difluoromethionine (DFM), into this site was accomplished. Although overproduction of the N-terminal catalytic fragment of AprA resulted in protein aggregates which could not be resolved, successful heterologous production of the entire AprA was accomplished in the presence and absence of the nonnatural amino acid. DFM incorporation was found to only slightly alter the enzyme kinetics of AprA. In addition, differential scanning calorimetry indicated no significant alteration in the thermal stability of the modified enzyme.

Conclusion: Although invariant in all metzincin proteases, the methionine 214 position in AprA can be successfully replaced by the nonnatural amino acid DFM resulting in little effect on protein structure and function. This study indicates that the increased size of the methyl group by the introduction of two fluorines is still sufficiently non-sterically demanding, and bodes well for the application of DFM to biophysical studies of protein structure and function in this class of protease.

Background

The metzincin superfamily of proteases includes the matrixins, astacins, reprotins, snapalysins, leishmanolysins and serralsins [1,2]. These zinc-dependent endoproteases may act on a large number of substrates, or they may be highly specific, targeting only one or a few proteins or oligopeptides. There are over 700 different enzymes that have been classified as metzincins and they are produced by organisms ranging from bacteria to plants and animals [3]. As diverse as the members of this superfamily are, surprisingly they all share a structurally similar catalytic site which includes a common zinc binding motif and an absolutely conserved methionine-con-

taining 1,4-tight β turn, termed the "Met-turn" in their active sites [4].

Many of the metzincin proteases are of medical significance. The matrixins, or matrix metalloproteinases (MMPs) include the collagenases, gelatinases and stromelysins [5,6]. Members of this subfamily not only degrade extracellular matrix for tissue remodeling and maturation, but they have also been found to release and activate several growth factors and modulate chemotactic signals [5]. In disease processes, however, they have been implicated in promoting tumour invasion and metastasis by degradation of matrix barriers, exacerbating periodontal disease,

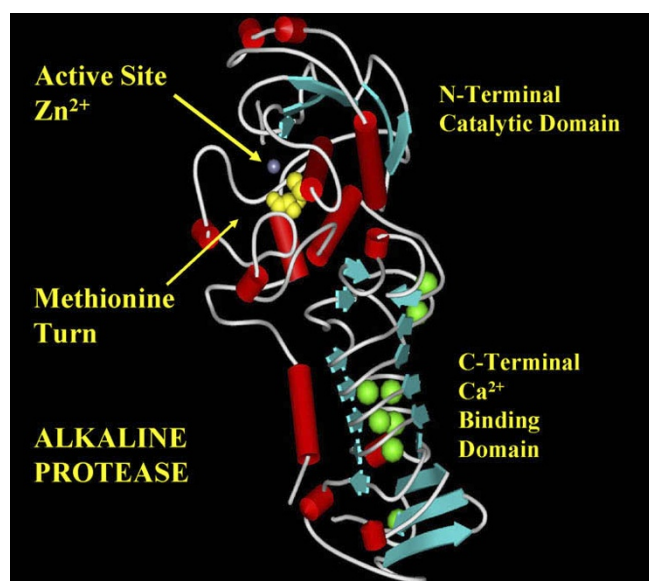


Figure 1
Ribbon representation of alkaline protease from *P. aeruginosa* (AprA). The active site zinc ion is shown in blue and the conserved methionine in yellow spacefill just below the zinc. Calcium ions in the C-terminal β -roll domain are green. Figure generated using WebLab Viewer Pro 3.7 using PDB entry 1KAP [15].

and destroying aggrecan and collagen in rheumatoid arthritis. For this reason, much interest has arisen in mechanistic and inhibitor studies of MMPs [7-9]. The astacins comprise a subfamily of enzymes involved in digestion, developmental regulation, and peptide processing. The repolysins or adamalysins are mainly responsible for the hemorrhagic effects and tissue necrosis typical of snake bites [2]. These effects are caused mainly by destruction of the extra-cellular matrix surrounding capillaries in conjunction with the inhibition of platelet aggregation by disintegrins. The N-terminal catalytic domains of members of this family are usually zinc and calcium dependent, while the C-terminal portion may be disintegrin, cysteine-rich, or lectin type domains with hemorrhagic or cell adhesion properties. Mammalian adamalysins play important roles in reproduction, myogenesis and cytodifferentiation as well as in disorders such as asthma, cardiac hypertrophy, and endotoxic shock [10-13]. The snapalysins are secreted proteases from several *Streptomyces* organisms and the membrane-bound leishmanolysins originate from a number of protozoan parasites. The serralsins, of which the alkaline protease from *P. aeruginosa* (AprA) is a member, are secreted by several pathogenic, gram-negative bacterial species [14]. They are, on average, 50 kDa proteins consisting of 2 domains; the C-terminal domain binds up to eight calcium ions in a

mainly structural role, but also contains the secretion signal. The catalytic site is located in a cleft on the N-terminal domain (Figure 1)[15]. The physiologic substrate(s) of these enzymes are not known with certainty, but are thought to be proteins of the extracellular matrix and possibly immune response elements such as interferon gamma and complement proteins [16,17].

To add a further dimension to the techniques applied to the analysis of protein structure and function, the incorporation of nonnatural amino acids into proteins is currently of great interest. One aspect of this approach is the incorporation of amino acids containing fluorine which would be applicable to not only probing subtle steric interactions in proteins (Van der Waals' radius of F is 1.47 Å vs. 1.20 Å for H), but also in applying ^{19}F spectroscopy to this area [18,19]. In order to probe the methionine position in this class of proteins (specifically AprA), the fluorinated analogue, S-difluoromethyl-L-homocysteine (L-difluoromethionine, (DFM)), was utilized to replace the methionine in the Met-turn and to determine its effects on catalytic activity and thermal stability.

Results

N-terminal catalytic fragment

Attempts to overproduce the N-terminal catalytic fragment of AprA which consisted of residues T40 to N258 of the 479 residue protein led to the production of aggregates in spite of attempts to increase soluble protein expression by control of temperature, isopropyl- β -D-thiogalactopyranoside (IPTG) concentration, co-expression of folding chaperones (GroES and GroEL) as well as secretion and fusion to a NusA solubility tag [20-23]. In several attempts at resolubilisation of these inclusion bodies (see Methods for details), semi-soluble aggregates of the truncated AprA were detected by gel filtration chromatography (Superose-12) having molecular weights higher than the exclusion limit of the resin ($>2 \times 10^6$ Da). Due to the difficulty of isolating and handling sufficient quantities of this truncated version of AprA, attention was then focused on overproduction of the full-length mature form of AprA which corresponds to residues G10 to V479.

Mature full-length AprA and DFM incorporation

The gene for the full-length mature form of AprA [24-26] which corresponds to residues G10 to V479 was isolated on to pET22b and overexpressed in *E. coli* BL21 (λ DE3), resulting in inclusion body formation. Isolation of the protein aggregates followed by solubilisation and refolding produced an active, stable and soluble enzyme of 49 500 Da, according to mass spectrometric analysis (Figure 2 and 3). L-DFM was synthesized as previously described [27,28] and incorporated into AprA using the methionine auxotrophic *E. coli* strain, B834 (λ DE3). The resulting inclusion bodies were refolded in an identical manner as

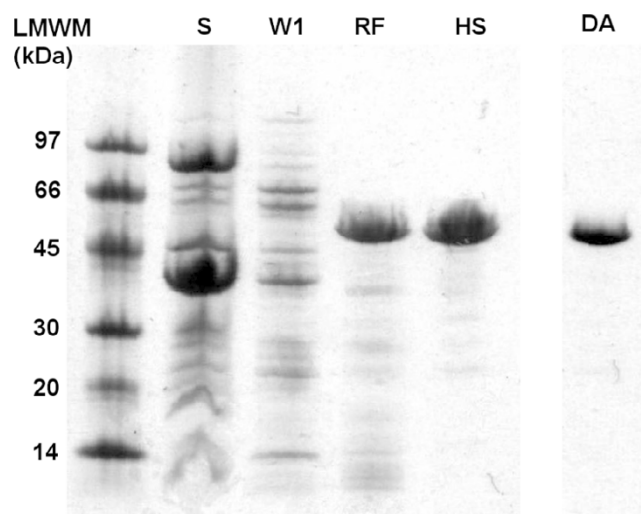


Figure 2
SDS-PAGE gel summary of the refolding of mature full-length AprA. S represents the supernatant of the crude lysate, W1 is the solution from the first wash of the inclusion body pellet, RF is the refolded protein and HS is the refolded protein after the high salt wash and DA is the refolded DFM-AprA.

the non-labelled protein, and the expected 49 535 Da protein, as determined by electrospray mass spectrometry (ESMS), was isolated (Figure 2 and 4).

Kinetic analysis and differential scanning calorimetry

Utilizing a previously reported spectrophotometric method for kinetic analysis of AprA, the mature, full-length recombinant protein showed similar kinetic properties for hydrolysis of Z-Arg-Arg-p-nitroanalide to those reported for the mature wild-type protein, isolated directly from *P. aeruginosa* growth media [29]. The k_{cat} values were determined to be $1.6 \pm 0.1 \text{ min}^{-1}$ versus $0.6 \pm 0.1 \text{ min}^{-1}$ and the K_m values were $12.6 \pm 2.4 \text{ }\mu\text{M}$ vs. $6 \pm 1 \text{ }\mu\text{M}$ for the enzyme produced by the cognate organism and the recombinant protein respectively.

Slightly improved assay conditions were found by increasing the amount of enzyme from 25 μg per assay to 100 μg in order to improve sensitivity, and by replacing the assay buffer from 5 mM TRIS to 20 mM HEPES to avoid possible metal binding effects of the TRIS buffer. The kinetic parameters of non-labelled and DFM-labelled AprA proteins were compared under the new conditions and found to be similar. The K_m was not significantly altered ($15.4 \pm 0.9 \text{ }\mu\text{M}$ for the unlabelled protein vs. $14 \pm 2 \text{ }\mu\text{M}$ for the DFM-incorporated), and the k_{cat} was only slightly affected by DFM incorporation, showing a decrease from $0.57 \pm 0.02 \text{ min}^{-1}$ to $0.44 \pm 0.02 \text{ min}^{-1}$.

Due to the proximity of the Met214 residue to the metal ligands which ligate the zinc ion (Figure 5), the effect of

substitution of this Met with the larger DFM residue on thermal denaturation was investigated. Replicate differential scanning calorimetry experiments were performed on both the mature full-length AprA and the DFM-labelled mature full-length AprA (DFM-AprA) in 20 mM HEPES (pH 7.8). The T_{max} for unlabelled and DFM-labelled AprA were determined to be $58.7 \pm 0.1^\circ\text{C}$ and $58.6 \pm 0.2^\circ\text{C}$ respectively (Figure 6).

^{19}F NMR spectroscopy

DFM-labelled mature full-length AprA was analyzed by ^{19}F nuclear magnetic resonance (^{19}F NMR) spectroscopy without proton decoupling to produce a complex set of ^{19}F resonance multiplets centered at approximately -93.8 ppm. Paramagnetic line broadening with addition of gadolinium(III)-N,N,N',N'-ethylenediaminetetraacetic acid ((GdEDTA)-) complex resulted in line broadening of all ^{19}F resonances. Proton-decoupled ^{19}F NMR experiments resulted in collapse of the ^{19}F multiplets, however complete resolution of the N-terminal DFM from the internal (DFM214) ^{19}F resonances was still incomplete at this field strength (564.5 MHz) (Figure 7).

Discussion

AprA is a member of the metzincin superfamily of metalloproteases and is one of several proteases secreted from *Pseudomonas aeruginosa* into tissue of infected patients [16,30,31] (Figure 1). It appears that a combination of AprA, elastase (LasB) and LasA protease and protease IV work in concert to produce tissue invasion [32,33]. AprA is biosynthesized in *P. aeruginosa* as a 479 residue polypeptide which is secreted via a C-terminal signal sequence directly into the extra cellular medium where a 9 amino acid N-terminal inhibitory propeptide is autocatalytically removed [26]. A critical aspect to the structure-function of AprA and the metzincins in general is the active-site arrangement of the metal ligands and the still incompletely understood methionine-containing 1,4-tight β turn at the base of the active site present in all members of the metzincin superfamily. Although much is known concerning the cellular biochemistry of a number of metzincins, detailed knowledge of the function of the components of the active site is still lacking. For example, methionine residues in proteins are less frequent in occurrence than many other residues (third least abundant after tryptophan and cysteine [34]) and it can frequently be replaced with other hydrophobic amino acids such as leucine [35,36]. However the surprising persistent presence of methionine in over 700 members of this class of protease has been noted [3,4,37,38]. Attempts to define the contribution that this seemingly quintessential amino acid plays in this class of enzyme have indicated that, depending upon the specific superfamily member studied, some flexibility in replacement may be possible, at least *in vitro*. For example, in the case of MMP-2 (gelati-

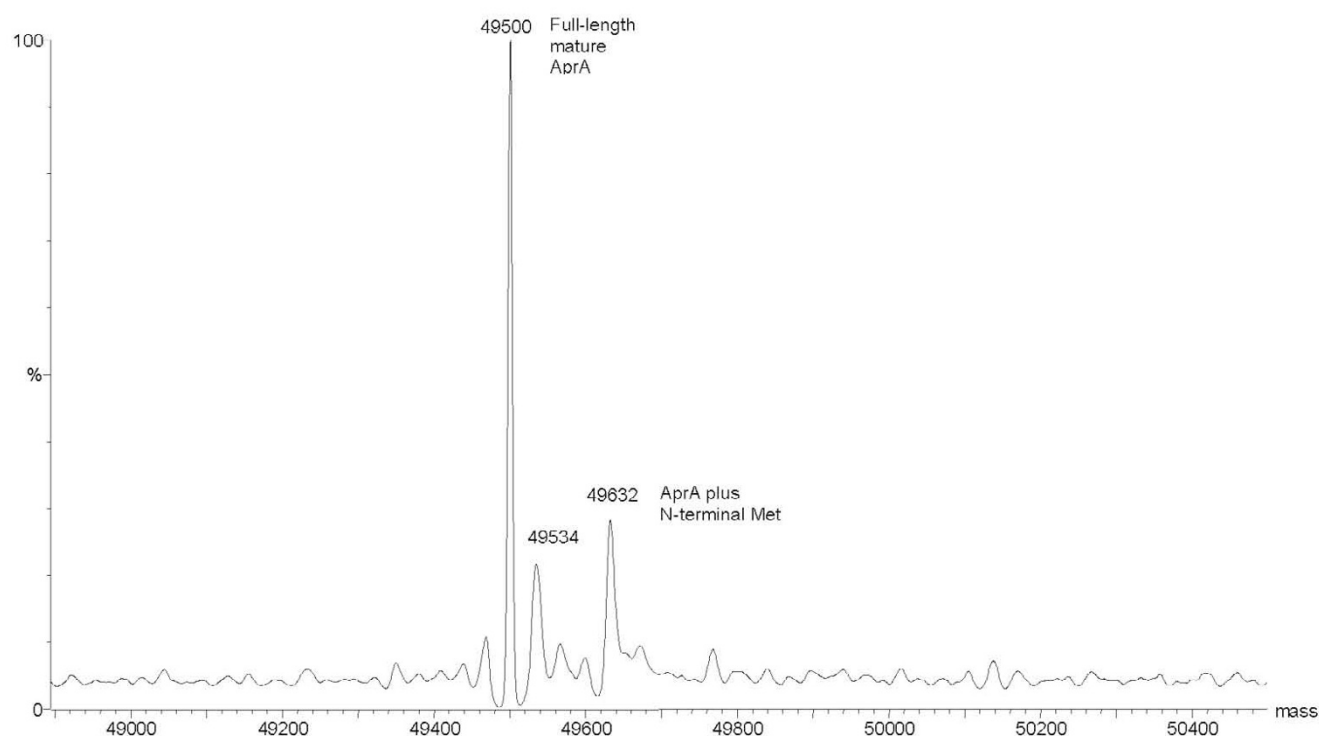


Figure 3

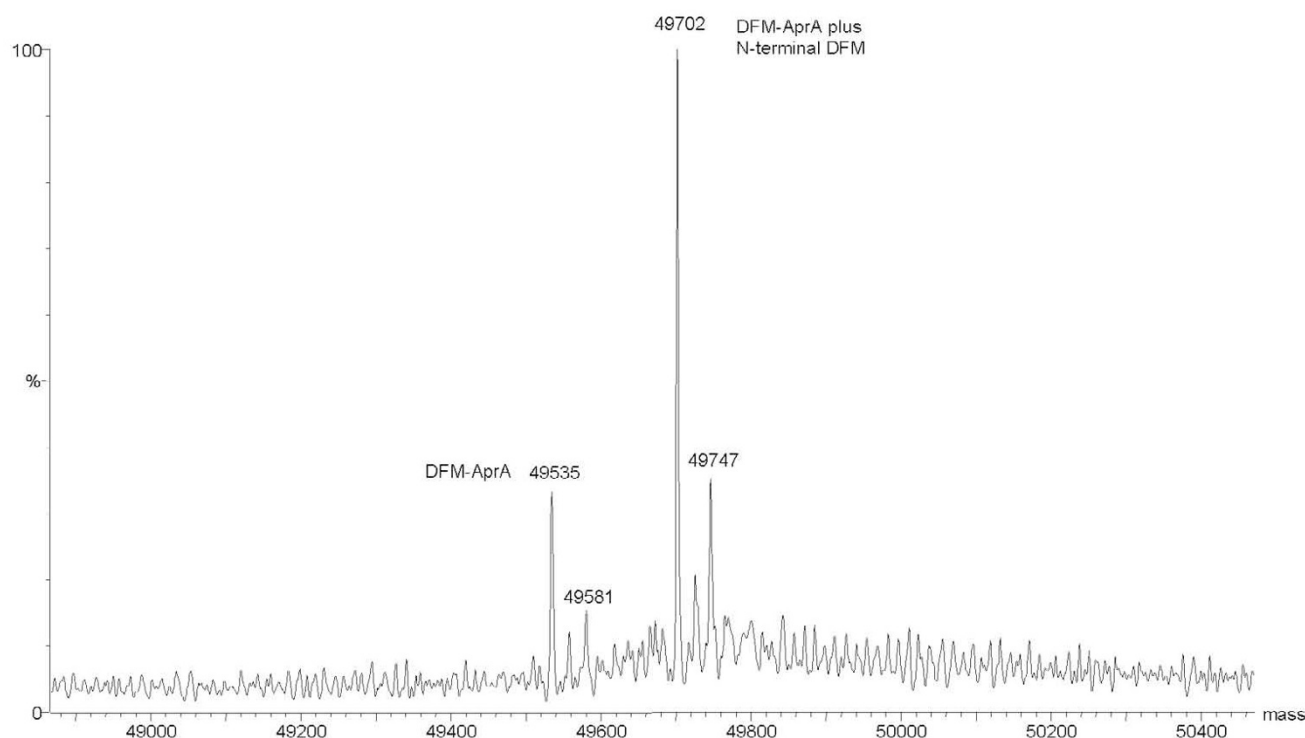
Positive ion mode electrospray mass spectrum of the refolded, full-length, mature AprA. The major peak in the spectrum (49 500 Da) corresponds exactly to the recombinant protein without the initiator methionine. The peak at 49 534 Da may represent a calcium adduct. The peak at 49 632 Da corresponds to AprA retaining the N-terminal methionine.

nase A), enzymes with replacements of the active site Met with either Leu or Ser have only minor perturbations in catalytic activity [39]. In the case of protease C from *Erwinia chrysanthemi*, replacement of the active site methionine (Met226) by leucine, alanine or isoleucine resulted in enzyme mutants sufficiently stable to be isolated, but this was not so with M226H, M226S or M226N mutations. However the enzyme kinetic properties of the M226L, M226A and M226I mutants were compromised and crystallographic studies indicated some alteration of the zinc binding histidines which might explain the effects of these mutations on the kinetic properties of the enzyme [40].

Substitution of the active site Met by selenomethionine (SeMet) in MMP-8 (human neutrophil collagenase) was also found to be somewhat perturbing. In the catalytic domain of MMP-8, SeMet appeared to decrease the protein's resistance to urea denaturation [41]. This loss of stability was accompanied by a considerable decrease in activity and an increase in K_m towards the substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH₂. In a previous study using the substrate (dinitrophenyl-

Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH₂), however, k_{cat} and K_m values were unaffected by SeMet replacement in MMP-8 [42,43].

Evidently additional investigations into the properties of the metzincins through modification of the methionine position may be of importance. However the usual ensemble of potential replacements of amino acid residues through site-directed mutagenesis is limited in general to replacement with the other nineteen amino acids. In order to explore the effects of alterations of the critical methionine in the metzincin class of proteases, the introduction of a sterically and electronically subtle methionine analogue DFM, was the focus of the present study. For these studies the alkaline protease from *Pseudomonas aeruginosa* was initially investigated as it is representative of the metzincin family of proteases, is important in tissue invasion by this pathogen and has only two methionines in its structure, the N-terminal methionine and the Met-turn methionine, M214. The presence of only two methionine residues in this metzincin (and only the active site methionine if a mature secreted AprA is produced) facilitates bioincorporation of DFM into the protein without the use of complex cell free in-vitro

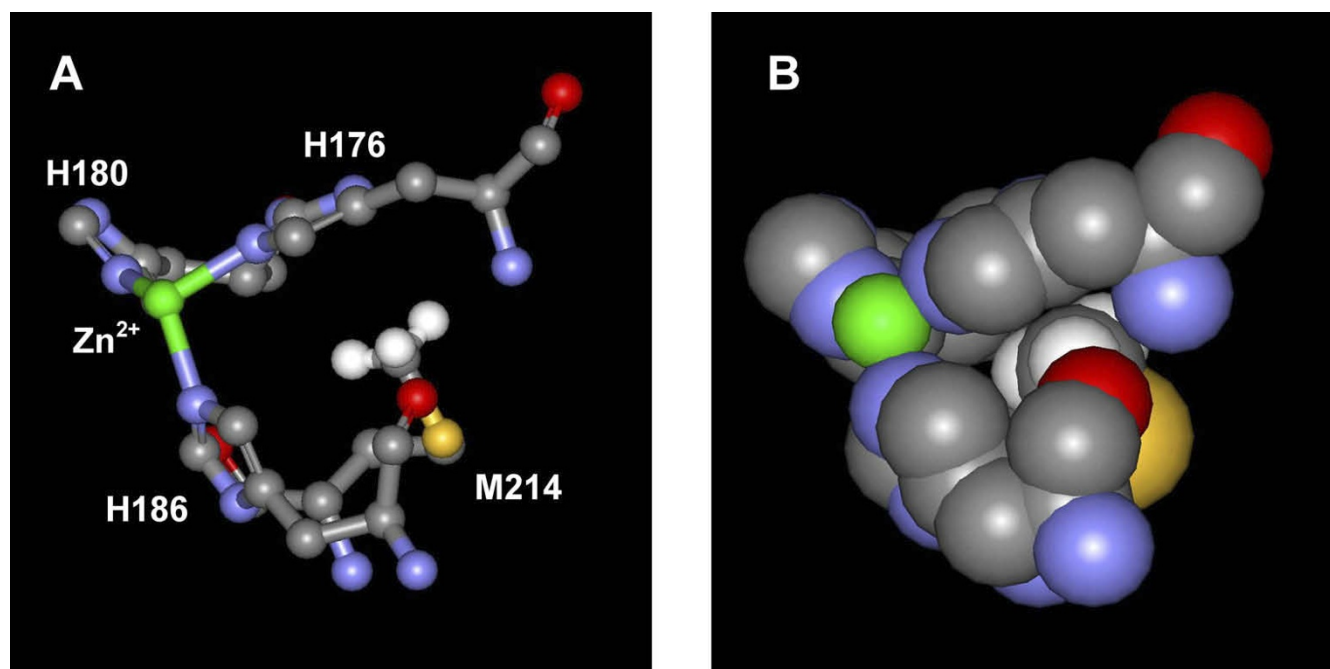
**Figure 4**

Positive ion mode electrospray mass spectrum of DFM-AprA. The calculated molecular weight of the labelled protein with the initiating residue was 49 702 Da, and without, 49 535 Da. Possible sodium adducts (2Na^+) are seen for both species at 49 747 Da and 49 581 Da.

translation system approaches [44-46]. As well, this choice of target protein for a preliminary study avoids complications with additional bioincorporation of the analogue into other methionine positions which may be present in other members of the metzincin superfamily.

DFM is a subtle derivative of methionine that has been shown to be useful as a ^{19}F -NMR probe in proteins [28,47]. Unlike leucine and isoleucine, this unbranched analogue may only produce a moderate increase in the steric properties of the methyl group due to the incorporation of two fluorine atoms. In addition, fluorination could profoundly affect the electron density of the sulfur atom [27]. As can be seen in Figure 5, the thiomethyl group of M214 is in close proximity to H176, H180, and H186 and may contribute to the proper positioning of these metal ligands. Hence it was of interest to explore the effects of this nonnatural amino acid on the catalytic activity and thermal stability of the modified protein by introduction of L-difluoromethionine into the critical methionine position in the Met-turn.

Previous studies on other metzincins such as stromelysin, MMP-8, and astacin have shown that the truncated enzymes containing only the catalytic domain of these enzymes could be expressed and were sufficiently stable to be studied [48-50]. In the current study, initial attempts were therefore focused on the overproduction of the smaller N-terminal catalytic domain of AprA (T40 to N258). Although evidence for cellular production of this fragment was observed, almost complete aggregation of this fragment occurred under a variety of experimental conditions designed for its control (Results). Previous reports by Guzzo and co-workers have shown that even minimal recombinant expression ($1\text{ }\mu\text{g/mL}$) of soluble, intact AprA protein required the co-expression of the entire secretion apparatus (AprD, AprE, AprF) [51]. When the structural gene for AprA was expressed alone, however, intracellular accumulation of AprA was barely detectable, most likely due to rapid degradation inside the cell [52]. In other reports, significant quantities of the enzyme could be found intracellularly, but only in the form of inclusion bodies, which are known to be highly resistant to degradation [24,53,54].

**Figure 5**

Active site of AprA showing the environment of the conserved methionine (M214). A: The three histidine ligands (labelled) are shown along with the zinc ion (in green). The spacefill representation (B) shows the closely packed arrangement around the methionine residue, as well as its proximity to the catalytic zinc and the three histidine residues. Protons (white) were added to the M214 methyl group for clarity. Based on structure from PDB entry 1KAP [15].

However successful production of mature full length (G10 to V479) AprA, was accomplished without the co-expression of the entire secretion apparatus. Although insoluble protein aggregates were produced, it was possible to resolubilise and refold the overexpressed protein, yielding approximately 40 mg of high purity enzyme per 1 L of culture (Figure 2). The ESMS analysis revealed that although the majority of the protein isolated from this protocol had $M_r = 49\,500$ Da (full length mature AprA, G10-V479), a small quantity of inseparable enzyme with an $M_r = 49\,632$ Da had maintained its N-terminal methionine (Met-G10-V479) from the cloning procedure (Figure 3). Kinetic analysis of this recombinant mature AprA compared favourably with values previously reported [29] for the wild type AprA secreted by *P. aeruginosa* (k_{cat}/K_m of 2140 ± 540 M⁻¹s⁻¹ and 1800 ± 300 M⁻¹s⁻¹ for wild type and recombinant enzymes respectively).

An almost 100% incorporation level of the fluorinated probe into AprA was readily accomplished by induction of protein expression in minimal media supplemented with 2 mM DFM. The labelled protein was also expressed as intracellular inclusion bodies which were resolubilised and refolded using the same protocol as the unlabelled recombinant protein, yielding approximately 16 mg of nearly homogeneous protein per 1 L of culture (Figure 2). The ESMS analysis indicated that, unlike the unlabelled

recombinant AprA, a majority of the DFM incorporated protein maintained the initiating residue ($M_r = 49\,702$ Da), while this DFM was cleaved in only about one quarter of the sample ($M_r = 49\,535$ Da) (Figure 4). The interesting observation that removal of the N-terminal DFM residue occurs to a lesser extent compared to methionine in this position indicates that the processing of fluorinated methionines by methionine aminopeptidase is a less efficient process. Related to this observation is a recent report by Budisa and coworkers indicating the lack of removal of trifluoromethionine from the N-terminus of a mutant green fluorescent protein[55]. It may be that the increased steric size of the fluorinated methyl groups in these analogues alters the correct positioning of the peptide bond of the substrate in the active site of the methionine aminopeptidase which could reduce hydrolytic efficiency by this enzyme. In any event, the presence of the N-terminal methionine (or DFM) is not expected to affect catalytic activity or thermal stability of the protein as the N-terminus is physically remote from the active site. The kinetic characteristics of the protein mixture with and without incorporated DFM were determined under the revised conditions described in the methods and results sections. The K_m was not significantly altered by replacement with DFM, the V_{max} and therefore k_{cat} were only slightly impaired, resulting in a decrease in k_{cat}/K_m from 620 ± 30 M⁻¹s⁻¹ to 530 ± 50 M⁻¹s⁻¹.

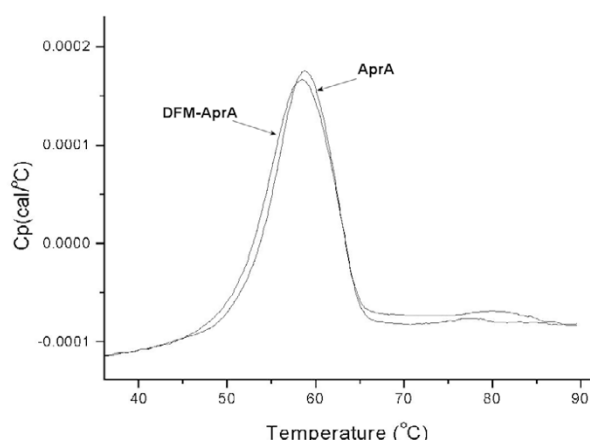


Figure 6
Differential scanning calorimetry data of both the unlabelled AprA and the DFM-incorporated AprA. All scans were performed at 1°C per minute in 20 mM HEPES (pH 7.8) and buffer scans were subtracted from sample runs.

In order to compare the effects of DFM on thermal stability of the protein, preliminary differential scanning calorimetry studies were undertaken on AprA and DFM-AprA. Under the conditions utilized in these experiments, the thermal denaturations of AprA and DFM-AprA were found to be irreversible. As shown in Figure 6, the thermal stability in 20 mM HEPES (pH 7.8) was not significantly altered upon fluorination of the Met-turn methionine. The T_{max} for unlabelled and DFM-labelled AprA were determined to be $58.7 \pm 0.1^\circ\text{C}$ and $58.6 \pm 0.2^\circ\text{C}$ respectively. This would indicate that the introduction of two fluorine atoms into the methyl group, which is in close contact to the critical histidines in the active site, can be accommodated by the protein such that the catalytic properties and thermal stability of AprA are not drastically perturbed.

Due to the lack of background ^{19}F resonances in most biological systems, the 100% natural abundance of the ^{19}F isotope and its sensitivity to NMR detection of 83 % to that of ^1H , the application of ^{19}F spectroscopy to studies in protein structure and function has been an important biophysical technique that has been shown to give critical information on the environment surrounding the fluorine nucleus [19,56-58]. Previous studies on DFM incorporation into the transglycosylase of bacteriophage λ and the leucine-isoleucine-valine (LIV) binding protein from *E. coli*, have detected separate sets of resonances for each DFM residue present in each protein and their presence

has been useful in detection of ligand binding [28,47]. As the two fluorine atoms in DFM are diastereotopic due to the presence of the chiral centre of the α -carbon of methionine, complex ^{19}F resonances can result. In fact, it has been shown that a complex octet of ^{19}F resonances is observed when a DFM residue is located in a conformationally restricted protein environment [28]. It was therefore of interest to investigate the ^{19}F NMR spectroscopy of DFM-AprA. The ^1H -coupled ^{19}F NMR spectrum of DFM-AprA (Figure 7A) is a composite of the ^{19}F resonances not only from the active site DFM at position 214, but also a contribution from the presence of protein molecules still maintaining the N-terminal DFM residue. Interestingly the two resonances overlap in the 564 MHz spectrum. It has previously been shown that application of paramagnetic line broadening agents, such as $\text{Gd}(\text{EDTA})^-$ will broaden the ^{19}F NMR resonances from fluorine nuclei in a protein in a distance-dependent manner and can serve to indicate which ^{19}F resonances originate from more solution exposed residues [56,58]. To possibly distinguish the signal contributions from each DFM, paramagnetic line broadening experiments utilizing $\text{Gd}(\text{EDTA})^-$ were undertaken. These experiments (Figure 7B) indicated that both DFM residues (DFM1 and DFM214) were equally susceptible to the line-broadening agent, indicating that close approach to the active site DFM by $\text{Gd}(\text{EDTA})^-$ was also possible in solution. Based on the crystal structure of AprA [15,59], the active site is open to solvent and could be expected to reasonably accommodate the rather large $\text{Gd}(\text{EDTA})^-$ molecule resulting in little discrimination between the two DFM residues. Although proton decoupling simplified the ^{19}F spectrum (Figure 7C), the resonances from the two DFM residues were still unresolved; the complexity of which may also result from contributions of the restricted environment of the active site DFM. Attempts to remove the N-terminal DFM with recombinant *E. coli* methionine aminopeptidase [60] under a variety of conditions resulted in complex mixtures due to proteolytic degradation by AprA. Additional attempts to produce a variant of AprA lacking the N-terminal DFM residue by introduction of the wild-type N-terminal self-cleaving propeptide sequence or a thrombin cleavage site resulted in extremely poor isolated yields of these forms of the protease. Further studies will be focused on the application of this NMR probe to investigating conformational changes in the active site upon ligand and inhibitor binding.

Conclusion

The ^{19}F biophysical probe, DFM, was successfully incorporated into the critical Met-turn of AprA and resulted in minimal catalytic or structural alteration of the enzyme. These findings are encouraging for the further application of DFM as a subtle probe for the investigation of the metzincin family of proteases.

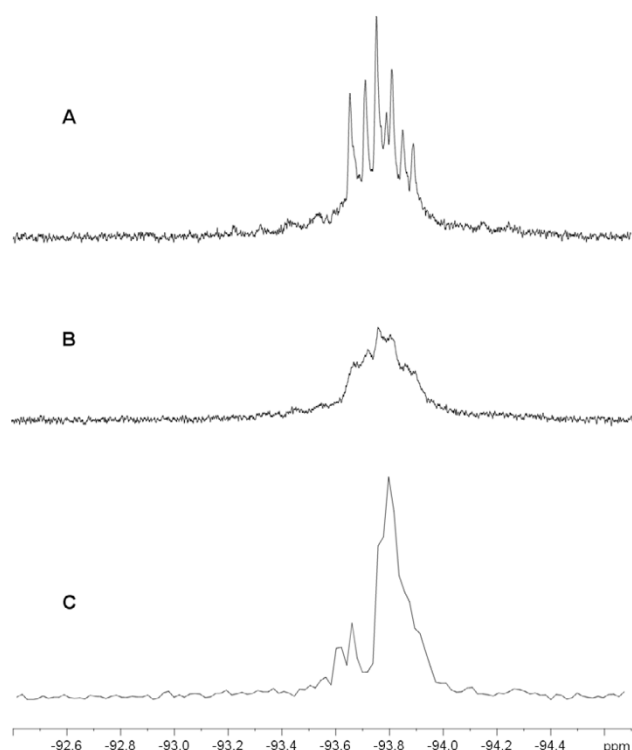


Figure 7
 ^{19}F NMR spectra of DFM-labelled AprA. The 564.7 MHz ^{19}F NMR spectrum of DFM-labelled AprA in D_2O with approximately 40 mM HEPES (A) and with the addition of 2 mM Gd(EDTA) (B). Proton-decoupled spectrum of DFM-labelled AprA (C).

Methods

Materials

Restriction endonucleases were purchased from New England BioLabs (Pickering, ON). The Expand[™] and Pwo polymerase kits as well as the alkaline phosphatase (from calf intestine) were Roche (Laval, QC) products and the Taq polymerase was from Qiagen (Mississauga, ON). The thrombin (from bovine serum) was purchased from Amersham Biosciences (Baie d'Urfé, QC). The peptide substrate, Z-Arg-Arg-pNA, was purchased from Bachem (King of Prussia, PA). The VP-DSC differential scanning microcalorimeter and Origin[™] software (Version 5.0) were from MicroCal[™] (Northampton, MA). ^{19}F NMR data were collected on Bruker Avance (Milton, ON) NMR spectrometers (500 and 600 MHz). Unless otherwise noted, all reagents and chemicals were of the highest commercial grade available and were used without further purification. L-Difluoromethionine was prepared by a previously reported method [28].

Cloning and bacterial expression

The genes of interest were amplified by PCR and digested using the primers and restriction endonucleases listed in Table 1. With the exception of pNusAPA, all PCR products were ligated in to appropriately digested pET22b(+) (Novagen Inc). The NusA fusion protein was produced from a pET43.1b(+) based vector (Novagen Inc). New constructs were confirmed by DNA sequencing (Mobix, Inc) in both directions and the protein products were analyzed by electrospray mass spectrometry (positive ionisation mode).

Unlabelled proteins were produced in the *E. coli* strain BL21 (λDE3), while strain B834 was used for DFM incorporation. A 10 mL starter culture (LB broth with 50 mg/mL carbenicillin) was inoculated from frozen stock and incubated overnight at 37°C with shaking. For the production of unlabelled proteins, the entire starter was used to inoculate 1 L of LB broth (with 50 mg/mL ampicillin) and the culture was grown to an OD_{600} of 0.6 to 0.8. IPTG was added directly to a final concentration of 1 mM and the culture was incubated at 37°C with shaking for an additional 4 hours. A similar procedure to that described by Vaughan *et al.* [28] for DFM incorporation into phage lysozyme was used to produce the L-DFM labelled AprA protein. In brief, a 10 mL starter culture of *E. coli* strain B834 harbouring plasmid pWApr was used to inoculate 1 L of M9 buffer (pH 7.2: 47.6 mM Na_2HPO_4 , 22 mM KH_2PO_4 , 8.5 mM NaCl, 18.7 mM NH_4Cl , 2 mM MgSO_4 , 0.1 mM CaCl_2) supplemented with 0.4 % glucose, 50 $\mu\text{g}/\text{mL}$ ampicillin, and 0.1 mM L-Met. The culture was grown at 37°C with shaking to an OD_{600} of approximately 0.7 and then harvested by centrifugation for 10 minutes at 5000 g. The cells were washed in M9 buffer and then resuspended in M9 supplemented with 0.4 % glucose and 50 $\mu\text{g}/\text{mL}$ ampicillin. After incubating at 37°C with shaking for 30 minutes, 2 mM L-DFM and 1 mM IPTG were added, and the culture was incubated (37°C with shaking) for a further 8 hours.

Refolding of the N-terminal catalytic domain

The expressed N-terminal catalytic domain segment that was initially produced was extracted and solubilised in much the same manner as the full-length protein described below. However, several variations to the refolding buffer were made in attempts to recover the soluble, active catalytic fragment. A range of pH conditions from 7.2 to 8.0 was used. ZnSO_4 was added to the refolding buffer with concentrations ranging from 0.1 μM to 0.1 mM. Glycerol (20 %) was also added to the refolding buffer and the L-arginine content was varied from 0.8 M down to 0.2 M. The arginine was replaced completely in several other attempts which instead involved 1 % Brij-35, 1 M non-detergent sulfobetaine 201 (NDSB-201), or 1 M KCl with 20 % glycerol. Finally, the dialysis buffer was

Table 1: Primers and endonucleases used in the cloning of the various constructs. Restriction endonuclease cut sites are underlined and the regions of the primers homologous to the gene of interest are in bold.

Construct	Primers	Restriction endonucleases	Protein product
pAPA'	N-terminal: 5' CCA GAA TTC <u>CAT ATG</u> ACC GTC GAC CAG GCG GCG GAG C 3' C-terminal: 5' CCA <u>GGA TCC</u> TTA GTT GGC CCC GTA GAG CTT CTG G 3'	<i>NdeI</i> <i>BamHI</i>	AprA catalytic fragment (APA')
pPelAPA'	N-terminal: 5' CCG AAT <u>TCC ATG G</u> CC GTC GCC GTC GAC CAG GCG GCG GAG C 3' C-terminal: Same as pAPA'	<i>NcoI</i>	APA' with pelB secretion signal
pNusAPA'	N-terminal: 5' G GGC GGG GAC GAA TTG GTC AAT GGC 3' C-terminal: Same as pAPA'	Blunt end ligation at N-terminus: no digestion required	APA' with NusA solubility tag and His ₆ tag
pWApr	N-terminal: 5' CCA GAA TTC <u>CAT ATG</u> GGT CGT AGC GAT GCG TAT ACC 3' C-terminal: 5' CCA <u>GGA TCC</u> TCA GAC GAC GAT GTC GGC CTG G 3'	<i>NdeI</i> <i>BamHI</i>	Mature AprA without pro-peptide

also varied to include 10–20 % glycerol, 0.05 % Brij-35 or 0.05 % Triton-X 100.

Solubilisation and refolding of the full-length AprA protein

Induced *E. coli* BL21 (λ DE3) cells harbouring plasmid pWApr were harvested in a 10 min spin at 5000 g, washed in M9 buffer (47.6 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.5 mM NaCl, 18.7 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂) then resuspended in lysis buffer (50 mM TRIS (pH 7.8), 1 mM EDTA, 0.1 % Triton-X-100) and disrupted by eight 20 second rounds of continuous sonication with cooling. The crude cell lysate was centrifuged for 10 minutes at 6000 g, and the pellet was washed twice in lysis buffer and then 3 times in lysis buffer without Triton.

The resulting inclusion body pellet was dissolved in a small amount of solubilisation buffer (50 mM TRIS, pH 7.8, 1 mM EDTA, 6 M guanidine hydrochloride) and incubated at 37°C for 1 hour. The solution was then centrifuged for 10 minutes at 13000 g and a 4 μ L aliquot was diluted to up to 1 mL in solubilisation buffer for an absorbance measurement at 280 nm. Protein concentration was estimated using a predicted extinction coefficient of 67 990 M⁻¹cm⁻¹ calculated by the ProtParam tool in ExPASy [61]. Additional solubilisation buffer was used to dilute the protein solution to 10 mg/mL. This solution was then passed through a 0.22 μ m syringe filter and added dropwise with stirring to ice cold refolding buffer (50 mM TRIS, pH 7.8, 1 mM CaCl₂, 0.8 M L-arginine), resulting in a 100-fold dilution (final refolding protein concentration: 0.1 mg/mL). After an overnight incubation with stirring at 4°C, the protein was dialysed against two changes of buffer with metals (20 mM HEPES, pH 7.8, 1 mM CaCl₂, 0.1 mM ZnSO₄), then one without CaCl₂ or ZnSO₄, but with 300 mM NaCl. Finally, the protein was concentrated using an Amicon pressure filtration device

with a 10 kDa cut-off filter and the high salt buffer exchanged to 20 mM HEPES (pH 7.8).

Mass spectrometry

Electrospray mass spectrometry of purified protein samples was carried out on a Micromass Q-TOF Global Ultima mass spectrometer in positive ion mode. Spectra were analyzed using the MaxEnt1 algorithm in the MassLynx (V4.0, Micromass Limited/Waters, Milford, Massachusetts) software. Buffer salts were removed from protein samples using a Nanosep microcentrifuge concentrator with a 10 kDa cut-off. Once in Milli-Q (Waters Corp.) purified water, samples were diluted with a solution of 50 % acetonitrile and 0.1 % formic acid.

Activity assay

Initial determinations of kinetic parameters using the substrate Z-Arg-Arg-p-nitro-analide were carried out under the conditions described by Louis *et al.* for wild type alkaline protease [29], using substrate concentrations ranging from 3 μ M to 200 μ M. The rate of p-nitroaniline released was calculated using the extinction coefficient of 8800 M⁻¹cm⁻¹ at 410 nm [29]. In order to avoid possible metal chelating effects of the TRIS buffer and to increase assay sensitivity, 20 mM HEPES buffer was used in place of the 5 mM TRIS, and 100 μ g of protein was used for each assay. Kinetic parameters, K_m and V_{max}, were determined by non-linear regression using the GraFit5™ software (Erithacus Software Limited).

Differential scanning calorimetry

A protein concentration of 0.5 mg/mL was used for each melting temperature determination. Initially, 50 mM TRIS (pH 7.8) was used as the buffer for these experiments, however, this was eventually replaced by 20 mM HEPES. Therefore, protein samples in 20 mM HEPES (pH 7.8) and

reference buffer (20 mM HEPES, pH 7.8) were degassed under vacuum just prior to analysis. Scanning was performed continuously between 15 and 90°C at a rate of 1°C per minute with a 1 minute hold in between each up and down scan. An initial buffer-buffer scan was performed in order to establish a thermal history for the system. The subsequent buffer-buffer scan was then used as a reference. Once the temperature was below 35°C again, the protein solution was loaded into the sample cell and scanned several times.

Data were analysed using the Origin5.0^(TM) software in the following manner. The reference buffer-buffer scan was subtracted from the subsequent protein scan, the data were normalised for protein concentration, and the temperature ($T_{\max}$) at the resulting peak was determined. Plots of heat capacity, C_p , versus temperature were then plotted. ($T_{\max}$ may be defined as the apparent temperature of maximum excess specific heat absorption [62,63]. The $T_{\max}$ values for both the mature wild type AprA and the DFM-incorporated proteins were determined in triplicate, averaged and the standard deviation determined.

¹⁹F NMR spectroscopy of DFM-labelled AprA

Protein was first dialysed against 5 mM HEPES (pH 7.8) and then lyophilised and stored at -20°C until needed. Approximately 4 mg of lyophilised protein was made up in 500 µL of D₂O for analysis. ¹⁹F NMR (564.5 MHz) data were collected using a Bruker Avance 600 MHz spectrometer fitted with a 5 mm dual ¹⁹F/¹H probehead tuned to ¹⁹F. Standard parameters were 11 261 Hz sweep width, 1.45 second acquisition time and a 0.5 second relaxation delay. A 1 Hz line broadening was applied. Spectra were recorded at 277 K and referenced to an external sample of trifluoroacetic acid (set at -76.53 ppm) or a solution of 0.05 % trifluorotoluene in CDCl₃ (set at -63 ppm) as external references. Gd(EDTA)-line broadening experiments were realized by adding 13 µL and later 26 µL of a stock solution of Gd(EDTA)- (80 mM GdCl₃, 500 mM EDTA, pH 7.1 with NaOH) to the sample.

Authors' contributions

PW carried out the cloning and protein isolation and characterization. Both authors drafted the manuscript. JFH conceived of the study, and participated in its design and coordination. Both authors read and approved the final manuscript.

Acknowledgements

The authors would like to gratefully acknowledge the financial support of the University of Waterloo and NSERC (Canada). The authors would also like to thank Dr. J. Lam for the gift of *P. aeruginosa* strain PA01, Dr. E. Daub and Ms. I. Bruni for initial work on AprA, Drs. T. Lowther and B. Matthews for the plasmid encoding the *E. coli* methionine aminopeptidase and Dr. Mark Vaughan for helpful discussions. The authors thank Dr. R. Smith for

mass spectrometry support and Ms. J. Venne and V. Robertson for NMR analysis.

References

1. Stocker W, Bode W: **Structural features of a superfamily of zinc-endopeptidases: the metzincins.** *Curr Opin Struct Biol* 1995, **5**:383-390.
2. Lipscomb WN, Strater N: **Recent Advances in Zinc Enzymology.** *Chem Rev* 1996, **96**:2375-2434.
3. Gomis-Ruth FX: **Structural aspects of the metzincin clan of metalloendopeptidases.** *Mol Biotechnol* 2003, **24**:157-202.
4. Bode W, Gomis-Ruth FX, Stocker W: **Astacins, serralsins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'.** *FEBS Lett* 1993, **331**:134-140.
5. McCawley LJ, Matrisian LM: **Matrix metalloproteinases: they're not just for matrix anymore!** *Curr Opin Cell Biol* 2001, **13**:534-540.
6. Woessner JF, Nagase H: **Matrix Metalloproteinases and TIMPs.** Oxford, U.K., Oxford University Press, Inc.; 2000.
7. Brew K, Dinakarandian D, Nagase H: **Tissue inhibitors of metalloproteinases: evolution, structure and function.** *Biochim Biophys Acta* 2000, **1477**:267-283.
8. Nagase H, Woessner JF: **Matrix metalloproteinases.** *J Biol Chem* 1999, **274**:21491-21494.
9. Ikejiri M, Bernardo MM, Meroueh SO, Brown S, Chang M, Fridman R, Mobashery S: **Design, synthesis, and evaluation of a mechanism-based inhibitor for gelatinase A.** *J Org Chem* 2005, **70**:5709-5712.
10. Primakoff P, Myles DG: **Penetration, adhesion, and fusion in mammalian sperm-egg interaction.** *Science* 2002, **296**:2183-2185.
11. Kheradmand F, Werb Z: **Shedding light on sheddases: role in growth and development.** *Bioessays* 2002, **24**:8-12.
12. Van Eerdewegh P, Little RD, Dupuis J, Del Mastro RG, Falls K, Simon J, Torrey D, Pandit S, McKenny J, Braunschweiger K, Walsh A, Liu Z, Hayward B, Folz C, Manning SP, Bawa A, Saracino L, Thackston M, Benchekroun Y, Capparelli N, Wang M, Adair R, Feng Y, Dubois J, Fitzgerald MG, Huang H, Gibson R, Allen KM, Pedan A, Danzig MR, Umland SP, Egan RW, Cuss FM, Rorke S, Clough JB, Holloway JW, Holgate ST, Keith TP: **Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness.** *Nature* 2002, **418**:426-430.
13. Liao JK: **Shedding growth factors in cardiac hypertrophy.** *Nat Med* 2002, **8**:20-21.
14. Mock WL, Yao J: **Kinetic characterization of the serralsins: a divergent catalytic mechanism pertaining to astacin-type metalloproteases.** *Biochemistry* 1997, **36**:4949-4958.
15. Baumann U, Wu S, Flaherty KM, McKay DB: **Three-dimensional structure of the alkaline protease of *Pseudomonas aeruginosa*: a two-domain protein with a calcium binding parallel beta roll motif.** *Embo J* 1993, **12**:3357-3364.
16. Kharazmi A: **Mechanisms involved in the evasion of the host defence by *Pseudomonas aeruginosa*.** *Immunol Lett* 1991, **30**:201-205.
17. Steadman R, Heck LW, Abrahamson DR: ***Pseudomonas aeruginosa* as an Opportunistic Pathogen.** In *Infectious agents and pathogens* Edited by: Campa MBMFH. New York, Plenum Press; 1993.
18. O'Hagan D, Rzepa HS: **Some influences of fluorine in bioorganic chemistry.** *Chem Commun* 1997:645-652.
19. Frieden C, Hoeltzli SD, Bann JG: **The preparation of ¹⁹F-labeled proteins for NMR studies.** *Methods Enzymol* 2004, **380**:400-415.
20. Thomas JG, Ayling A, Baneyx F: **Molecular chaperones, folding catalysts, and the recovery of active recombinant proteins from *E. coli*. To fold or to refold.** *Appl Biochem Biotechnol* 1997, **66**:197-238.
21. Tsumoto K, Ejima D, Kumagai I, Arakawa T: **Practical considerations in refolding proteins from inclusion bodies.** *Protein Expr Purif* 2003, **28**:1-8.
22. Vuillard L, Rabilloud T, Goldberg ME: **Interactions of non-detergent sulfobetaines with early folding intermediates facilitate in vitro protein renaturation.** *Eur J Biochem* 1998, **256**:128-135.
23. Davis GD, Elisei C, Newham DM, Harrison RG: **New fusion protein systems designed to give soluble expression in *Escherichia coli*.** *Biotechnol Bioeng* 1999, **65**:382-388.

24. Atsumi Y, Yamamoto S, Morihara K, Fukushima J, Takeuchi H, Mizuki N, Kawamoto S, Okuda K: **Cloning and expression of the alkaline proteinase gene from *Pseudomonas aeruginosa* IFO 3455.** *J Bacteriol* 1989, **171**:5173-5175.
25. Okuda K, Morihara K, Atsumi Y, Takeuchi H, Kawamoto S, Kawasaki H, Suzuki K, Fukushima J: **Complete nucleotide sequence of the structural gene for alkaline proteinase from *Pseudomonas aeruginosa* IFO 3455.** *Infect Immun* 1990, **58**:4083-4088.
26. Duong F, Lazdunski A, Cami B, Murgier M: **Sequence of a cluster of genes controlling synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationships to other secretory pathways.** *Gene* 1992, **121**:47-54.
27. Houston ME, Harvath L, Honek JF: **Syntheses of and chemotactic responses elicited by (Met-Leu-Phe analogs containing difluoro- and trifluoromethionine.** *Bioorg Med Chem Lett* 1997, **7**:3007-3012.
28. Vaughan MD, Cleve P, Robinson V, Duewel HS, Honek JF: **Difluoromethionine a a Novel 19F NMR Structural Probe for Internal Amino Acid Packing in Proteins.** *J Am Chem Soc* 1999, **121**:8475-8478.
29. Louis D, Kohlmann M, Wallach J: **Spectrophotometric assay for amidolytic activity of alkaline protease from *Pseudomonas aeruginosa*.** *Anal Chim Acta* 1997, **345**:219-225.
30. Avidano MA, Cotter CS, Stringer SP, Schultz GS: **Analysis of protease activity in human otitis media.** *Otolaryngol Head Neck Surg* 1998, **119**:346-351.
31. Lomholt JA, Poulsen K, Kilian M: **Epidemic population structure of *Pseudomonas aeruginosa*: evidence for a clone that is pathogenic to the eye and that has a distinct combination of virulence factors.** *Infect Immun* 2001, **69**:6284-6295.
32. Suter S: **The role of bacterial proteases in the pathogenesis of cystic fibrosis.** *Am J Respir Crit Care Med* 1994, **150**:S118-22.
33. Matsumoto K: **Role of bacterial proteases in pseudomonal and serratal keratitis.** *Biol Chem* 2004, **385**:1007-1016.
34. Voet D, Voet JG, Pratt CW: **Fundamentals of Biochemistry.** 2nd edition. Hoboken, New Jersey, John Wiley & Sons, Inc.; 2005.
35. Lipscomb LA, Gassner NC, Snow SD, Eldridge AM, Baase WA, Drew DL, Matthews BW: **Context-dependent protein stabilization by methionine-to-leucine substitution shown in T4 lysozyme.** *Protein Sci* 1998, **7**:765-773.
36. Ohmura T, Ueda T, Hashimoto Y, Imoto T: **Tolerance of point substitution of methionine for isoleucine in hen egg white lysozyme.** *Protein Eng* 2001, **14**:421-425.
37. Hooper NM: **Families of zinc metalloproteases.** *FEBS Lett* 1994, **354**:1-6.
38. Stocker W, Grams F, Baumann U, Reinemer P, Gomis-Ruth FX, McKay DB, Bode W: **The metzincins--topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases.** *Protein Sci* 1995, **4**:823-840.
39. Butler GS, Tam EM, Overall CM: **The canonical methionine 392 of matrix metalloproteinase 2 (gelatinase A) is not required for catalytic efficiency or structural integrity: probing the role of the methionine-turn in the metzincin metalloprotease superfamily.** *J Biol Chem* 2004, **279**:15615-15620.
40. Hege T, Baumann U: **The conserved methionine residue of the metzincins: a site-directed mutagenesis study.** *J Mol Biol* 2001, **314**:181-186.
41. Pieper M, Betz M, Budisa N, Gomis-Ruth FX, Bode W, Tschesche H: **Expression, purification, characterization, and X-ray analysis of selenomethionine 215 variant of leukocyte collagenase.** *J Protein Chem* 1997, **16**:637-650.
42. Stack MS, Gray RD: **Comparison of vertebrate collagenase and gelatinase using a new fluorogenic substrate peptide.** *J Biol Chem* 1989, **264**:4277-4281.
43. Qoronfleh MW, Ho TF, Brake PG, Banks TM, Pulvino TA, Wahl RC, Eshraghi J, Chowdhury SK, Ciccarelli RB, Jones BN: **Production of selenomethionine-labeled recombinant human neutrophil collagenase in *Escherichia coli*.** *J Biotechnol* 1995, **39**:119-128.
44. Hendrickson TL, de Crecy-Lagard V, Schimmel P: **Incorporation of nonnatural amino acids into proteins.** *Annu Rev Biochem* 2004, **73**:147-176.
45. Hoshaka T, Abe R, Shiraga K, Sisido M: **Incorporation of fluorescently labeled nonnatural amino acids into proteins in an *E. coli* in vitro translation system.** *Nucleic Acids Res Suppl* 2003:271-272.
46. Cornish VW, Benson DR, Altenbach CA, Hideg K, Hubbell WL, Schultz PG: **Site-specific incorporation of biophysical probes into proteins.** *Proc Natl Acad Sci U S A* 1994, **91**:2910.
47. Salopek-Sondi B, Vaughan MD, Skeels MC, Honek JF, Luck LA: **(19)F NMR studies of the leucine-isoleucine-valine binding protein: evidence that a closed conformation exists in solution.** *J Biomol Struct Dyn* 2003, **21**:235-246.
48. Ye QZ, Johnson LL, Hupe DJ, Baragi V: **Purification and characterization of the human stromelysin catalytic domain expressed in *Escherichia coli*.** *Biochemistry* 1992, **31**:11231-11235.
49. Schnierer S, Kleine T, Gote T, Hillemann A, Knauper V, Tschesche H: **The recombinant catalytic domain of human neutrophil collagenase lacks type I collagen substrate specificity.** *Biochem Biophys Res Commun* 1993, **191**:319-326.
50. Reyda S, Jacob E, Zwilling R, Stocker W: **cDNA cloning, bacterial expression, in vitro renaturation and affinity purification of the zinc endopeptidase astacin.** *Biochem J* 1999, **344** Pt 3:851-857.
51. Guzzo J, Murgier M, Filloux A, Lazdunski A: **Cloning of the *Pseudomonas aeruginosa* alkaline protease gene and secretion of the protease into the medium by *Escherichia coli*.** *J Bacteriol* 1990, **172**:942-948.
52. Guzzo J, Pages JM, Duong F, Lazdunski A, Murgier M: ***Pseudomonas aeruginosa* alkaline protease: evidence for secretion genes and study of secretion mechanism.** *J Bacteriol* 1991, **173**:5290-5297.
53. Miyajima Y, Hata Y, Fukushima J, Kawamoto S, Okuda K, Shibano Y, Morihara K: **Long-range effect of mutation of calcium binding aspartates [correction of asparates] on the catalytic activity of alkaline protease from *Pseudomonas aeruginosa*.** *J Biochem (Tokyo)* 1998, **123**:24-27.
54. Carrio MM, Villaverde A: **Construction and deconstruction of bacterial inclusion bodies.** *J Biotechnol* 2002, **96**:3-12.
55. Budisa N, Pipitone O, Siwanowicz I, Rubini M, Pal PP, Holak TA, Gelmi ML: **Efforts towards the design of "Teflon" proteins: in vivo translation with trifluorinated leucine and methionine analogues.** *Chem Biodivers* 2004, **1**:1465-1475.
56. Luck LA, Falke JJ: **Open conformation of a substrate-binding cleft: 19F NMR studies of cleft angle in the D-galactose chemosensory receptor.** *Biochemistry* 1991, **30**:6484-6490.
57. Bordo D, Argos P: **Suggestions for "safe" residue substitutions in site-directed mutagenesis.** *J Mol Biol* 1991, **217**:721-729.
58. Duewel HS, Daub E, Robinson V, Honek JF: **Elucidation of solvent exposure, side-chain reactivity, and steric demands of the trifluoromethionine residue in a recombinant protein.** *Biochemistry* 2001, **40**:13167-13176.
59. Miyatake H, Hata Y, Fujii T, Hamada K, Morihara K, Katsube Y: **Crystal structure of the unliganded alkaline protease from *Pseudomonas aeruginosa* IFO3080 and its conformational changes on ligand binding.** *J Biochem (Tokyo)* 1995, **118**:474-479.
60. Lowther WT, Zhang Y, Sampson PB, Honek JF, Matthews BW: **Insights into the mechanism of *Escherichia coli* methionine aminopeptidase from the structural analysis of reaction products and phosphorus-based transition-state analogues.** *Biochemistry* 1999, **38**:14810-14819.
61. ProtParam: [<http://us.expasy.org/tools/protparam.html>].
62. Ladbury JE, Chowdhry BZ: **Biocalorimetry: applications of calorimetry in the biological sciences.** Chichester, John Wiley and Sons, Ltd.; 1998:345.
63. Fersht A: **Structure and Mechanism in Protein Science.** New York, W. H. Freeman and Company; 1999.