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Characterization of Aminopeptidase in the Free-living Nematode *Panagrellus redivivus*: Subcellular Distribution and Possible Role in Neuropeptide Metabolism

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Abstract: Aminopeptidase was detected in homogenates of the free-living nematode *Panagrellus redivivus* with the aminoacyl substrate L-alanine-4-nitroanilide. Subcellular distribution of activity was 80% soluble and 20% membrane-associated. Aminopeptidases in the two fractions differed in affinity for Ala-4-NA, with Km's of 0.65 mM (soluble) and 2.90 mM (membrane). Specific activities (units/mg) at pH 7.8, 27°C were 9.10 (soluble) and 14.30 (membrane). Each enzyme was competitively inhibited by amastatin (90% at 100 µM inhibitor, IC_{50} = 3.7 µM) and inhibited by puromycin (30% at 500 µM) and 1,10-phenanthroline ($IC_{50's}$; 148 µM, soluble; 89 µM, membrane). Activity was restored by Zn^{++}, with maximum recoveries of 50% (soluble) and 90% (membrane), each at 23 µM $ZnCl_2$. Estimated molecular masses for each were ~150 kDa. FMRFamide-like neuropeptides behaved as competitive inhibitors. Modification of the N-terminal F of FMRFamide weakened inhibition by 95%, suggesting that the N-terminus is essential for binding to the enzyme. Two nematode FMRFamides, APKPFIRFa and RNKFEFIRFa, were the most potent tested. This is the first biochemical characterization of aminopeptidase in a free-living nematode other than *Caenorhabditis elegans* and demonstrates the high selectivity of the *P. redivivus* enzymes for neuropeptide substrates.

Key words: FMRFamide-like peptide, inhibitor; membrane, metallopeptidase, neuropeptide, protease

Nematodes, like other eukaryotic organisms, depend upon proteolytic enzymes for the regulation of essential developmental, metabolic and physiological events. Aminopeptidases are members of clans of proteases involved in many of these diverse processes (Barrett et al., 2004), are present in both the cytoplasm and as membrane components, and comprise a wide variety of structures and activities (Taylor, 1993).

In nematodes, aminopeptidases are associated with intestinal digestion and nourishment for embryogenesis (Joshua, 2001; Laurent et al., 2001; Brooks et al., 2003), neuropeptide processing and signal attenuation (Sajid and Isaac, 1995; Sajid et al., 1996, 1997; Masler, 2002), molting (Hong and Bouvier, 1993; Rhoads et al., 1997; Rhoads and Fetterer, 1998) and reproduction (Brooks et al., 2003; Lilley et al., 2005). Investigation of the interactions of aminopeptidases with neuropeptides has intensified because of the growing interest in neuropeptides as targets for parasite control (Maule et al., 2002) and because of growing evidence for the presence in nematodes of large neuropeptide families including the FMRFamide-related peptides (Li et al., 1999; Kim and Li, 2004; McVeigh et al., 2005), insulins (Pierce et al., 2001) and others (Nathoo et al., 2001). The FMRFamide peptides have been the most widely studied group in nematodes because of their ubiquity and their involvement in the regulation of muscle contraction, with its effects upon feeding, locomotion and reproduction (Day and Maule, 1999; Maule et al., 2002; Rogers et al., 2003).

Panagrellus redivivus Goodey is a bacteriophagous, oviviparous, free-living soil nematode, being one of the few that do not lay eggs, but hatch juveniles internally (Perry, 2002). It is gonochoristic, producing equal numbers of males and females, in contrast to the widely studied hermaphrodite, *Caenorhabditis elegans* Maupas. *Panagrellus redivivus* thus presents developmental contrasts with other nematodes and provides useful comparisons for studying basic physiological and biochemical mechanisms. Aminopeptidase and the digestion of peptides have been described in *P. redivivus* (Masler et al., 2001; Masler, 2002). The present report characterizes aminopeptidases in cytosolic and membrane fractions from *P. redivivus* and demonstrates substrate preferences among a variety of nematode and non-nematode neuropeptides.

Materials and Methods

Animals and sample preparation: Panagrellus redivivus was grown axenically in liquid medium (Chitwood et al., 1995), and 1-wk old cultures containing mixed stages (approximately 1.3 µl worms/10 µl medium) were harvested. Nematodes were rinsed 5 times with autoclaved water using 10 to 15 ml water/ml of settled worms for each rinse, then stored at -80°C until use. Extracts were prepared by homogenizing frozen nematodes in 10 volumes of autoclaved HPLC-grade water per 1 volume of nematodes using a methanol-washed ground glass homogenizer. Homogenates were centrifuged (40,000*g*, 5°C), and the supernatants were collected. Pellets were washed 3 times in water by vigorous agitation, followed each time by centrifugation as above. Washes were pooled with the original supernatant, and the pool was designated as the soluble fraction. Aliquots were dried in a SpeedVac (Savant Instruments, Farmingdale, NY) and stored at –20°C. Aliquots of washed pellets were dried and stored as above.

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Membranes were prepared by processing dried pellets using a Native Membrane Protein Extraction Kit (Calbiochem, San Diego, CA) following the manufacturer's protocol for frozen tissue. The protocol was modified to accommodate small processing volumes (50–100 µl), and the proprietary solubilization and wash buffers were tested in the enzyme assay and found not to interfere with activity. The extraction yielded solubilized membrane proteins, designated as the membrane fraction. This fraction was used immediately or stored at 5°C overnight. Total proteins were estimated by microBCA assay (Pierce Chemical Co., Rockford, IL) following the manufacturer's protocol.

Protease assay and inhibitor screening: Aminopeptidase activity was measured using methods modified from Masler et al. (2001). Reaction buffer used to prepare all reaction components was 100 mM Tris-HCl, pH 7.8. Reactions were initiated by the addition of the substrate L-alanine-4-nitroanilide (Ala-4-NA: Fluka/Sigma, St. Louis, MO) to enzyme (soluble or membrane) in a 96-well microtiter plate (Corning Plastics, Corning, NY) in a total reaction volume of 180 µl. Substrate concentrations varied from 0.5 mM to 8 mM, depending upon the experiment. Typical enzyme sample amounts were 5.5 µg (soluble) and 6.4 µg (membrane)/reaction. Progress of the reactions was monitored over time by increased absorbance (410 nm, 27°C) from the production of para-nitroaniline (pNA). A reference standard of pNA (Sigma) was determined by linear regression to absorb 30.74 mAU/nMol at 410 nm. Using this value, 1 Unit of *P. redivivus* aminopeptidase activity is defined as 1 nMol pNA produced/min at 27°C. Kinetic characteristics (Vmax and Km) were determined using Lineweaver-Burk double reciprocal plots. pH tolerance was estimated by preparing 100 mM Tris-HCl buffers as a pH series between 6.0 and 9.2. Samples and substrate were each prepared in the different buffers, and the assay was performed as described.

Thermal tolerance of the enzymes was determined by incubating individual samples, prepared in 100 mM Tris-HCl buffer, pH 7.8, for 1 hr in a thermal gradient from 22°C to 48°C. The temperature-treated samples were allowed to equilibrate to 27°C and were transferred to a microtiter plate for aminopeptidase assay.

Protease inhibitors (amastatin, epiamastatin, bestatin, epibestatin and puromycin; Sigma) were each prepared in reaction buffer, mixed with enzyme samples and incubated for 30 min at 27°C prior to addition of substrate. 1,10-phenanthroline (Sigma) in reaction buffer was mixed with enzyme and incubated for 60 min at 27°C prior to adding substrate. $ZnCl_2$ (GFS Chemicals, Columbus, OH) was added with substrate. Total reaction volumes were maintained at 180 µl. Neuropeptides were screened in the same way, except that there was no pre-incubation with enzyme. Amidated neuropeptides screened were nAcFnLRFa, FLFQPQRFa, FMRFa, FMRDFa, FMDRFa, FDMRFa, DFMRFa, FLRFa, pEDFLRFa, pEGRFa, pESLRWa, YGGFMRRVa (Sigma), KHEYLRFa, KPNFLRFa, RNKFEFIRFa, KNEFIRFa and APKPKFIRFa (gifts of D. P. Thompson, Pfizer Animal Health, Kalamazoo, MI).

Molecular mass estimates: Native molecular mass of the soluble enzyme was estimated by high-performance size exclusion chromatography (HP-SEC) using Progel 3000 size exclusion (300 x 7.8 mm) and Progel 3000 guard (75 x 7.8 mm) columns (Phenomenex, Torrance, CA). Elution buffer was 100 mM Tris-HCl, pH 7.8, with 300 mM NaCl. Flow rate was 0.5 ml/min. Fractions were collected in 96-well plates and assayed directly. The column was calibrated using Dextran blue and protein markers ranging from 18 kDa to 240 kDa (Sigma). Supplementary molecular size estimates were done on both the soluble and membrane-associated enzymes using non-denaturing PAGE. Test samples were dissolved in PAGE sample buffer (10 mM Tris-HCl, pH 8.8, 0.0025% bromophenol blue, 10% glycerol) and separated on gradient gels (4–20%; NOVEX/Invitrogen, Carlsbad, CA) using pH 8.6 running buffer (24 mM Tris, 192 mM glycine). Following electrophoresis, sample lanes were cut sequentially into 2.5 mm sections, and each was incubated overnight at 27°C in 300 µl reaction buffer containing 8 mM Ala-4-NA. Reference marker proteins applied to adjacent lanes were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa) (Amersham/Pharmacia, Piscataway, NJ).

Data analysis: Data were analyzed by linear and nonlinear regressions using Excel (Microsoft, Redmond, WA), and means were compared by *t*-test using SigmaStat (Systat, Point Richmond, CA).

Results

Aminopeptidase activity was detected in both soluble and membrane fractions prepared from *P. redivivus*. Maximum specific activities (Units/mg total protein) were observed at pH 7.8 for the soluble enzyme and pH 7.3 for the membrane form (Fig. 1). Each enzyme was sensitive to alkaline pH, since activities showed distinct declines by pH 8. Significant losses of activity, relative to maximum values, were observed at pH 8.6 for the soluble enzyme ($P < .01$, n = 4–5) and at pH 8.2 for the membrane aminopeptidase ($P < .001$, n = 3). Approximately 50% of activity was lost at pH 8.6 (soluble) and pH 8.7 (membrane), and affinity for the substrate, determined with the soluble enzyme, was also affected by pH. Change in pH from 7.8 to 8.1 caused a significant increase in Km, from 0.63 ± 0.12 mM Ala-4-NA to 1.35 ± 0.36 mM ($P = 0.001$; n = 7–8).

Aminopeptidase activity in both soluble and membrane fractions declined steadily after 1 hr of exposure to temperatures above 30°C (Fig. 2). The loss of activity reached 50% for the membrane and soluble forms at 36.7°C and 39.8°C, respectively. Lost activity was most

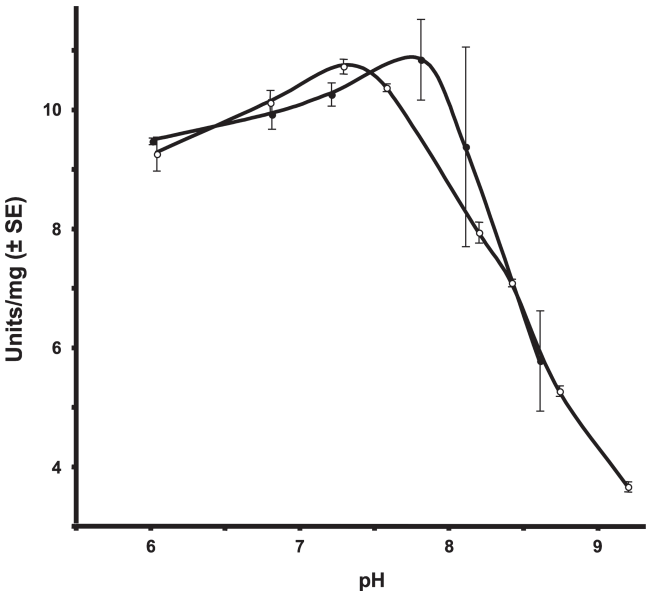

FIG. 1. Effect of pH on aminopeptidase activity in extracts of *Panagrellus redivivus*. Extracts were prepared as described, and aliquots of soluble (●) and membrane (○) fractions were dissolved in a series of 100 mM Tris-HCl buffers prepared from pH 6.0 through 9.2. Activity is expressed as mean Units of aminopeptidase activity/mg protein ± SE of 4 to 6 separate assays.

likely due to aminopeptidase denaturation, since activity was not restored after extended incubation with substrate at 27°C. Curiously, nearly all activity was lost from the membrane fraction at 47°C, whereas maximum loss of activity from the soluble fraction was 80%, even at 48°C (Fig. 2).

Soluble aminopeptidase applied to the HP-SEC column eluted primarily as a single peak with an estimated molecular mass of 150 kDa (Fig. 3, peak 1) and with a second apparent peak of activity of 100 kDa (Fig. 3, peak 2). Total aminopeptidase activity recovered in peaks 1 and 2 was 43%. When both soluble and membrane enzymes were analyzed using native PAGE, they co-migrated in the gel (not shown), suggesting similar molecular masses. Aminopeptidase activity from each fraction was located between the albumin (67 kDa) and lactate dehydrogenase (140 kDa) markers.

The distribution of aminopeptidase activity between the soluble (cytosolic) and membrane fractions was unequal. Significantly more ($P = 0.004$; n = 4) activity was detected in the cytosol (77.7%) than in the membrane fraction (23.3%). Also, substrate affinity was more than 4-fold greater for the soluble enzyme (Km = 0.65 ± 0.07 mM Ala-4-NA; n = 12) than for the membrane enzyme (2.90 ± 0.55; n = 4) (Fig. 4, $P = 0.001$). Specific activity (Units/mg) of the membrane enzyme was greater (14.30 ± 1.63; n = 4) than for the soluble enzyme (9.10 ± 0.65; n = 11) ($P = 0.001$).

The activities of both enzymes were sharply reduced by the aminopeptidase inhibitor amastatin (Table 1). Each was inhibited 90% at 100 µM amastatin, with IC_{50} = 3.7 µM. Amastatin was a competitive inhibitor of each aminopeptidase and increased Km from 0.65 mM Ala-4-NA to 6.73 mM for the soluble enzyme (3.7 µM amastatin) and from 2.90 mM Ala-4-NA to 3.58 mM (3.7 µM amastatin) and to 7.08 mM (37 µM amastatin) for the membrane enzyme. Epiamastatin was less effective

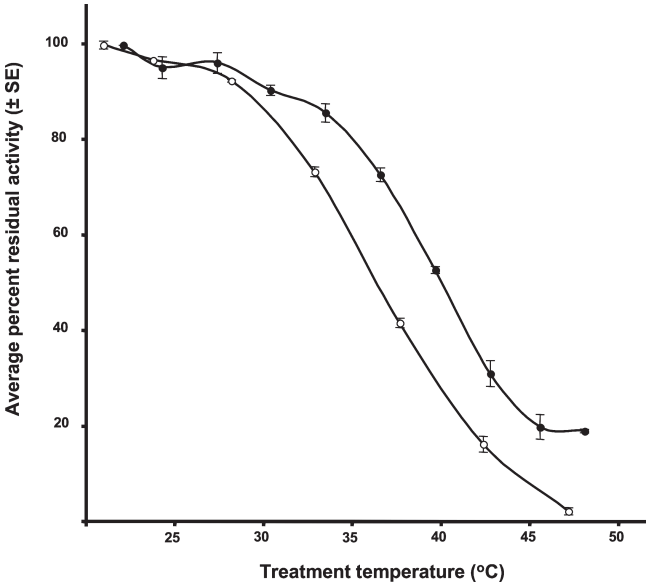

FIG. 2. Thermotolerance of *Panagrellus redivivus* aminopeptidase. Extracts were prepared as described, and aliquots of soluble (●) and membrane (○) fractions were dissolved in 100 mM Tris-HCl, pH 7.8, and then incubated for 1 hr at temperatures from 22°C to 48°C. Samples were then assayed for enzyme activity as described. Data are expressed as the percent of aminopeptidase activity detected relative to non-heat treated control extracts. Each data point is the mean ± SE of 3 to 4 replicates.

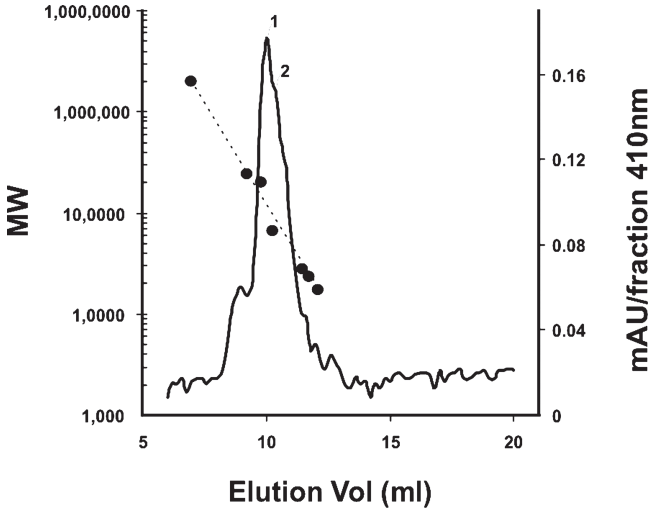

FIG. 3. Estimate of *Panagrellus redivivus* aminopeptidase molecular mass by HP-SEC. Extract (soluble fraction) was dissolved in 100 mM Tris-HCl containing 300 mM NaCl, pH 7.8. Proteins were injected onto the size-exclusion column in 79 µg samples (see Materials and Methods) and eluted at 0.5 ml/min in sample buffer, and 240 µl fractions were collected. Aliquots of each fraction were assayed as described (210 µl final reaction volume, 4 mM substrate). Solid line: Absorbance (410 nm) of fractions following aminopeptidase assay. Dotted line: Molecular mass standards regression used to calibrate column.

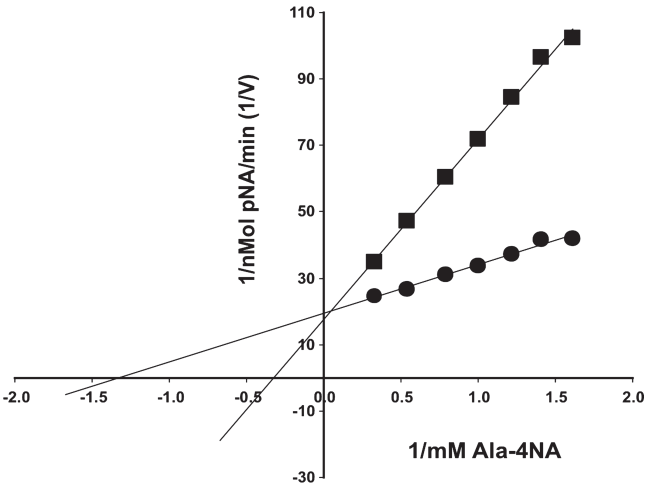

FIG. 4. Typical activities of aminopeptidase from *Panagrellus redivivus* soluble (●) and membrane (■) fractions. V = nMol pNA/min.

as an inhibitor of soluble aminopeptidase than amastatin; bestatin, epibestatin and puromycin were ineffective (Table 1) except when tested (e.g., puromycin) at a relatively high concentration.

Aminopeptidase activities in each fraction were inhibited nearly 90% by 330 µM 1,10-phenanthroline, with IC_{50} of 148 µM for the soluble enzyme and 89 µM for the membrane form (Table 1). The two forms differed in their response to added Zn^{++} (Fig. 5). In the presence of 330 µM 1,10-phenanthroline, soluble activity was restored maximally (48%) at 23 µM $ZnCl_2$. In contrast, membrane aminopeptidase activity was restored to 50% at 6 µM $ZnCl_2$ and 87% at 23 µM.

A broad array of amidated neuropeptides screened against both the soluble and membrane enzymes (Table 2) showed that aminopeptidase activity in each preparation was reduced by the presence of a number of different peptides. Generally, inhibition of the soluble aminopeptidase was marginally higher, with a few exceptions, than that of membrane aminopeptidase. Peptides with modified (DFMRF, nAcFnLRFa) or blocked (pEDPFLRFa, pEGRFa, pESLRWa) N-terminals were poor inhibitors of either enzyme. Peptide length bore no correlation to efficacy as an inhibitor. However, position of residue modification was critical. Substitution of D-amino acids in FMRFa decreased

TABLE 1. Effect of protease inhibitors on aminopeptidase activities in *Panagrellus redivivus*.

Inhibitor	µM	Percent inhibition[a] Soluble	Percent inhibition[a] Membrane	IC_{50} (µM) Soluble	IC_{50} (µM) Membrane
Amastatin	100	92	90	3.7	3.7
″	20	89	—[b]	—	—
Epiamastatin	100	78	—	—	—
″	20	40	—	—	—
Bestatin	100	16	—	—	—
Epibestatin	100	3	—	—	—
Puromycin	500	30	30	—	—
″	100	11	4	—	—
1,10-phenanthroline	330	88	87	148	89

[a] Percent inhibition is average of two separate experiments.
[b] — = not tested.

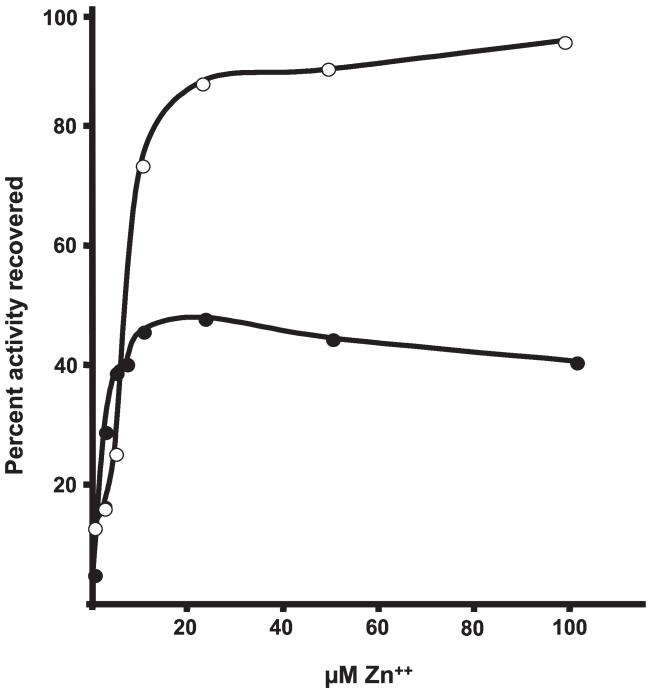

FIG. 5. Rescue of *Panagrellus redivivus* soluble aminopeptidase activity from inhibition by 1,10-phenanthroline with by Zn^{++}. Extracts were prepared as described. Aliquots of soluble (●) and membrane (○) fractions were dissolved in 100 mM Tris-HCl, pH 7.8, and incubated with 330 µM 1,10-phenanthroline for 1 hr to inhibit aminopeptidase. Subsequently, aminopeptidase activity was measured (27°C, 3 mM Ala-4-NA) in the presence of varying concentrations of $ZnCl_2$. Results are expressed as the means (2–3 experiments) of the percent aminopeptidase activity recovered vs. control (no 1,10-phenanthroline, no $ZnCl_2$).

TABLE 2. Effect of amidated neuropeptides on apparent aminopeptidase activities in *Panagrellus redivivus*.

Peptide[b]	Percent inhibition[a] Soluble	Percent inhibition[a] Membrane	Origin[c]
pEDPFLRFa	24	19	Mollusk
pEGRFa	13	0	Sea anemone
pESLRWa	16	25	Sea anemone
nAcFnLRFa	9	—	Synthetic analog
YGGFMRRVa	35	28	Mammal
FLFQPQRFa	30	31	Mammal
FMRFa	63	38	Mollusk
FMRDFa	48	30	Synthetic analog
FMDRFa	17	20	Synthetic analog
FDMRFa	19	21	Synthetic analog
DFMRFa	5	19	Synthetic analog
FLRFa	59	35	Mollusk
KHEYLRFa	40	27	Nematode^{Ce,Pr,apn,ppn}
KPNFLRFa	53	34	Nematode^{Ce,apn}
RNKFEFIRFa	58	36	Nematode^{Ce,apn,ppn}
KNEFIRFa	52	26	Nematode^{Ce,apn,ppn}
APKPKFIRFa	65	30	Nematode^{Ce,ppn}

[a] Average of 2 to 4 separate experiments.
[b] Concentration of peptide in all experiments was 100 µM.
[c] Identified/predicted in *Caenorhabditis elegans*^{Ce}, *P. redivivus*^{Pr}, animal-parasitic nematodes^{apn}, and plant-parasitic nematodes^{ppn}.

the effectiveness of the peptide as an aminopeptidase inhibitor, and this decrease was more pronounced as the substitution occurred closer to the N-terminus (Table 2). With both the soluble and membrane enzymes, substitution of D-phenylalanine at the C-terminus decreased the value of the peptide as an inhibitor by 20 to 30%. Efficacy was further reduced by substitutions at arginine and methionine and was lowest when N-terminal phenylalanine was substituted, where effects were reduced 50% with the membrane aminopeptidase and 90% with the soluble enzyme.

Inhibition of *P. redivivus* aminopeptidase activity by neuropeptides was competitive (Fig. 6). FMRFa and FLRFa each increased apparent Km by 5-fold at 50 µM peptide (Km = 0.65 mM Ala-4-NA, control; 3.3 mM, FMRFa; 3.4 mM FLRFa) and 10-fold at 100 µM peptide (7.0 mM Ala-4-NA, FMRFa; 9.2 mM, FLRFa). In addition, both FMRFa and FLRFa had identical IC_{50} values (200 µM peptide). The IC_{50} for four other neuropeptides, each of which scored high in the peptide screen (Table 2) and have been identified in nematodes, were determined: KHEYLRFa (400 µM), KPNFLRFa (250 µM), APKPKFIRFa (80 µM) and RNKFEFIRFa (75 µM).

Discussion

Nematode aminopeptidases reflect the biochemical diversity of this large group of proteases (Taylor, 1993; Rawlings and Barrett, 2004). In animal-parasitic nematodes, a secreted ~260 kDa glycoprotein oligomer with aminopeptidase activity was cloned from *Acanthocheilonema vitae* (Harnett et al., 1999), a soluble 290 kDa metalloaminopeptidase heterodimer was isolated from *Ascaris suum* (Rhoads and Fetterer, 1998) and a 240 kDa monomeric aminopeptidase was purified from *A. suum* membranes (Sajid et al., 1997). This latter aminopeptidase metabolized amidated neuropeptides and, like the *P. redivivus* enzymes, was sensitive to N-terminal modifications of the peptide substrates. A 110 kDa membrane aminopeptidase monomer cloned from *Haemonchus contortus* (Smith et al., 1997) contained a conserved zinc binding motif and was sensitive to 1,10-phenanthroline inhibition. Aminopeptidases characterized from the free-living *C. elegans* include a 69 kDa soluble zinc metalloenzyme (AP-1; Baset et al., 1998), a 52 kDa leucine aminopeptidase (Joshua, 2001) with the conserved zinc binding motif, a soluble puromycin-sensitive 105 kDa monomer (PAM-1; Brooks et al., 2003) inhibited by 1,10-phenanthroline and rescued by Zn^{++}, and a soluble 71 kDa zinc-dependent monomer (AP-P; Laurent et al., 2001) that metabolizes neuropeptides. In contrast to other nematode aminopeptidases, AP-P activity inhibited by 1,10-phenanthroline could not be rescued with divalent metal cations (Laurent et al., 2001). Aminopeptidases detected in soluble fractions of *C. elegans* and *P. redivivus* extracts (Masler, 2002) had similar substrate affinities and specific activities, but had clearly different sensitivities to aminopeptidase inhibitors. Comparisons between aminopeptidases from *C. elegans* and the plant-parasitic nematode *Heterodera glycines* (Masler et al., 2001; Masler, 2004) revealed similarities in substrate affinities, but differences in subcellular distribution and response to divalent metal cations. Leucine aminopeptidase has been reported in eggs of *H. glycines* (Tefft and Bone, 1985), and an *H. glycines* aminopeptidase with similarity to *C. elegans* PAM-1 has been cloned (Lilley et al., 2005). The predicted 882 residue enzyme contains a conserved zinc-binding motif and appears to be a cytosolic aminopeptidase.

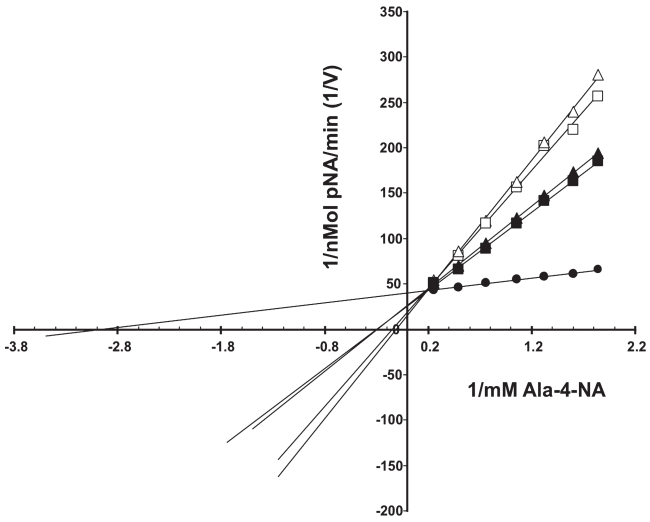

Fig. 6. Effect of FMRFa and FLRFa on apparent Km of *Panagrellus redivivus* aminopeptidase activity. Peptides were mixed with samples just prior to adding substrate. Each was tested at 3.7 µM (FLRFa = ■; FMRFa = ▲) and 37 µM (FLRFa = □; FMRFa = △). Controls (●) contained no peptide. V = nMol pNA/min.

The aminopeptidase activities detected in *P. redivivus* homogenates suggest neutral aminopeptidases (Taylor, 1993), with selective sensitivities to aminopeptidase inhibitors and distribution between at least two subcellular fractions. Each *P. redivivus* aminopeptidase appears to be a metalloenzyme and neither is highly susceptible to puromycin. This latter characteristic they share with *C. elegans* PAM-1, found to be much less sensitive to puromycin than aminopeptidase homologs from non-nematodes (Brooks et al., 2003).

The estimated molecular mass of the *P. redivivus* aminopeptidases is within the broad range reported for a variety of nematode aminopeptidases (Sajid et al., 1997; Smith et al., 1997; Rhoads and Fetterer, 1998; Harnett et al., 1999; Joshua, 2001). The moderate recovery of the soluble form following chromatography may be due to a combination of factors including physical loss, effect of salt used in the elution buffer and reduced exposure to co-factors (e.g., divalent metal cations) following fractionation.

Heat lability of both the soluble and membrane en-

zymes caused gradual declines in activity with similar shapes for the loss curves. However, the soluble form consistently exhibited a slightly greater (2–3°C) stability to heat than the membrane enzyme, a characteristic that should be explored further with purified enzymes. The residual 20% activity of the soluble enzyme at 48°C is a bit curious, since nearly all of the membrane enzyme activity was lost by 47°C. However, at 60°C (data not shown), essentially all soluble aminopeptidase activity was lost, similar to what has been reported for *C. elegans* and *H. glycines* soluble aminopeptidases (Masler et al., 2001). Although heat-induced loss of measurable activities in *P. redivivus* aminopeptidases was gradual, there was a sharp drop in substrate affinity, determined with the soluble form. Km estimates revealed a two-fold decrease in affinity from 27°C to 37°C, suggesting a structural change occurred in the soluble aminopeptidase. The responses of the enzymes to temperature are of physiological interest. Laws et al. (2005) demonstrated that *P. redivivus* grown at 25°C was able to survive for several days at 37°C, but was not able to produce viable progeny, and reported that development of progeny was suppressed at 31 to 32°C. The parallels between the decline of activity and substrate affinity of *P. redivivus* aminopeptidase and the survival and development of the nematode are interesting.

The inhibition of *P. redivivus* soluble and membrane aminopeptidases by 1,10-phenanthroline indicates that they are metalloproteases, and activity rescue by $ZnCl_2$ places them in the M1 family of zinc-dependent exopeptidases (Rawlings and Barrett, 2004). The differential responses of the two *P. redivivus* enzymes to zinc rescue was striking and may be an important biochemical feature deserving of future analysis. It is interesting that a soluble aminopeptidase from *C. elegans*, which was completely inhibited by 1,10-phenanthroline, was restored to 80% activity by addition of Zn^{++} (Brooks et al., 2003). This is in contrast to the 50% recovery by the *P. redivivus* soluble aminopeptidase. However, the *C. elegans* aminopeptidase was a purified recombinant enzyme, while the *P. redivivus* aminopeptidase was present in a crude fraction, so comparative results should be interpreted cautiously. Nevertheless, the contrast between the *P. redivivus* membrane and soluble enzyme responses to Zn^{++} remains compelling. Finally, it is of interest to note that, following chromatographic fractionation, less than 50% of *P. redivivus* soluble aminopeptidase activity was recovered. Perhaps reduced access to Zn^{++} may account for some of this loss.

The presence of soluble and membrane forms of aminopeptidase in *P. redivivus* has been suggested (Masler et al., 2001), and the distribution between the two fractions reported here is similar to that found in another free-living nematode, *C. elegans* (Masler et al., 2001). Soluble (Harnett et al., 1999; Laurent et al., 2001; Brooks et al., 2003) and membrane-associated (Sajid et al., 1997; Smith et al., 1997) forms of aminopeptidase have been characterized from a number of nematodes. Functions of membrane-associated aminopeptidases in animals include cleavage of peptide fragments that are products of digestion of larger proteins in the gut and attenuation of neuropeptide signals. These aminopeptidases may be integral membrane proteins or merely membrane-associated and removed from the membrane through rather mild treatment (Sajid et al., 1997). The *P. redivivus* membrane aminopeptidase appears not to be weakly associated with membranes since the procedure used to obtain the enzyme was designed to solubilize membrane proteins. Furthermore, pellets were thoroughly washed prior to the membrane extraction.

During the extraction procedure, an initial solubilizing wash of the pellet was performed. Over 40% of all solubilized pellet protein was in this wash. In contrast, nearly 90% of aminopeptidase activity remained with the membranes and was released following a more aggressive second extraction step. Consequently, the *P. redivivus* membrane aminopeptidase appears to be an integral membrane protein. The soluble aminopeptidase may be cytosolic or secreted, since both are present in nematodes, but present information is insufficient to assign *P. redivivus* aminopeptidase to one or the other of these categories. Nevertheless, aminopeptidase activities in soluble and membrane fractions from *P. redivivus* clearly suggest differences in substrate affinities, solubilities and responses to rescue by $ZnCl_2$ of the enzymes involved. These observations provide a strong argument for at least two different aminopeptidases in *P. redivivus*.

Some indication of the physiological function of proteases can be deduced from their biochemical characterization, and, while physiological roles for aminopeptidases in nematodes are varied, one of the most important is the metabolism of neuropeptides. Nematodes possess large numbers of genes coding for numerous families of neuropeptides (Nathoo et al., 2001; Masler, 2006), many of which are involved with neuromuscular coordination and its effects upon feeding, reproduction and survival. The largest and most diverse family of nematode neuropeptides comprises the FMRFamide-related peptides, characterized by a C-terminal amidated RF motif ubiquitous among invertebrates (McVeigh et al., 2005). Although FMRFamide is common among insects and mollusks, neither it nor its analog FLRFamide have been discovered in nematodes, although the number of variations on the MRFamide, LRFamide and RFamide motifs in nematodes is enormous (McVeigh et al., 2005). This variety attests to their importance, and they have been characterized from parasitic and free-living species.

The metabolism of FMRFamide-related peptides by endo- and exopeptidases has been demonstrated in free-living *C. elegans* (Laurent et al., 2001) and the animal parasite *Ascaris suum* (Sajid et al., 1996, 1997), and

neuropeptide digestion by *P. redivivus* homogenates has been reported (Masler, 2002). The present work demonstrates the interaction between FMRFamide-like peptides and *P. redivivus* aminopeptidase. Results using FMRFa and FLRFa indicate that FMRFamide-related peptides can act as competitors with the Ala-4-NA substrate for interaction with the aminopeptidase, presumably as alternative substrates.

Not surprisingly for an aminopeptidase, N-terminally blocked or modified peptides are poor substrates. What is notable for this *P. redivivus* enzyme is its sensitivity to substitutions in the N-terminus. Apparently, binding to the active site of the enzyme, as assessed by the ability of the peptide to compete with or displace Ala-4-NA, is severely hindered by substitution of D-Phe for L-Phe at position 1 (N-terminal). This result agrees with observations of *A. suum* aminopeptidase, where digestion of FMRFamide was prevented by the same substitution (Sajid et al., 1997). Sensitivity of the *P. redivivus* aminopeptidase active site to N-terminal structure is further illustrated by the gradual decline in the inhibition of aminopeptidase activity as the D-substituted amino acid in the competing FMRFamide is moved closer to the C-terminus.

Selectivity of the *P. redivivus* enzyme is clearly demonstrated by its response to a series of FMRFamide-like peptides. Two sequences identified in nematodes (KHEYLRFa and KPNFLRFa) had higher IC_{50} than the reference FMRFa and FLRFa. Two other nematode sequences (RNKFEFIRFa and APKPKFIRFa) were 2 to 2.5-fold more preferred by the enzyme than the reference sequences.

It is of interest to note that a penultimate proline can protect neuropeptides from degradation by many aminopeptidases (Mentlein, 1988), and that KPNFIRFamide, isolated from *P. redivivus*, is an example of such a peptide (Kubiak et al., 1996). Also, *C. elegans* AP-P (Laurent et al., 2001) required a penultimate proline to specifically cleave the N-terminal residue from peptides. In *P. redivivus*, both KPNFLRFa and APKPKFIRFa can apparently serve as substrates at least as well as KHEYLRFa and RNKFEFIRFa.

The D-amino acid data and the sequence IC_{50} data describe an aminopeptidase highly responsive to, and selective of, potential neuropeptide substrates. In addition, the manner in which neuropeptides with and without penultimate prolines are both effective in competing with Ala-4-NA for binding to *P. redivivus* aminopeptidase presents intriguing questions about enzyme specificity and regulation. Since experiments with *P. redivivus* were done using a heterogeneous fraction, some caution must be exercised in interpreting the peptide substrate experiments. Endoproteases in the extract could produce cleavage products that affect the interaction between added peptide substrate and aminopeptidase. With the availability of purified enzyme, and the identification of cleavage products (e.g., by mass spectrometry), such biochemical questions will be resolved.

The rapidly expanding number and diversity of neuropeptides predicted and described in nematodes indicates the importance of these regulatory molecules for nematode development and growth. Examining the regulation of neuropeptide titer and signal attenuation, through responsive and selective proteolytic action, will be essential to understanding how these peptides perform their roles.

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