Proteolytic Degradation of Hemoglobin in the Intestine of the Human Hookworm 

Necator americanus

Najju Ranjit,1,3 Bin Zhan,5 Brett Hamilton,2 Deborah Stenzel,3 Jonathan Lowther,4 Mark Pearson,1 Jeffrey Gorman,2 Peter Hotez,5 and Alex Loukas1

1Helminth Biology Laboratory and 2Protein Discovery Centre, Division of Infectious Diseases, Queensland Institute of Medical Research, and 3School of Life Sciences, Queensland University of Technology, Brisbane, and 4Institute for the Biotechnology of Infectious Diseases, University of Technology, Sydney, Australia; 5Department of Microbiology, Immunology, and Tropical Medicine, George Washington University, Washington, DC

Blood-feeding parasites use mechanistically distinct proteases to digest hemoglobin (Hb), often as multienzyme cooperative cascades. We investigated the roles played by 3 distinct proteases from adults of the human hookworm Necator americanus. The aspartic protease Na-APR-1 and the cysteine protease Na-CP-3 were expressed in catalytically active form in yeast, and the metalloprotease Na-MEP-1 was expressed in catalytically active form in baculovirus. Antibodies to all 3 proteases were used to immunolocalize each native enzyme to the intestine of adult N. americanus. Recombinant Na-APR-1 cleaved intact human Hb. In contrast, Na-CP-3 and Na-MEP-1 could not cleave Hb but instead cleaved globin fragments that had been hydrolyzed by Na-APR-1, implying an ordered process of hemoglobinolysis. Seventy-four cleavage sites within Hb/H9251- and H9252-chains were characterized after digestion with all 3 proteases. All of the proteases demonstrated promiscuous subsite specificities within Hb; noteworthy preferences included aromatic and hydrophobic P1 residues and hydrophobic P1’ residues for Na-APR-1 and hydrophobic P1 residues for Na-MEP-1. We conclude that Hb digestion in N. americanus involves a network of distinct proteases, some of which act in an ordered fashion, providing a potential mechanism by which some of these hemoglobinases exert their efficacy as recombinant vaccines against hookworm infection.

More than 700 million persons in developing countries are infected with the human hookworms Necator americanus and Ancylostoma duodenale [1]. The main pathology associated with hookworm infection stems from intestinal blood loss, which can lead to iron deficiency anemia in heavy infections. Hookworms are voracious blood feeders, causing an estimated loss of up to 9 mL of blood per day in heavily infected subjects [2]. Although anthelmintic chemotherapies are generally effective at removing adult worms from the gut, reinfection occurs rapidly, leading to concerns about the long-term viability of such practices [3]. Unlike other human helminthiases, clear-cut protective immunity does not occur in most individuals exposed to hookworms [4]. This has culminated in efforts to develop a prophylactic vaccine against hookworm infection as a sustainable solution for long-term control [5, 6].

Hematophagous parasites depend on the catabolism of hemoglobin (Hb) for survival. Schistosome blood flukes and the malarial parasite Plasmodium falciparum digest Hb by means of cooperative proteolytic cascades. In schistosomes, the Hb tetramer is cleaved initially by aspartic proteases [7, 8] and to a lesser degree by papainlike cysteine proteases; the cysteine proteases are thought to exert most of their activity downstream of the aspartic protease by further catabolizing globin fragments [8]. On the other hand, treatment of schistosomes with double-stranded RNA for the gastrodermal cathepsin B cysteine protease SmCB1 interrupted the ability of worms to digest serum albumin [8],

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Reprints or correspondence: Dr. Alex Loukas, Helminth Biology Laboratory, Div. of Infectious Diseases, Queensland Institute of Medical Research, 300 Herston Rd., QLD 4006, Australia (Alex.Loukas@qimr.edu.au).

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suggesting that the order in which the different proteases act differs for each distinct protein substrate.

There are degrees of redundancy in hemoglobinolysis in blood-feeding parasites. The roles and hierarchical positions of various Schistosoma and Plasmodium proteases in the hemoglobinolytic processes have been debated, but the application of gene knockout (Plasmodium) and gene silencing (Schistosoma) technologies have resolved some of these issues. For example, hemoglobinase knockout and double-knockout P. falciparum have been used to show that Hb degradation is partially redundant and relies on dual protease families with overlapping functions [9]. For Schistosoma mansoni, silencing of the mRNAs for different hemoglobinases has shown an ordered and somewhat less redundant pathway of Hb degradation [8, 10].

Gene silencing techniques are available for schistosomes and plasmidia but have not yet been successfully used with hookworms; indeed, some have questioned whether parasitic nematodes have the required enzymes to process double-stranded RNA [11, 12]. We have therefore taken a different approach to explore hemoglobinolysis in blood-feeding hookworms. We previously identified proteases involved in the digestion of Hb in the canine hookworm Ancylostoma caninum and used recombinant enzymes to reveal a semiordered cascade of proteolysis in vitro [13]. Two of the enzymes involved in this cascade were then shown to confer protection as recombinant proteins against hookworm infection in dogs [14, 15].

To characterize the hemoglobinolysis pathways used by human hookworms, we isolated intestinal tissue from adult N. americanus and identified the most highly expressed mRNAs [16], including homologues of some of the A. caninum hemoglobinases. Here, we describe the recombinant expression of intestinal aspartic proteases, cysteine proteases, and metalloproteases from the gut of N. americanus. We provide evidence for an ordered cascade of hemoglobinolysis whereby aspartic proteases (Na-APR-1), cysteine proteases (Na-CP-3), and metalloproteases (Na-MEP-1), in that order, digest Hb and subsequent globin fragments, and provide a map of the cleavage sites. Our findings shed light on the molecular mechanisms used by hookworms to obtain nutrition from human blood and further support the pursuit of hemoglobinases as targets for the development of new drugs and vaccines against blood-feeding parasites of humans and livestock.

**METHODS**

cDNA cloning. Na-apr-1 was reported earlier [17] and assigned GenBank accession number JAC00543. Na-cp-3 was reported recently [18] and assigned GenBank accession number ABL52373. The Na-mep-1 cDNA was initially identified as an expressed sequence tag (EST; BG467946) by using Ac-mep-1 as the query in a BLAST search of the Parasite Genomes Database (http://www.ebi.ac.uk/blast2/parasites.html). Gene-specific primers were designed for 5' and 3' rapid amplification of cDNA ends (RACE) using adult N. americanus RNA (GeneRacer; Invitrogen), as described elsewhere [19]. The full-length cDNA sequence of Na-mep-1 was obtained by creating a consensus consisting of the 5' and 3' RACE products and the BG467946 EST sequence. The cDNA sequence of Na-mep-1 has been deposited in GenBank with accession number EU523699.

**Protein expression and purification.** Na-APR-1 was expressed in the yeast Pichia pastoris, as described for Ac-APR-1 [14]. Expression of Na-CP-3 in P. pastoris was reported by us recently [20]. Attempts to express Na-MEP-1 in yeast were unsuccessful (data not shown), so we expressed it in baculovirus. The entire open reading frame (ORF) minus the predicted signal peptide was expressed as a secreted protein with an N-terminal melittin signal peptide and a C-terminal 6×His tag, and recombinant protein was expressed and purified by nickel–nitrilotriacetic acid (Ni-NTA) chromatography using methods described elsewhere [21]. Protein concentration was determined using a bicinchoninic acid assay kit (Pierce). Na-MEP-1 was also expressed in Escherichia coli to obtain sufficient protein for antibody production. The entire ORF minus the signal peptide was ligated into pBAD/Thio-TOPO (Invitrogen), and E. coli strain BL21-DE2 (Invitrogen) was transformed with ligation product. Subsequent induction of recombinant protein was performed as described in the manufacturer’s instructions (Invitrogen). The cell pellet was collected 4–6 h after induction by centrifugation at 25,000 g for 20 min and resuspended in 20 mmol/L NaH2PO4 and 500 mmol/L NaCl. The cells were subjected to 2 cycles of disruption in a French press (SLM Instruments). Lysates were centrifuged at 20,000 g for 15 min, supernatant was retained, and pellets were incubated in 6 mol/L guanidine HCl, 50 mmol/L Tris, and 10 mmol/L imidazole for 2 h at room temperature (RT) and then centrifuged again. Solubilized pellet was purified under denaturing conditions on a Ni-NTA agarose column, in accordance with the manufacturer’s instructions (Novagen). Purified protein was electrophoresed through SDS-PAGE gels to assess purity, and the identity of the purified protein was confirmed by Western blot analysis using an anti-6×His antibody (Invitrogen).

**Catalytic activity of recombinant hemoglobinases.** Recombinant Na-MEP-1, expressed in both E. coli and baculovirus, was electrophoresed on precast gelatin zymogen gels (Invitrogen), in accordance with the manufacturer’s instructions with slight modifications: 10 mmol/L CaCl2 or 10 mmol/L ZnCl2 were added to the zymogram developing buffer, and the pH of the developing buffer was titrated in single pH unit increments from 3 to 7. Excretory/secretory proteins of adult A. caninum were used as a positive control for the zymogram, and 1,10-phenanthroline (final concentration, 10 μmol/L) was included in some reactions to inhibit metalloprotease activity. Zymograms were incubated overnight at 37°C and stained with Coomassie brilliant blue followed by destaining. Catalytic activity of
Na-APR-1 toward the fluorogenic substrate 7-methoxycoumarin-4-acetyl-GKPILF\(\text{H9262}\)FRLK(DNP)-D-Arg-amide (MoCaC-GKPI\(\text{H9262}\)FRLK; Sigma) (\(\downarrow\) represents the site where the peptide is cleaved) [22] was assayed in a FLUOstar Optima microplate reader (BMG Labtech) at 37°C. Rates of hydrolysis were monitored by recording the increase in fluorescence at excitation and emission wavelengths of 330 and 390 nm, respectively. The optimum pH for enzyme (1.0 \(\mu\)g) activity was determined by assaying in 50 mmol/L sodium acetate buffers at half-unit pH increments from 2 to 6. For determining kinetic parameters, the final substrate concentration was 1.0 mmol/L, and the final volume of each reaction was 100 \(\mu\)L. Enzyme (0.105 mmol/L) efficiency was assessed at pH 3.5 by measuring initial rates over a range of substrate concentrations (0.2–25 mmol/L). The catalytic constants \(k_\text{cat}\), \(K_m\), and \(k_\text{cat}/K_m\) were determined from the resulting Michaelis-Menten plot. The catalytic activity of Na-CP-3 against Z-Phe-Arg-aminomethylcoumarin has been described by us elsewhere [20].

**Antibody production and immunolocalization.** All animal work was conducted with the approval of the Queensland Institute of Medical Research Animal Ethics Committee. In female BALB/c mice, antibodies were raised against Na-MEP-1 expressed in *E. coli*, using methods described elsewhere [20]. Production of anti-Na-CP-3 serum has been reported elsewhere [20]. A rabbit antiserum to recombinant Na-APR-1 was produced as described elsewhere for Ac-APR-1 [14]. Immunolocalization with fixed adult *N. americanus* worms was performed as described elsewhere [23], with minor modifications. Briefly, sections were rehydrated in PBS and blocked with 5% fetal calf serum/PBS for 1 h at RT. Sections were incubated with antisera (dilutions of 1:100 to 1:1000) in 1% bovine serum albumin (BSA) for 1 h at RT. Anti–mouse IgG Alexa Fluor 555 (Invitrogen) was used at a dilution of 1:500 in 1% BSA/PBS/0.05% Tween 20 (PBST); sections were incubated for 1 h at RT. Sections were washed in PBST and air dried, and cover slips were applied with mounting medium (Sigma). Slides were viewed and photographed using a Leica IM 100 fluorescent microscope.

**Proteolysis of Hb by Na-APR-1, Na-CP-3, and Na-MEP-1.** Human Hb was prepared as described elsewhere [7]. Hb was incubated with individual recombinant proteases at molar ratios of 72:1 Hb to Na-APR-1, 5:1 and 50:1 Hb to Na-CP-3, and 14:1 and 140:1 Hb to Na-MEP-1, in 0.1 mol/L sodium acetate, 0.5 mmol/L dithiothreitol, and 10 mmol/L CaCl\(_2\) at half-unit pH increments from 3 to 7 for up to 18 h at 37°C. Hb was also incubated with various combinations of the recombinant proteases at 37°C for 6–18 h. Each sample was electrophoresed on a 15% SDS-PAGE gel under native conditions to observe the amount of intact Hb that remained.

**Analysis of Hb hydrolysates by liquid chromatography (LC)—mass spectrometry (MS) and tandem MS (MS/MS).** Hb samples that had been incubated with various combinations of hookworm proteases were subjected to reverse-phase high-performance LC, and the peptides were identified by LC-MS. LC-MS analysis was performed using an UltiMate 3000 Nano LC system (Dionex) with a capillary LC flow splitter and a variable-wavelength UV-visible detector scanning at 214 nm, coupled to a quadrupole time-of-flight mass spectrometer (MicrOTOF-Q; Bruker Daltonics) operated with a low-flow electrospray needle. The column used was a Vydac monomeric C18 column (300 Å, 3 \(\mu\)m, 150 \(\mu\)m × 150 mm; flow rate, 1.2 \(\mu\)L/min). The mobile-phase buffers used for the gradient program were water with 0.1% formic acid (buffer A) and acetonitrile-water (4:1) with 0.1% formic acid (buffer B). The gradient program consisted of 5% buffer B for 5 min, linear ramping to 55% buffer B over 29 min, linear ramping to 90% buffer B over 1 min, holding at 90% buffer B for 9 min, ramping back to 5% buffer B over 1 min, and holding at 5% buffer B for 20 min. The mass spectrometer scanned 50–3000 \(m/z\) (mass-to-charge ratio) and acquired data for 50 min of each analysis.

Data acquisition was facilitated with HyStar software (version 3.2; Bruker) and processed with Data Analysis software (version 3.2; Bruker). The mass spectrometer used an AutoMSn feature that collected MS/MS spectra for the 2 most intense ions in each full-scan spectrum. The scan time was set to 0.5 s for the survey scan, and the MS/MS spectra recorded were the result of 2 microscans, giving an overall duty cycle of 2.5 s. The data were analyzed using Data Analysis and BioTools software (version 2.2; Bruker). Mascot searches were performed using the Swiss-Prot database with a 20-ppm tolerance on the precursor, 0.2-Da tolerance on the product ions, methionine oxidation as a variable modification, and charge states 1, 2, and 3. Searches were performed using no enzyme, so that novel protease cleavage sites generated by the hookworm hemoglobinases could be detected.

Data were entirely dependent on mascot identity, and an individual ion score of >30 was used as a threshold to elucidate false positives.
identities. The relative entropy between the observed and background distributions of each amino acid at the P4–P4' subsites for each enzyme was calculated using the WebLogo program [24].

RESULTS

cDNAs encoding N. americanus hemoglobinases. Cloning of Na-apr-1 [17] and Na-cp-3 [18] has been reported elsewhere. Na-MEP-1 ORF contained 846 aa (excluding the signal peptide of 26 residues) with a predicted molecular mass of 96.77 kDa. Na-MEP-1 belonged to the M13 peptidase family. The closest homologues included predicted M13 proteases from other hookworms (Ancylostoma species) and the free-living nematode Caenorhabditis elegans. Na-MEP-1 was 56% identical at the amino acid level to Ac-MEP-1 from A. caninum (data not shown). Table 1 lists orthologues of each of these 3 N. americanus hemoglobinases from 3 other blood-feeding parasites: the dog hookworm A. caninum, the blood fluke S. mansoni, and the malaria parasite P. falciparum.

Expression and purification of Na-APR-1 and Na-MEP-1. Na-APR-1 was expressed in yeast and secreted as a zymogen into culture medium. The purified recombinant protein was produced at an approximate yield of 2.0 mg/L (figure 1A). Na-CP-3 was expressed in yeast and purified as described elsewhere [20]. Na-MEP-1 was expressed in baculovirus/Hi5 cells, secreted into culture medium, and purified at a yield of 0.2 mg/L (figure 1B). The identities of the 3 recombinant proteins were confirmed by Western blot analysis using anti-6×His antibodies (data not shown). Na-MEP-1 was also expressed in E. coli but required denaturing conditions to solubilize. The protein was purified under denaturing conditions and verified by Western blot anal-
ysis using anti-6×His antibody (figure 1C). The yield of purified, denatured protein was 1.5 mg/L. Attempts to refold denatured Na-MEP-1 expressed in E. coli were unsuccessful (data not shown).

Catalytic activity assays. Na-APR-1 was secreted from P. pastoris in its pro form and underwent autoactivation at acid pH. Optimal cleavage of MoCac-GKPIFFRLK was at pH 3.5 (data not shown). MoCac-GKPIFFRLK was hydrolyzed by 0.105 nmol/L recombinant Na-APR-1 at pH 3.5, with $K_m = 15.4 \pm 0.7 \mu$mol/L, $k_{cat} = 32.2 \pm 0.7$, and $k_{cat}/K_m = 2,090,909$ L/(mol·s) (figure 2A). As little as 5.0 ng of recombinant Na-MEP-1 displayed gelatinolytic activity in the presence of CaCl$_2$ (figure 2B); catalytic activity was not observed when CaCl$_2$ was replaced with ZnCl$_2$ (data not shown). Activity was observed at pH 4.5–6.5 (data not shown), and addition of 1,10-phenanthroline completely inhibited catalytic activity.

Immunolocalization. Immunofluorescence experiments were conducted with tissue sections of adult N. americanus. Antibodies to Na-APR-1, Na-CP-3, and Na-MEP-1 all localized to the brush border of the intestine of adult worms, confirming earlier reports of intestinal localization for Na-APR-1 [17] and Na-CP-3 [20] (figure 3). Prevaccination control serum did not bind to any structures.

Hb degradation and LC-MS analysis of Hb hydrolysates. Human Hb was readily degraded by Na-APR-1 within 5 min at pH 3.5 and 4.5; digestion was slower at pH 5.5 but still occurred (figure 4A). In contrast, Na-CP-3 and Na-MEP-1 did not digest intact Hb (figure 4B), even after 24-h incubation (data not shown), but they did act downstream of Na-APR-1 in the hemoglobinolysis pathway, digesting globin fragments after initial digestion of Hb with Na-APR-1 (figure 5A). Nineteen cleavage sites were detected in the α-chains and another 18 in the β-chains of Hb after digestion with Na-APR-1 (figure 5B). Neither Na-CP-3 nor Na-MEP-1 digested Hb when assessed by LC-MS/MS (data not shown). However, when Na-APR-1 and Na-CP-3 were added to Hb together, 4 additional cleavage sites were detected in Hb-α and 9 in Hb-β. Na-MEP-1 alone did not digest Hb, but when added to Hb in the presence of Na-APR-1 and Na-CP-3, another 16 cleavage sites in the α-chain and 8 in the β-chain were detected (figure 5). None of the proteases displayed a distinct preference for defined residues at the P4–P4’ subsites. However, Na-APR-1 showed a preference for aromatic and hydrophobic residues at P1, cleaving at 8 sites with P1 Phe, 10 with P1 Leu, and 5 with P1 Ala. Na-APR-1 preferred hydrophobic P1’ residues, notably Leu and Ala, but nonhydrophobic residues were also accommodated. Na-CP-3 was also promiscuous in its preferred P1 residues, again mostly cleaving after hydrophobic and neutral residues. Na-MEP-1 preferred P1 hydrophobic residues including Ala (5 sites) but also readily cleaved hydrophobic and neutral residues. Na-MEP-1 showed a preference for aromatic and hydrophobic residues at P1, cleaving at 8 sites with P1 Phe, 10 with P1 Leu, and 5 with P1 Ala. Na-APR-1 preferred hydrophobic P1’ residues, notably Leu and Ala, but nonhydrophobic residues were also accommodated. Na-CP-3 was also promiscuous in its preferred P1 residues, again mostly cleaving after hydrophobic and neutral residues. Na-MEP-1 preferred P1 hydrophobic residues including Ala (5 sites) but also readily cleaved sites with P1 Lys, Ser, and Asp (figure 6).

DISCUSSION

To liberate Hb into the gut lumen, hookworms must first lyse the ingested erythrocytes via pore formation [25]. Hookworms then, like other blood-feeding parasites, digest Hb via a cooperative and often redundant network of proteases [26]. Here we show that the human hookworm N. americanus uses a pathway of distinct proteases (hemoglobinases) to digest Hb and the sub-
sequent globin peptides. The pathway is similar to that used by *P. falciparum* [27] in that it consists of at least 3 distinct classes of endopeptidases—aspartic, cysteine, and metalloenzymes—and is a major target for the development of new therapies.

Plasmodia are no more closely related to nematodes than they are to humans, which implies that convergent evolution has resulted in phylogenetically distant pathogens adopting similar proteolytic cascades of Hb hydrolysis. *P. falciparum* makes initial

**Figure 5.** Cleavage of hemoglobin (Hb) by various *Necator americanus* recombinant proteases for 18 h at 37°C and pH 4.5. In panel A, colored lines represent the UV trace (214 nm) of Hb, and black lines represent the total ion count (i, overlapping UV trace of all 4 samples; ii, Hb only; iii, Hb and Na-APR-1; iv, Hb, Na-APR-1, and Na-CP-3; v, Hb, Na-APR-1, Na-CP-3, and Na-MEP-1). Panel B shows a map of Hb α- and β-chains highlighting the cleavages made by each recombinant hemoglobinase when all 3 enzymes were coincubated with Hb at pH 4.5. Black arrows indicate Na-APR-1 cleavage sites, green arrows indicate Na-CP-3 cleavage sites, red arrows indicate Na-MEP-1 cleavage sites, and stars indicate hinge regions. AU, absorbance units; Intens., mean signal intensity.
cleavages of Hb with aspartic proteases, called “plasmepsins.” Four distinct plasmepsins cleave Hb in a semiordered fashion [28], but *P. falciparum* clones with plasmepsin gene deletions remained viable despite prolonged doubling times, suggesting that at least the early phases of the hemoglobinolysis process are redundant [9, 27]. This is in contrast to the *P. falciparum* cysteine hemoglobinases, termed “falcipains” (falcipain-1, -2, -2’, and -3); falcipain-3 is essential for Hb digestion and parasite growth, and falcipain-2 knockout parasites were shown to be >1000-fold more sensitive to the aspartic protease inhibitor, pepstatin, than wild-type controls, highlighting the cooperative action of cysteine and aspartic hemoglobinases in this parasite [29]. A combination of pepstatin and E64, inhibitors of plasmepsins (aspartic proteases) and falcipains (C1 family cysteine proteases), respectively, inhibit 98% of globin proteolysis by *P. falciparum* digestive vacuole extract [30]. Although the hemoglobinolysis process in this species is redundant to some extent, these 2 major groups of proteases initiate digestion of the blood meal.

In contrast to *Plasmodium* species, hemoglobinolysis is less redundant in the hematophagous flatworms [31]. Current data suggest that only 1 aspartic protease and 3 clan CA cysteine proteases are present in the intestine of schistosomes, and only the aspartic and 2 of the cysteine proteases (SmCB1 and SmCL1) have been shown to digest the intact Hb tetramer [7, 32, 33]. Further evidence of an ordered cascade in schistosomes comes from studies using RNA interference [8]. Silencing of the *S. mansoni* mRNA encoding cathepsin D aspartic hemoglobinase made larval worms unable to digest Hb in vitro, and none of the cathepsin D dsRNA–treated worms matured to adulthood when injected into mice [10]. Suppression of mRNA encoding the cathepsin B cysteine protease SmCB1 did not overly affect Hb degradation but slowed parasite growth considerably [34].

The degree of redundancy, in terms of both numbers of proteases and overlapping functions/substrate preferences, has not been thoroughly explored in hookworms or other parasitic nematodes. Progress in this area has been hampered by a number of roadblocks, including the lack of a hookworm genome sequence and, until recently, a substantive number of ESTs. Additionally, neither robust RNA interference nor transgenesis technologies have been developed for hookworms and most other parasitic nematodes. As a result, studies addressing the functions of parasitic nematode proteases has been restricted to in vitro observations only.

*Na-APR-1* is not the only hemoglobinase that cleaves intact Hb in *N. americanus*. A pepsinlike aspartic protease, *Na-APR-2*, also cleaves Hb and does so at distinct sites to *Na-APR-1* [35]. Moreover, redundancy exists among the hookworm gut cysteine proteases. *Na-CP-3* did not cleave Hb but did cleave globin peptides; in contrast, *A. caninum* Ac-CP-2 cleaved canine Hb [13]. We recently reported a family of 5 cathepsin B cysteine proteases from the gut of *N. americanus* [20], most of which were identified from just 480 ESTs. Only 1 of these enzymes, *Na-CP-3*, was expressed in active form, precluding analyses of their hemoglobinolytic properties. However, *Na-CP-3* but not the other *N. americanus* intestinal cathepsins B had a C-terminal insertion in the same region as the falcipain-2 “Hb-interacting motif” [36], suggesting that not all of the *N. americanus* intestinal cathepsins B are involved in Hb digestion [29]. Indeed, adult hookworms can be kept alive for numerous months in medium containing just serum in the absence of Hb or blood (D. Smyth, A.L., unpublished data), implying that Hb is a major food source required by hookworms for survival but not the only one, in vitro at least.

*N. americanus* intestinal cells produce an array of proteolytic enzymes [16], and our study has now attributed a hemoglobinolytic function to several of these. This is by no means the com-

![Figure 6. P4–P4’ subsite specificities of Na-APR-1 (A), Na-CP-3 (B), and Na-MEP-1 (C). Tandem mass spectrometry data compiled from Mascot searches were used to create an alignment of peptide cleavage sites, which was submitted to the WebLogo program to generate the image shown. Plots depict relative entropy between the observed and background distributions of amino acids at each subsite. The larger the letter denoting each amino acid, the more frequently that residue occurred. Hydrophobic residues are black, polar residues are green, basic residues are blue, and acidic residues are red.](https://academic.oup.com/jid/article-abstract/199/6/904/2192290)
plete set of enzymes involved in the pathway, but our findings serve to highlight the complexity of the process and show that at least some level of order exists. The pathway is similar to that described for the canine hookworm *A. caninum*, but several key differences exist. *Na*-CP-3 was incapable of cleaving intact Hb and instead cleaved globin peptides generated by hydrolysis with *Na*-APR-1; however, *Ac*-CP-2 is the only cysteine hemoglobinase from *A. caninum* that cleaves intact Hb identified to date. Numerous cysteine proteases are present in the gut of *N. americanus* [16], so we cannot yet discount the presence of an intestinal cathepsin B that digests intact Hb.

In addition to the proteases mentioned above, which have mainly endopeptidase and oligopeptidase activities, it has been suggested that aminopeptidases and other proteases with exopeptidase functions play a vital role in the blood digestion process in hematophagous parasites. They are thought to exert their peptidase functions play a vital role in the blood digestion process downstream of the endopeptidases, removing terminal amino acids and dipeptides for transport into the intestinal cells (helminths) or in the cytosol (*Plasmodium* species). Aminopeptidase activity was recently described in *P. falciparum* trophozoites, suggesting that this activity was responsible for generating free amino acids from Hb peptides after prior digestion with the endopeptidases [37]. *S. mansoni* and *Schistosoma japonicum* express an intracellular leucine aminopeptidase in the gastrodermal cells, which has been proposed to function in removing terminal amino acids from internalized globin peptides [38]. Hookworm ESTs encoding aminopeptidases and carboxypeptidases are present in public databases, and we are currently exploring their roles in the Hb-digestion process (T. Don, A.L., unpublished data).

Another blood-feeding nematode, *Haemonchus contortus*, is highly pathogenic to sheep [39]; its gut contains a plethora of mechanistically distinct endo- and exopeptidases [26, 40, 41], but most have not been unequivocally assigned homoglobinolytic functions. Complexes that are enriched for these intestinal proteases are highly efficacious vaccines [41, 42], further supporting the case for dissecting the molecular basis of hemoglobinolysis in blood-feeding nematodes. Like these *Haemonchus* enzymes, the hookworm hemoglobinases described in the present study are expressed in the gut lumen of the parasite and are therefore exposed to host antibodies when the worm ingests blood. Antibodies to *Ac*-APR-1 and *Ac*-CP-2 bind to the gut of feeding hookworms, neutralize the catalytic activity of the target enzymes, and damage the epithelial surface [14, 15]. Given the involvement in hemoglobinolysis of the *N. americanus* proteases described in the present study, they are potential targets for vaccines that interrupt hematophagy. Because of the potential redundancy in the hemoglobinolysis cascade, we are now considering multivalent vaccines consisting of fragments or epitopes, which are the targets of neutralizing antibodies from multiple hemoglobinases, such as those described here.

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**References**


