

## A Multi-enzyme Cascade of Hemoglobin Proteolysis in the Intestine of Blood-feeding Hookworms\*

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Blood-feeding pathogens digest hemoglobin (Hb) as a source of nutrition, but little is known about this process in multicellular parasites. The intestinal brush border membrane of the canine hookworm, *Ancylostoma caninum*, contains aspartic proteases (APR-1), cysteine proteases (CP-2), and metalloproteases (MEP-1), the first of which is known to digest Hb. We now show that Hb is degraded by a multi-enzyme, synergistic cascade of proteolysis. Recombinant APR-1 and CP-2, but not MEP-1, digested native Hb and denatured globin. MEP-1, however, did cleave globin fragments that had undergone prior digestion by APR-1 and CP-2. Proteolytic cleavage sites within the Hb  $\alpha$  and  $\beta$  chains were determined for the three enzymes, identifying a total of 131 cleavage sites. By scanning synthetic combinatorial peptide libraries with each enzyme, we compared the preferred residues cleaved in the libraries with the known cleavage sites within Hb. The semi-ordered pathway of Hb digestion described here is surprisingly similar to that used by *Plasmodium* to digest Hb and provides a potential mechanism by which these hemoglobinases are efficacious vaccines in animal models of hookworm infection.

In mammals, gastric digestion of proteins derived from food involves a cascade of mechanistically distinct proteases. One of the major food sources of blood-feeding parasites, hemoglobin (Hb), is presumed to be degraded in a similar manner by a cascade of proteases that cleave the intact Hb tetramer into successively smaller fragments that are used to provide nutrients for anabolic processes such as maturation and reproduction. To date, the only hematophagous parasite in which such a proteolytic cascade of Hb digestion has been identified is the

malaria parasite, *Plasmodium falciparum*, where a semi-ordered catalytic pathway exists including members of at least three different mechanistic classes of endoproteases. Hb is degraded in the *P. falciparum* digestive vacuole initially by aspartic proteases termed plasmepsins (1, 2), a step that can be inhibited by the addition of the aspartic protease inhibitor pepstatin A (3). *P. falciparum* also expresses vacuolar cysteine proteases, termed falcipains, which are able to digest native Hb and denatured globin (4–6). After initial digestion with plasmepsins and falcipains, a *P. falciparum* metalloprotease, falcilysin, digests globin fragments even further (7).

Hematophagous helminths include representatives from diverse phyla including nematodes and platyhelminths. Their life cycles are complex, and so to ensure transmission in the face of environmental adversity, helminths release vast quantities of fertilized eggs, a physiologic process that necessitates a protein-rich diet, such as blood. This is evident in the increased consumption of blood by female schistosomes (8) and the over-expression of mRNAs encoding hemoglobinolytic proteases in female schistosomes (9). Aspartic and cysteine proteases are present in the gut lumen and surrounding gastrodermis of blood-feeding adult schistosomes, and although an ordered pathway of Hb degradation is assumed, it has yet to be characterized in any detail (10, 11).

Hookworms are blood-feeding nematodes that reside in the small intestine of infected mammalian hosts (12). An estimated 740 million people are infected with the human hookworms *Ancylostoma duodenale* and/or *Necator americanus* (13). Many hookworm-infected people, particularly women and children, develop subclinical or clinical hookworm disease, including anemia and impaired physical and cognitive development, as a direct result of blood loss at the site of attachment in the intestine (12). The impact of this blood loss in chronically infected populations is enormous. For example, in a single village of ~800 people in South China, it was estimated that hookworms caused an annual blood loss of 200 liters (14). To obtain an unimpeded blood meal, hookworms secrete potent anticoagulants, the actions of which have been well characterized (15, 16). Once blood is ingested by adult hookworms, they lyse erythrocytes using a pore-forming, membrane-bound hemolysin (17), releasing the red cell contents into the intestinal lumen for proteolytic degradation. The pH of the hookworm intestine is not definitively known; however, it is presumed to be acidic in nature (18). All of the proteases identified from this anatomic site have acidic pH optima (19–21); Hb digestion by

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hookworm secretory extracts is optimal at pH 5–7 (22). Moreover, the intestinal contents from the related blood-feeding helminth, *Schistosoma mansoni*, are acidic pH (11).

mRNAs encoding hookworm proteases of different mechanistic classes, including aspartic (19, 20, 23), cysteine (24), and metalloproteases (25, 26), have been cloned, and the expression sites of some have been localized to the intestinal brush border of the blood-feeding adult stage (for a review, see Ref. 18). Until now, only the aspartic proteases, APR-1 and APR-2, had been shown to digest Hb *in vitro* (19, 20). Moreover, these hookworm proteases were more efficient at digesting Hb from permissive host species than from nonpermissive hosts, despite sharing identical active sites residues; this highlights the exquisite co-evolution of these enzymes with host substrates (19, 20, 27). We have suggested that hookworms likely employ a cascade of hemoglobinolysis utilizing multiple proteases of distinct mechanistic classes (18). A cathepsin B-like cysteine protease, Ac-CP-2 (24), was recently shown to be expressed in the intestinal brush border of adult *Ancylostoma caninum* hookworms, and immunization of dogs with the recombinant protease resulted in an anti-fecundity effect on female hookworms (21). In addition, a neprilysin-like metalloendopeptidase was identified from the intestinal brush border of adult *A. caninum* (25); however, the recombinant molecule was not expressed in catalytically active form, precluding the determination of a functional role for this enzyme.

Here we show that the canine hookworm digests Hb in a semi-ordered cascade that consists of at least aspartic, cysteine, and metalloproteases, which act in synergy at acidic pH. The classes of proteases involved and the order of their actions are strikingly similar to those used by *P. falciparum* to digest Hb in the food vacuole.

#### EXPERIMENTAL PROCEDURES

**Protease cDNAs and Protein Expression**—Cloning of cDNAs encoding the proteases described here has been reported elsewhere: Ac-APR-1 (23) (GenBank™ accession no. U34888); Ac-CP-2 (24) (GenBank™ accession no. U18912); Ac-MEP-1 (25) (GenBank™ accession no. AF273084). Expression and purification of recombinant APR-1 in baculovirus (20) and CP-2 in the yeast *Pichia pastoris* (21) have been reported previously. To express MEP-1 in baculovirus, the entire open reading frame minus the predicted signal peptide was amplified by PCR using a MEP-1 pBluescript plasmid as the template. The 5' PCR primer incorporated a SacI restriction site and was designed so that the recombinant protein was fused in-frame with the honey bee melittin signal peptide encoded by pMelBac (Invitrogen). The 3' primer incorporated an NcoI restriction site preceded by nucleotides encoding a hexa-His tag and stop codon. Amplicons were ligated into pMelBac by restriction cloning, and plasmids were generated by heat shock transformation of TOP 10 *Escherichia coli* (Invitrogen). Plasmid preparation, sequencing, and protein expression were performed as described for APR-1 (20). Secretion of the recombinant protein was confirmed using an anti-hexa-His monoclonal antibody (Invitrogen) and a polyclonal antiserum raised to insoluble MEP-1 expressed in *E. coli* (25). After generation of a high titer tertiary viral stock, *Trichoplusia ni* High 5 cells were infected with recombinant virus and maintained in shaker flasks at 27 °C with constant shaking at 100 rpm as described previously (20).

**Purification of Recombinant Ac-MEP-1**—Supernatant from 500 ml of Ac-MEP-1 baculovirus culture was concentrated to 100 ml using a 10-kDa molecular weight cut-off membrane (Pall Scientific), and the buffer was exchanged into 2 liters of Tris-buffered saline, pH 7.5, plus 100 mM CaCl<sub>2</sub> (gelatin binding buffer) and concentrated to a final volume of 100 ml. The concentrated supernatant was bound to gelatin-Sepharose (Amersham Biosciences) previously equilibrated in gelatin binding buffer. Binding occurred overnight on a rotating wheel at 4 °C. The MEP-1 supernatant/gelatin-Sepharose mix was applied to a column and allowed to settle. The column was then washed with 100 ml of gelatin binding buffer, and Ac-MEP-1 was eluted in 15 × 1.0-ml fractions of 2% Me<sub>2</sub>SO in H<sub>2</sub>O (elution buffer). Elution fractions were analyzed by SDS-PAGE, and gels were stained with Coomassie Brilliant Blue. Fractions containing recombinant proteins were pooled and concentrated, and buffer was exchanged into gelatin binding buffer.

**Assessment of Enzymatic Activity and pH Optima**—The pH optimum and substrate preferences for Ac-APR-1 have been previously described (20, 27). Recombinant Ac-CP-2 cleaved the substrate benzoyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarin with an optimal pH range of 5.0–7.0 as described previously (21). Optimal assay conditions for Ac-MEP-1 were determined using pre-cast gelatin gels (Invitrogen) and the Novex® zymogram gels developing system per the manufacturer's guidelines (Invitrogen). To determine the optimal pH for proteolytic activity, zymogram gels were cut into strips and incubated in developing buffer (Invitrogen; containing 10 mM CaCl<sub>2</sub> or ZnCl<sub>2</sub>) overnight at single pH value increments from pH 1.0 to 8.0 in the presence/absence of 10 μM 1,10-phenanthroline. Gelatin gels were stained with Coomassie Brilliant Blue to observe clear zones of hydrolysis.

**Immunohistochemistry**—Adult *A. caninum* were prepared for immunostaining as described previously (19). Nonspecific binding sites were blocked with 5% fetal bovine serum in phosphate-buffered saline, 0.05% Tween 20 (PBS-T) for 1 h. Dog antisera were produced against each protease formulated with the adjuvant AS03 as described for CP-2 (21); animals were bled after the third vaccination but before parasite challenge. Antisera (diluted 1:100, 1:500, 1:1000 in phosphate-buffered saline) were applied to each tissue section and incubated for 2 h at room temperature in a humidity chamber. Subsequent steps were as described elsewhere (19).

**Determination of Subsite Preferences Using Positional Scanning Synthetic Combinatorial Libraries**—The P1-P4 specificities of recombinant Ac-CP-2 were investigated using a combinatorial fluorogenic peptide library established by the Proteomics group at the University of California, San Francisco, as described previously (28). Briefly, aliquots of 25 nM (in the appropriate activity buffer for each protease) from the 20 respective residues of each of the P1-P4 sub-libraries were added to wells (8000 compounds of diversity/well) of a 96-well Microfluor-1 U-bottom plate (Dyrex Technologies). The assay was performed in 100 mM sodium acetate, pH 5.5, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.01% Brij-35, and 1% Me<sub>2</sub>SO (from the substrates). Assays were initiated by the addition of enzyme and monitored fluorimetrically with a SpectraMax Gemini fluorescence spectrometer (Molecular Devices) with excitation at 380 nm and emission at 460 nm. All assays were performed in triplicate. Aspartic and metalloproteases usually exert their specificities at the prime side of the cleavage site; to determine the P1'-P4' subsite preferences for APR-1 and MEP-1, peptide library analysis was carried out as described previously (29). The substrate peptide library acetyl-XXX-amide contains an equimolar mixture of the 19 naturally occurring amino acids, excluding cysteine, at each position. The peptide mixture (1 mM) was digested with Ac-MEP-1 in 20 mM HEPES, pH 7.4, 140 mM NaCl, 10 mM CaCl<sub>2</sub> or with Ac-APR-1 in 0.1 M NaOAc, pH 5.0, to ~5% completion. Digests (10 μl) were subjected to N-terminal sequencing on a Prociase 494 automated Edman sequencer (Applied Biosystems). The molar proportion of each amino acid residue at a given position was determined by dividing the amount of each residue present in the corresponding sequencing cycle by the amount present in the starting mixture.

**Proteolysis of Hb by Ac-APR-1, Ac-CP-2, and Ac-MEP-1**—Whole dog Hb was prepared by lysis of red blood cells as described previously (20). One hundred micrograms of Hb was incubated for 5, 10, and 15 min with various combinations of 1.0 μg each of Ac-APR-1, Ac-CP-2, and Ac-MEP-1 ± inhibitors (1.0 μM pepstatin A, 10 μM E-64, 10 μM 1,10-phenanthroline, respectively) in 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, pH 6.0 activity buffer (total volume 250 μl). Twenty-five microliters of each sample was electrophoresed on a 15% SDS-polyacrylamide gel under native conditions (20). Hemoglobinolysis was also monitored at 410 nm using a spectrophotometer and reversed phase (RP)-HPLC.<sup>1</sup>

**RP-HPLC, MALDI-TOF, and MS-MS Analysis of Hb Hydrolysates**—Hb that had been incubated for 15 min with various combinations of hookworm proteases in the presence/absence of specific protease inhibitors were subjected to RP-HPLC analysis, and the respective masses of Hb-derived peptides were determined by MALDI-TOF. Briefly, aliquots of 50 μl from each hydrolysate were separated by RP-HPLC using a Vydac C18 TP54 150 × 4.6-mm column (Bodman) and a linear gradient of water/acetonitrile (both containing 0.1% trifluoroacetic acid) from 15 to 65% for 50 min. The HPLC separation was monitored by a UV detector set at 215 nm. Fractions were collected automatically every minute and analyzed by MALDI-TOF (Axima CFR

<sup>1</sup> The abbreviations used are: RP, reversed phase; HPLC, high pressure liquid chromatography; MS, mass spectroscopy; MS-MS, tandem mass spectroscopy; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

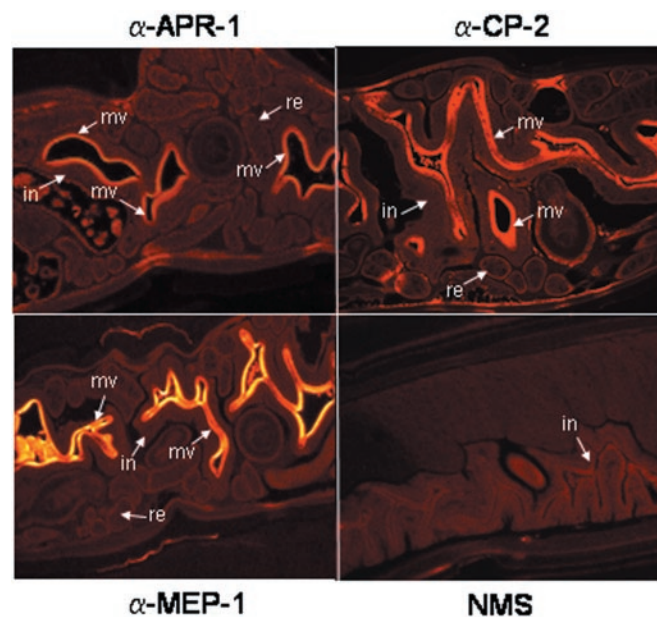


FIG. 1. Recognition of aspartic (APR-1), cysteine (CP-2), and metalloproteases (MEP-1) in the intestine of adult *A. caninum* hookworms using specific antisera raised to each recombinant protease. Normal mouse serum (NMS) did not bind to any structures. *in*, intestine; *mv*, microvillar surface of intestine; *re*, reproductive organs.

plus, Kratos) either in linear or reflectron mode, using  $\alpha$ -cyano-4-hydroxycinnamic as MALDI matrix. Aliquots of 20  $\mu$ l from each sample were also analyzed by LC-MS. The instrumental set up consisted of an HPLC (HP 1100, Agilent) connected to an electrospray-ion trap mass spectrometer (LCQ, ThermoFinnigan). Chromatographic conditions for the HPLC-MS analyses were as follows: flow rate of 50  $\mu$ l/min, obtained with a microflow processor (Acurate, LC Packing) set to a split ratio 1:10, Vydac 238MS  $1 \times 250$ -mm column, mobile phases were water (A) or 100% acetonitrile (B) both containing 0.01% heptafluorobutyric acid and 1% acetic acid. The separation was carried out at 10% B for the first 5 min followed by a linear gradient from 10 to 50% B for 40 min. Mass spectrometric analysis was performed either in full scan mode or in data-dependent mode (MS-MS). The latter analytical approach was used to perform the selective fragmentation of the ionic species that in any given scan (from 300 to 2000 atomic mass units) yielded the highest peak intensity, allowing both molecular mass and structural information to be obtained for chromatographically separated peptides. MS-MS data were then analyzed using TurboSequest 2.0 software (30) (ThermoFinnigan).

## RESULTS

**Aspartic, Cysteine, and Metalloproteases Localize to the Intestinal Microvilli of Adult Hookworms**—We previously localized APR-1 (20), CP-2 (21), and MEP-1 (25) to transverse sections of adult *A. caninum* hookworms. Here we confirm that each protease bound to the intestine only of longitudinal sections of adult hookworms (Fig. 1), whereas control serum did not bind to any structures. Both male and female worms were probed, with no significant differences in localization observed between the two sexes (not shown).

**Expression and Substrate Affinity Purification of Catalytically Active MEP-1**—Ac-MEP-1 was expressed by baculovirus in High 5 cells and secreted into culture medium at a yield of 4 mg-liter<sup>-1</sup>. The protease was successfully purified to homogeneity (as determined by Coomassie Brilliant Blue staining of SDS-polyacrylamide gels) using gelatin-Sepharose (Fig. 2A) but did not bind to nickel-nitrilotriacetic acid-agarose (not shown) despite the presence of a hexa-His tag confirmed by Western blotting using an anti-hexa-His monoclonal antibody (not shown). The purified protein migrated with an apparent molecular mass of 105 kDa (Fig. 2A).

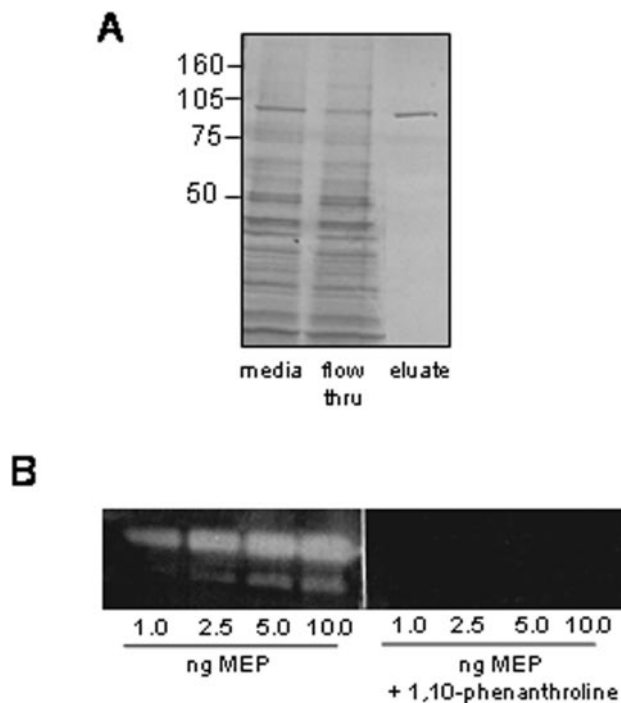


FIG. 2. A, expression of recombinant Ac-MEP-1 in baculovirus and purification of the recombinant protease from culture supernatant. B, gelatin-zymogram gel showing catalytic activity of recombinant MEP-1 at pH 5.5 and its inhibition by 1,10-phenanthroline.

**Ac-MEP-1 Is Enzymatically Active and Degrades Gelatin**—Ac-MEP-1 readily degraded gelatin (with as little as 1.0 ng of recombinant enzyme), and activity was completely inhibited by 1,10-phenanthroline (Fig. 2B). Activity was detected at a broad range of pH values; the most intense zones of hydrolysis were seen at pH 2.0 and again at pH 5.0–7.0 (not shown). Interestingly, gelatinolytic activity was not detected at pH 3.0 and only weakly at pH 4.0. Gelatinolytic activity was detected at similar levels using both 10 mM CaCl<sub>2</sub> and ZnCl<sub>2</sub> (not shown).

**Subsite Specificities of Hookworm Proteases Using Synthetic Combinatorial Libraries**—Aspartic and metalloproteases normally exert their specificities C-terminally to the scissile bond, suggesting that P1–P4 fixed positional libraries would not be suitable for subsite determinations. Papain-like cysteine proteases, however, are most selective N-terminal to the cleavage site, notably at the P2 position (31). The P1–P4 specificities of CP-2 were determined, revealing a preference for basic residues at P1, notably Lys, and a fairly unusual preference for Pro at P2 (Fig. 3). Papain-like enzymes usually prefer bulky, hydrophobic amino acids in this position (31). Cysteine proteases are typically less selective at P3 and P4; and CP-2 preferred Thr and Arg at P3 and displayed less specificity at P4 but prominently cleaved acidic residues. The prime side specificities of APR-1 and MEP-1 were assessed (29) using a peptide library technique to analyze the substrate pool selected by the proteases from a completely random mixture of peptide dodecamers (Fig. 3). For APR-1, hydrophobic residues were well tolerated at P1', with the major selections being for Phe, Tyr, Ile and Met, and Ala and Ser. Other P1' residues were deselected. There were also subtle preferences for hydrophobic residues at P2' with Val being the most favored amino acid. No significant selectivity was seen beyond P2'. MEP-1 was most selective at the P1' position, where it excluded charged residues as well as Pro and Trp. Although the protease tolerated a wide spectrum of amino acids at P1', it displayed a significant preference for Met, Ile, Phe, and Ala at that position. MEP-1 was less selective at positions downstream of P1', but subtle

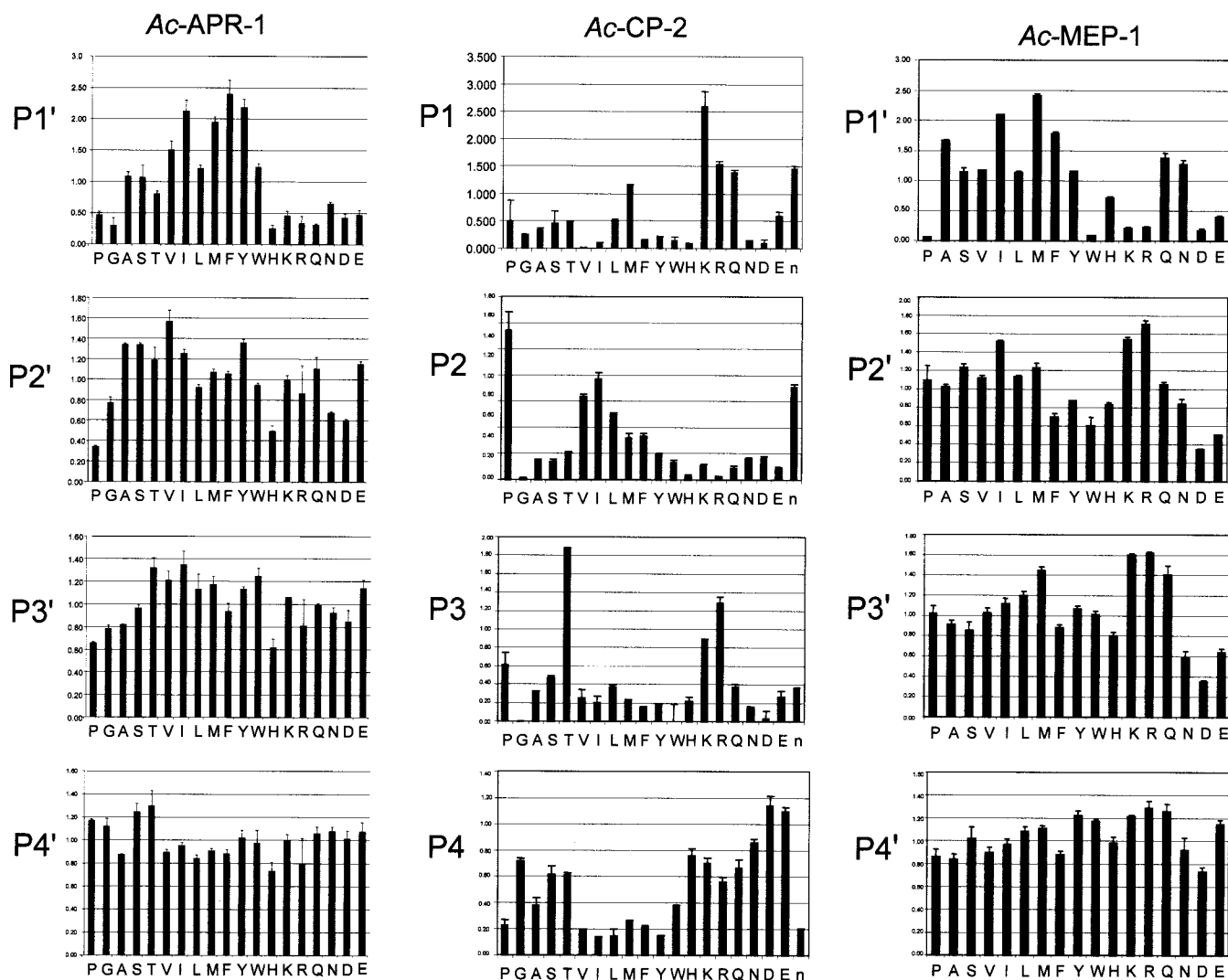


FIG. 3. Subsite specificities of APR-1 ( $P1'$ - $P4'$ ), CP-2 ( $P1$ - $P4$ ), and MEP-1 ( $P1'$ - $P4'$ ) using random combinatorial synthetic peptide libraries. The preferences for APR-1 and MEP-1 were determined by Edman sequencing of a partial digest of an N-terminally blocked peptide dodecamer. Data were normalized so that a value of 1 would be the average amount of each amino acid present in the substrate pool at a given position. Data represented are the average of two runs with standard deviation.

preferences, for example for basic residues at the  $P2'$  and  $P3'$  positions, were apparent from the digestion profile. No significant selectivity was seen at positions downstream of the  $P4'$  position (data not shown).

**Ac-APR-1, Ac-CP-2, and Ac-MEP-1 Degrade Hb in a Semi-ordered Cascade**—Native canine Hb was digested for 15 min with the different enzymes in isolation or in various combinations in the presence/absence of reducing agents with and without protease inhibitors at pH 6.0. As shown in Fig. 4, both APR-1 and CP-2 (only in the presence of reductant) digested native Hb. Class-specific protease inhibitors (pepstatin A for APR-1 and E-64 for CP-2) abolished hemoglobinolytic activity as expected (not shown). Hb was readily denatured by millimolar concentrations of dithiothreitol, so to test the ability of CP-2 to digest native Hb in the presence of physiologically relevant reductants, we assessed the catalytic activity of the enzyme against Hb in the presence of 1 or 10 mM reduced glutathione (GSH). CP-2 was equally effective at digesting Hb in the presence of 10 mM dithiothreitol, 1 mM GSH, and 10 mM GSH (not shown). Physiologically relevant levels of GSH (1 mM) did not inhibit APR-1 activity but slowed the rate of catalysis  $\sim$ 4-fold (not shown) as determined by measuring the  $A_{410}$  of native Hb. The types and quantities of reductants found in the hookworm intestine are unknown, but we presumed that the ingestion of

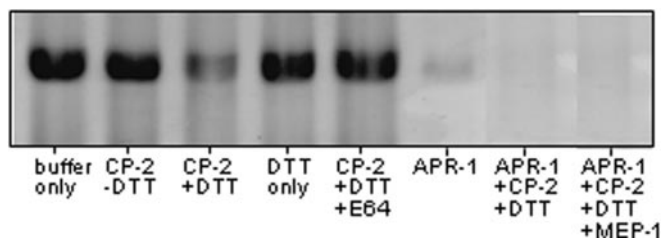


FIG. 4. Digestion of canine hemoglobin in a semi-ordered pathway of catalysis at pH 6.0 after incubation for 15 min with Ac-APR-1, Ac-CP-2, and Ac-MEP-1 in the presence/absence of specific inhibitors and 10 mM dithiothreitol (DTT) as indicated. Digests were resolved by nondenaturing and nonreducing SDS-PAGE and stained with Coomassie Brilliant Blue.

large quantities of blood and subsequent lysis of red cells would result in concentrations of  $\leq$ 1.2 mM GSH (32). MEP-1 did not digest native Hb (Fig. 4) or heat-denatured globin (not shown). After digestion of Hb with APR-1 and CP-2, SDS-PAGE analysis of hydrolysates was not sufficient to determine whether MEP-1 further cleaved globin fragments after proteolysis with the other enzymes. To observe the digestion profiles and determine the masses and sequence identities of proteolytically degraded Hb peptides, hydrolysates were separated by RP-HPLC

and submitted to MALDI-TOF for analysis. To confirm the identity of the peptide fragments, the samples were also analyzed by LC-MS. As observed by SDS-PAGE, APR-1 alone and CP-2 alone (not shown) readily degraded Hb into numerous fragments, but MEP-1 did not. Furthermore, MEP-1 did not digest Hb when combined with just APR-1 or just CP-2 alone (not shown). However, MEP-1 did digest globin fragments after initial proteolysis with both APR-1 and CP-2 together (Fig. 5). By considering previously reported cleavage sites for APR-1 (20) and additional sites identified here, as well as cleavage sites for CP-2 and MEP-1, we developed a map of Hb catalysis by hookworm proteases (Fig. 6). For APR-1, we detected 38 and 44 cleavage sites in the  $\alpha$  and  $\beta$  chains of Hb, respectively. In agreement with subsite preference data using combinatorial libraries, APR-1 was relatively promiscuous but favored hydrophobic residues at P1'. Peptides that were generated by cleavage of Hb with APR-1 were compared with Hb peptides digested with both APR-1 and CP-2; CP-2 cleaved the  $\alpha$  chain at 15 sites and the  $\beta$  chain at 16 sites that were not cleaved by APR-1. In contrast to the subsite preference data obtained for CP-2 using synthetic combinatorial libraries, basic residues in Hb were not favored at the P1 subsite; instead, there was little selectivity, with P1-Ser, -Leu and -Asp being identified three times each. Only one basic P1 residue,  $\beta$ Arg-30, was identified from CP-2-digested Hb hydrolysates. By library scanning, CP-2 was predicted to favor Pro and, to a lesser extent, the hydrophobic residues Ile, Val, and norleucine at the P2 site. Hb P2 subsites favored by CP-2 included Thr, Ile, and Leu; surprisingly, P2-Pro was not identified. It should be noted, however, that not all cleavage sites would be detected using the methods employed here. Moreover, the secondary structure of Hb may mask some sites predicted to be ideal, especially if they contain proline. MEP-1 cleaved globin fragments from the  $\alpha$  chain at 10 sites and the  $\beta$  chain at 8 sites. Using prime side peptide libraries, MEP-1 favored largely hydrophobic P1' residues; in agreement with these data, the preferred P1' residues in degraded globin (after digestion with APR-1 and CP-2) for MEP-1 were Leu and Ala. It was more difficult to predict the preferred P2' residues because many of these sites contained cleavage sites for the other two enzymes (Fig. 6), both of which are thought to act upstream of MEP-1 in the hemoglobinolysis cascade described here. The preferred P1 residues for MEP-1 in Hb were Asp and Leu. MEP-1 digested peptides that contained between one and six residues N-terminal to the scissile bond and between one and four C-terminal residues. It cannot be discounted, however, that peptides unique to the final Hb digest (cleaved with all three proteases) were generated by secondary catalysis with APR-1 or CP-2 after MEP-1 had begun to act.

#### DISCUSSION

We recently described the first nematode recombinant proteases to digest Hb, the cathepsin D-like enzymes *Ac*-APR-1 from *A. caninum* and *Na*-APR-1 from the human hookworm, *N. americanus* (20). We now show that two additional enzymes from the intestinal microvilli, a cathepsin B-like cysteine protease, CP-2 (33), and a neprilysin-like metalloprotease, MEP-1 (25), also digest Hb in a semi-ordered pathway of catalysis. APR-1 and CP-2 digest native and denatured Hb, whereas MEP-1 acts downstream of these two enzymes and digests globin peptides generated by prior proteolysis with APR-1 and CP-2.

The cascade described here is remarkably similar to that used by *P. falciparum* to digest Hb in the food vacuole of the parasite (2, 3, 7). Two aspartic proteases, plasmepsins I and II, have been implicated in the initial steps of Hb degradation in *Plasmodium*. Plasmepsin I, like hookworm APR-1, cleaves the

Hb tetramer at the hinge region, unraveling the molecule for subsequent enzymatic proteolysis (1). Plasmepsin II is capable of digesting native Hb but is more active against denatured or fragmented globin such as that produced by the action of plasmepsin I (34). In addition to *Na*-APR-1, we identified a second aspartic protease from the human hookworm, *N. americanus*, *Na*-APR-2; APR-2 was most similar to a family of nematode-specific aspartic proteases, termed nemepsins (19). APR-2 digested Hb but at very different sites than APR-1, suggesting that in *N. americanus*, at least, multiple aspartic proteases are responsible for digesting Hb in the intestine of adult worms. In like fashion, *P. falciparum* digestive vacuoles contain at least four distinct aspartic proteases (35). After extensive *in vitro* and *in silico* screening of *A. caninum* cDNA libraries and expressed sequence tags, respectively, we did not identify an orthologue of *Na*-APR-2 from *A. caninum*.<sup>2</sup>

Cysteine proteases of *P. falciparum* digest both native Hb and reduced globin (5, 6, 36, 37). Although the reducing environment of the digestive vacuole is an unresolved issue, erythrocytes contain 1.2 mM GSH and 0.006 mM cysteine (32). The reductant concentration of the hookworm intestine is unknown, but given the large volume of blood that the worms ingest and subsequently lyse (17, 38), we have assumed that the concentration is similar to that found in blood. *Ac*-CP-2, like other papain family enzymes, was dependent on a reducing agent for activity. Physiological concentrations of GSH (at least intraerythrocytic) were sufficient for CP-2 to digest Hb but were not too high to inhibit APR-1 from digesting Hb, albeit at a slower rate.

Cysteine proteases exert much of their substrate selectivity at the S2 position of the active site pocket. CP-2 displayed an unusual, but not unprecedented, preference for Pro at P2 for a clan CA protease (Merops classification). Papain-like enzymes with similar P2-Pro specificities have been reported from other parasites including trypanosomes (39) and liver flukes (40) as well as mammalian cathepsin K (41) and ginger rhizome cysteine proteases (42). Although CP-2 readily digested P2-Pro from combinatorial peptide libraries, this substrate preference was not detected in Hb hydrolysates, despite the presence of multiple prolines in canine Hb  $\alpha$  and  $\beta$  chains. However, many of the Pro residues in Hb were one or two residues either side (both prime and nonprime) of the scissile bonds at APR-1 cleavage sites (Fig. 6), possibly accounting for the absence of P2-Pro sites in Hb hydrolysates detected here.

*Ac*-MEP-1, like falcilysin from *P. falciparum*, did not digest Hb or heat-denatured globin but did further digest globin fragments generated by initial cleavage with aspartic and cysteine proteases, implying a downstream role for this enzyme in the hemoglobinolysis cascade. The sheep barber's pole worm, *Haemonchus contortus*, expresses a family of metalloproteases in the intestine (43, 44); although these enzymes have not been expressed in active form, it is thought that they are involved in Hb digestion. MEP-1 displayed a broad pH range when assessed in zymograms. Although gelatinolytic activity is relatively crude and nonquantitative, the strongest regions of hydrolysis were observed at two distinct pH values with peaks at pH 2.0, no activity at pH 3.0, and a second peak of activity at pH 5.0–7.0. Falcilysin was recently shown to be expressed in both the food vacuole and within vesicular structures elsewhere in *P. falciparum* (45). Moreover, the enzyme was shown to have dual specificity, displaying globinolytic activity at acidic pH and a distinctly different substrate preference at neutral pH, prompting the authors to suggest that falcilysin functions as two different proteases in different anatomic loca-

<sup>2</sup> B. Zhan, A. Loukas, and P. Hotez, unpublished observation.

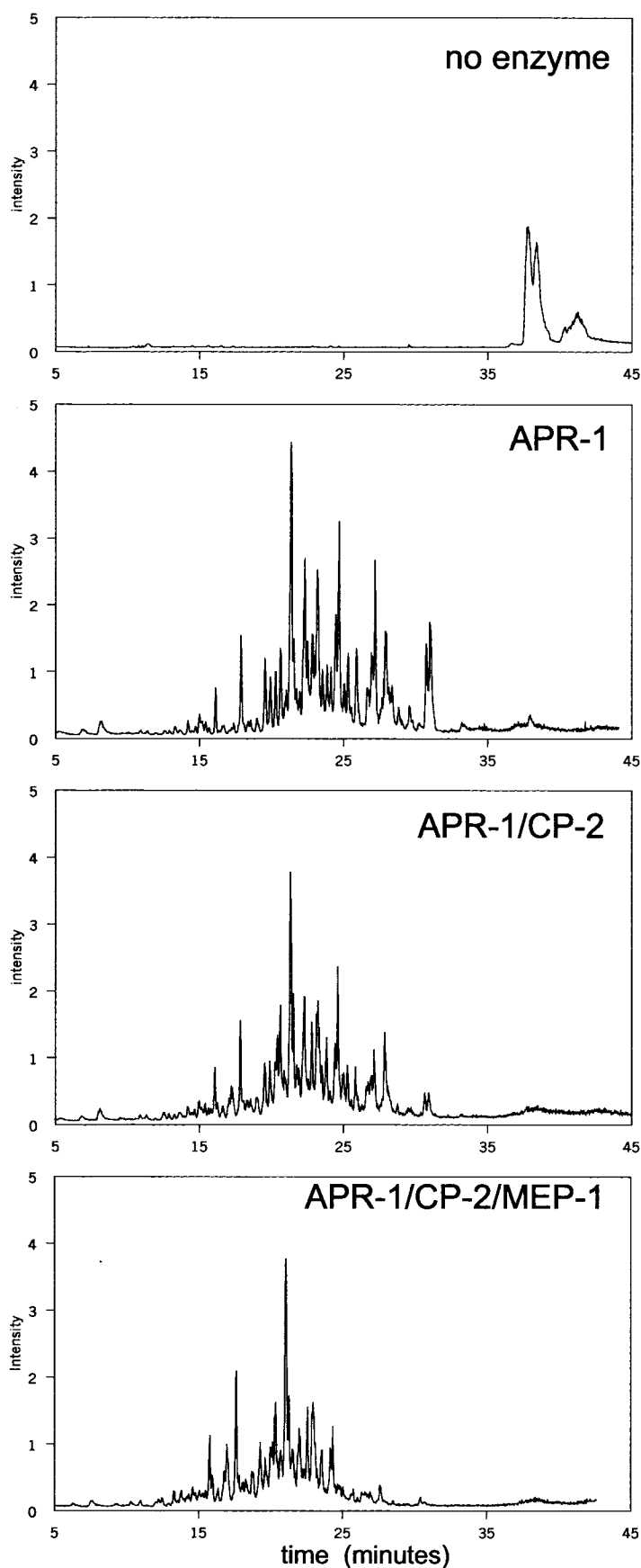


FIG. 5. RP-HPLC traces of hemoglobin digested at pH 6.0 with APR-1 alone (*no enzyme*), APR-1/CP-2, and APR-1/CP-2/MEP-1. Each sample was incubated for 15 min in the presence (not shown) or absence of different class-specific protease inhibitors. Samples were then applied to a Vydac C18 TP54 column for RP-HPLC.

tions within the parasite (45). Although dual peaks in gelatinolytic activity were seen for MEP-1, the protease was only detected in the intestine of adult worms, suggesting a single

function in Hb digestion. Further investigations are warranted to determine the substrate preferences of MEP-1 at different pH values before a potential dual role beyond hemoglobinolysis



FIG. 6. Cleavage map of dog hemoglobin  $\alpha$  and  $\beta$  chains showing sites in each molecule where the different hookworm hemoglobinases cleave the substrates. Letters above arrows refer to cleavage sites for APR-1 (A), CP-2 (C), and MEP-1 (M) as determined by HPLC-MS and MS-MS. Boxed residues denotes the hinge region of native hemoglobin.

can be entertained for this protease.

Hydrostatic pressure of the nematode pseudocoelom ensures that blood ingested by adult hookworms has a rapid passage time through the intestine and out of the anus (38). It is therefore important for the parasite to quickly lyse ingested erythrocytes (17) and employ fast acting hemoglobinases in the intestinal lumen. Complete digestion of the Hb tetramer by APR-1 and CP-2 was evident after just 15 min *in vitro*, and the three proteases together cleaved the Hb  $\alpha$  and  $\beta$  chains in at least 131 places. It is unlikely that the digestive cascade consists of just these three enzymes; the related human hookworm, *N. americanus*, expresses at least two hemoglobinolytic aspartic proteases in the intestine (19, 20), and unpublished scans of the *N. americanus* expressed sequence tag data set revealed a family of at least five distinct cysteine proteases from adult parasites.<sup>3</sup> Moreover, we have identified an aminopeptidase from *A. caninum*<sup>4</sup> that might be involved in Hb catalysis, given that similar aminopeptidases from *P. falciparum* trophozoites (46) and *S. mansoni* gastrodermal cells (47) are thought to be involved in the downstream proteolytic degradation of Hb. Other exopeptidases including carboxypeptidases are also likely to be involved in the release of free amino acids in hookworms.

Although an ordered pathway of hemoglobinolysis has not yet been determined for the trematode parasite, *S. mansoni*, intestinal aspartic and cysteine proteases have been cloned, expressed, and shown to digest Hb in isolation (48–51). Hookworms, schistosomes and plasmodia are all endoparasites, with the latter being intra-erythrocytic during its blood-feeding periods. Interestingly, exoparasites that feed on blood, namely biting insects and acarines, rely predominantly on serine proteases to digest the blood meal (52–54). Nevertheless, gut-specific expression and secretion of cysteine proteases, similar to that seen in the free-living nematode *Caenorhabditis elegans* and parasitic helminths, occur in many insects. Moreover, feeding by these insects on plants genetically engineered to produce cysteine protease inhibitors results in a switch to serine proteases as digestive enzymes, suggesting that the two protease families are redundant at least in insects (55).

Hemoglobinases are proving to be excellent targets for vac-

cines against blood-feeding nematodes (18, 56). *Haemonchus* galactose-containing glycoprotein is a protein complex from the intestine of the sheep barber's pole worm, *H. contortus*. Vaccination of sheep with H-gal-GP confers high levels of protection against parasite challenge; the complex is predominantly made up of aspartic, cysteine, and metalloproteases (57), and recent evidence suggests that the purified enzymes from *Haemonchus* galactose-containing glycoprotein are responsible for the vaccine efficacy (44, 58, 59). We recently described the efficacy of Ac-CP-2 as an anti-hookworm vaccine in dogs experimentally challenged with *A. caninum* (21). Vaccinated animals produced antibodies that neutralized the proteolytic activity of the recombinant enzyme and resulted in decreased growth and fecundity of female worms. Moreover, antibodies from vaccinated dogs were found adhered to the intestines of worms recovered from those dogs. It is likely that the protective mechanism afforded to animals vaccinated with hemoglobinases is the generation of neutralizing antibodies that interrupt digestion of the blood meal, affecting adult worm growth, survival, and fecundity (a blood meal is required to produce eggs). Elucidation of the molecular mechanisms by which hematophagous worms digest blood will hopefully lead to the production of new generation control strategies. Perhaps hemoglobinases will form part of a mixture vaccine against hookworms. By targeting potential antigens from hookworm infective larvae and adult blood feeders, such as the hemoglobinases described here, recombinant vaccines against these complex multicellular pathogens are a realistic near term goal (12, 18).

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<sup>3</sup> A. Loukas, unpublished observation.

<sup>4</sup> T. Don and A. Loukas, unpublished observation.

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