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Broad-Spectrum Antimicrobial Activity of Hemoglobin

Craig A. Parish,[a] Hong Jiang,[a] Yoshi Tokiwa,[a] Nina Berova,[a] Koji Nakanishi,[a,*] Denise McCabe,[b] Warren Zuckerman,[b] Ming Ming Xia[b] and Joëlle E. Gabay[b,*]

[a]*Department of Chemistry, Columbia University, 3000 Broadway, New York, NY 10027, USA*
[b]*Department of Microbiology, College of Physicians & Surgeons, 701 West 168th Street, New York, NY 10032, USA*

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Abstract—While hemoglobin is one of the most well characterized proteins due to its function in oxygen transport, few additional properties of hemoglobin have been described. While screening serum samples for novel antimicrobial factors, it was found that intact hemoglobin tetramers, including that from human, exhibited considerable activity against gram-positive and gram-negative bacteria, and fungi. To further characterize this surprising activity, the antimicrobial potency of sections of human hemoglobin was tested against a panel of microorganisms. In all cases separate testing of the alpha and beta subunits provided activity at least as potent as the intact tetramer. This activity is derived from the protein portion of hemoglobin since removal of the heme prosthetic group did not lead to decreases in potency. In addition, cyanogen bromide cleavage of both subunits provided fragments that still contained substantial antimicrobial activity. It has been possible to map specific regions of the human hemoglobin molecule that are responsible for significant antimicrobial activity. The carboxyl terminal thirty amino acids of the β subunit, which form a cationic α-helix based on the crystal structure of the intact tetramer, were active against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. In view of the fact that different hemoglobin-derived peptide fragments exhibit diverse antibiotic activities, it is conceivable that, in addition to its role in oxygen transport, hemoglobin functions as an important multi-defense agent against a wide range of microorganisms.

Introduction

Broad-spectrum antimicrobial proteins and peptides are ubiquitous in nature. Many animals, plants, and even microorganisms are known to produce compounds that serve to limit the growth of or kill bacteria and fungi as a defense mechanism.[1–4] In higher animals and plants these molecules serve as important components of an innate immune system.[5] These bioactive compounds are frequently present in blood, skin and mucosa as well as in secretions.[6–9]

Antimicrobial peptides are classified based on their origin and size. For example, defensins are small cysteine-stabilized, β-sheet peptides derived from vertebrate leukocytes and epithelial cells, and play a major role in innate immunity.[5,10,11] The defensins are produced by the proteolytic processing of larger primary translated sequences.[12] While there are several types of human defensins present in different tissues,[13] whether or not different types play specialized roles is unclear. Magainins are a family of positively-charged, amphipathic α-helical peptides that are found in the skin of *Xenopus laevis* and display broad-spectrum antimicrobial activity.[14,15] The α-helical structure of magainins appears to be important for their binding to and permeabilization of target membranes.[16] Serprocidins are larger proteins that bear sequence similarity to serine proteases and in some instances possess enzymatic activity, which is not required for their antimicrobial effect.[17] Both groups of antimicrobial proteins exhibit a broad spectrum of activity against bacteria and fungi.

In this report, we present evidence that intact hemoglobin (Hb) and its fragments possess potent antimicrobial activity that is quantitatively comparable to the well-characterized antimicrobial proteins described above. These results, along with the observation that hemoglobin is processed in vivo to peptides, suggest that the large amount of hemoglobin in blood may provide a significant reservoir of antimicrobial activity.

Results and Discussion

In an initial attempt to characterize any antimicrobial factor(s) that may be present in the serum of *Alligator*

*Corresponding author. Tel.: +1-212-854-2169; fax: +1-212-932-8273; e-mail: kn5@columbia.edu

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mississippiensis (American alligator), which seldom get infected, we have found that fractions enriched in antimicrobial activity contained significant amounts of hemoglobin. To clarify this activity, additional hemoglobins were obtained from a commercial source (equine, reptile, and human) or by standard purification from fresh lysed erythrocytes (alligator).[18] The antimicrobial activity of hemoglobin was measured using a standard radial diffusion assay.[19] The antimicrobial activities are reported as MIC (minimum inhibitory concentration) values that are obtained by determining the intercept of the curves shown in Figure 1 with the abscissa (i.e. zero inhibition). It was found that all four of the purified hemoglobins exhibited activity on *E. coli* and three of the four hemoglobins were active on *C. albicans*. The MIC values were in the same range as well-characterized antimicrobial peptides, including defensins and magainins, and proteins, such as azurocidin.[20,21]

In order to determine whether human hemoglobin was bacteriostatic or bacteriocidal, a standard killing assay[21] was used where the number of colony forming units (CFU) was determined before and after incubation with various concentrations of hemoglobin. Values for the amount of human hemoglobin required to cause a 50% reduction in the number of CFU (LD_{50}) of *E. coli* (8 μg/mL) and *C. albicans* (6 μg/mL) were determined. At higher concentrations, all of the CFU were eliminated, indicating that human hemoglobin is bacteriocidal and fungicidal at the concentrations used in the radial diffusion assays.

Several factors were considered to explain the surprising antimicrobial activity of hemoglobin. One possibility is that the heme moiety could act either as an iron chelator or as an oxidant, leading to damage of the bacterial and fungal cell walls. Alternatively, the globin polypeptide, which is highly helical in nature, could itself inflict lethal damage. To distinguish between these possible mechanisms, the α and β chains of human hemoglobin were purified and the antimicrobial activities of the isolated globin chains in the presence and absence of the heme group were evaluated. All antimicrobial data for hemoglobin and its isolated components are provided in Table 1 and are summarized below.

(1) Entries 1–4

Intact hemoglobin tetramers from various species had differing activities against the microorganisms tested. While all species were very active against *E. coli*, only snake Hb was active against *Streptococcus faecalis*, and snake, horse and human Hb were active against *C. albicans* in the radial diffusion assay.

(2) Entries 2, 5, and 6 (Hb versus α subunit)

The isolated human α chain with or without heme (entries **5** and **6**) exhibited enhanced activity as compared to intact hemoglobin (entry **2**). Indeed, the human α chain was very active on both *S. faecalis* (MIC 2–3 μg/mL) and *Staphylococcus aureus* (MIC 15).

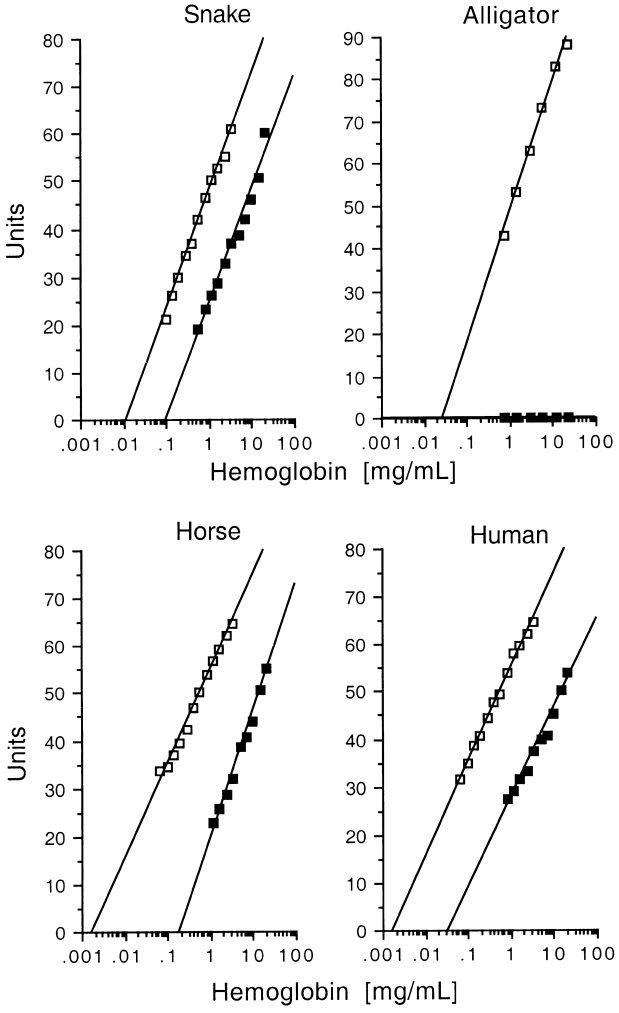

Figure 1. Antibacterial and antifungal activities of hemoglobin from various species: Radial diffusion assays were performed to measure the effects of various hemoglobins. Antibacterial activity was monitored using an *E. coli* K-12 strain (MIC4100), provided by Howard Shuman, Columbia University, Department of Microbiology, and antifungal activity was monitored using a clinical isolate of *C. albicans* from the New York-Presbyterian Hospital. The indicated amounts of hemoglobins were added to wells punched in agarose and the plates were incubated for 3 h at 37 °C. The agarose was then overlaid with a mixture of the relevant growth medium and agar. The plates were then incubated at 37 °C overnight and zones of clearing were measured. The graphs show the concentration of hemoglobin present in the wells on the horizontal axis and the diameter of the cleared zone in mm multiplied by a factor of 10 (units) on the vertical axis. Open squares (□) denote the activity against *E. coli*, while closed squares (■) denote the activity against *C. albicans*.

(3) Entries 2, 5, and 6

Intact human hemoglobin was largely inactive on both gram-positive targets (*S. faecalis*—MIC 1500 and *S. aureus*—20,000). Against *C. albicans*, the isolated α chain was also more active (MIC 7–15) than the intact hemoglobin (entry **2**—MIC 50). These results indicate that the presence or absence of heme in the free α globin makes little difference in its antimicrobial activity, thus showing that the heme moiety plays no role in the antimicrobial activity. Thus, the separation of α and β subunits unmasks antimicrobial activity on gram-positive bacteria that is not exhibited by intact hemoglobin.

Table 1. Antimicrobial activities of hemoglobins and related peptides

Entry	Test substance	Target organisms			
		Escherichia coli Gram negative	*Streptococcus faecalis* Gram positive	*Staphylococcus aureus* Gram positive	*Candida albicans* Fungus
Intact hemoglobins:					
1	Alligator Hb	**25**[a]	ND[b]	ND	20,000
2	Human Hb	**2**	1500	20,000	**50**
3	Horse Hb	**2**	20,000	20,000	250
4	Snake Hb	**10**	**15**	20,000	**100**
Human hemoglobin fragments:					
α peptides					
5	α + heme	**1**	**2**	ND	**15**
6	α-heme	**2**	**3**	15	7
7	I α (1–32)	(> 100)	(> 100)	(> 100)	(> 100)
8	II α (33–76)	**100**	100	(> 350)	**70**
9	III α (1–76)	(> 470)	300	(> 470)	(> 470)
10	IV α (77–141)	(> 100)	(> 100)	(> 100)	**30**
β peptides					
11	β + heme	**3**	**15**	ND	10,000
12	β-heme	**4**	**20**	200	300
13	V β (56–146)	**5**	**8**	**60**	**25**
14	VI β (1–55)	**100**	**15**	**60**	**25**
15	β (116–146)[c]	**2**	2500	**50**	**10**
16	β (56–72)[d]	**20**	10,000	ND	1500

[a]The values are given in μg/mL and correspond to the minimum inhibitory concentration (MIC) obtained as the intercept on the horizontal axis from results similar to those shown in Figure 1. MIC values less than or equal to 100 μg/mL are shown in bold. Each assay was performed in triplicate several times. The results in the Table are derived from a single determination performed in triplicate. Values in parentheses indicate that the sample had no activity at the indicated highest concentration that was tested.
[b]Not determined.
[c]β (116–146) sequence: HHFGKEFTPPVQAAYQKVVAGVANALAHKYH.
[d]β (56–72) sequence: GNPKVKAHGKKVLGAFS.

(4) Entries 2, 11, and 12 (Hb versus β subunit)

The isolated β chain without heme exhibited activity on all four target organisms, supporting the hypothesis that the heme plays no role in the antimicrobial activity and that subunit separation leads to enhanced activity. Thus, although the hemoglobin tetramer (entry **2**) is only negligibly active against the two gram-positive organisms (MIC 1500 and 20,000), the activity of the isolated β globin chain (entry **12** — MIC 20 and 200) is greatly enhanced. In the case of β + heme (entry **11**), antimicrobial activity was observed on two of the bacterial targets (*E. coli* — MIC 3 and *S. faecalis* — MIC 15) but not on *C. albicans* (MIC 10,000). These results indicate that the heme moiety is not required for antimicrobial activity and that some aspects of the individual globin molecules are responsible for bactericidal and fungicidal activities. As in the case of the α chain (entries **5** and **6**), the activity of free β chain (entries **11** and **12**) on gram-positive bacteria is greater than the activity of the intact hemoglobin molecule (entry **2**). In summary, the results with isolated subunits indicate that tetramer dissociation exposes additional bioactive peptidic surfaces.

Based on the finding that isolated subunits without the heme exhibited potent activity, the question as to whether specific regions within the α and β globin chains were responsible for the antimicrobial activity was addressed. The isolated α and β chains were cleaved by cyanogen bromide (CNBr) and the fragments were purified by HPLC (Fig. 2). The identities of the fragments were confirmed by MALDI-TOF mass spectrometry (Table 2). The activity of the purified fragments was

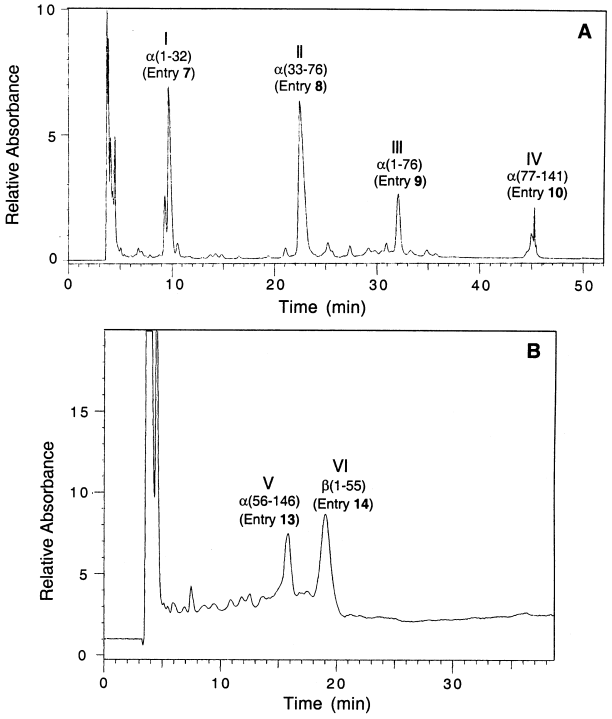

Figure 2. Isolation of α and β hemoglobin cyanogen bromide fragments. (A) The peptide fragments resulting from cyanogen bromide treatment of the Hb-α subunit were purified by reverse-phase HPLC (see Experimental). The identities of each fraction were confirmed by MALDI-TOF mass spectrometry (see Table 2): I, Hb-α (1–32); II, Hb-α (33–76); III, Hb-α (1–76); IV, Hb-α (77–141). (B) The peptide fragments resulting from cyanogen bromide treatment of the Hb-β subunit were purified by reverse-phase HPLC (see Experimental). The identity of each fraction was confirmed by MALDI-TOF mass spectrometry: V, Hb-β (56–146); VI, Hb-β (1–55). Entry numbers refer to the corresponding antimicrobial data in Table 1.

measured using the radial diffusion assay (Table 1). Since the α globin polypeptide contains two methionine residues, three peptide products would be expected. However, four peptide products were observed by RP-HPLC due to incomplete cleavage at methionine-32.

(5) Entries 7–10 (CNBr fragments of the α subunit)

Peptide α (33–76) (entry **8**) was active against *E. coli* (MIC 100), *S. faecalis* (MIC 100), and *C. albicans* (MIC 70), whereas peptide α (77–141) (entry **10**) exhibited activity only on the fungus (MIC 30). The α (1–32) peptide (entry **7**) exhibited no antimicrobial activity at the highest concentration tested (100 μg/mL). The product of incomplete CNBr digestion, α (1–76) (entry **9**), exhibited modest activity only on *S. faecalis* (MIC 300). These results indicate that CNBr cleavage destroys critical sequences required for potent antimicrobial activity of the α chain. A different proteolytic cleavage may provide fragments with the same activities exhibited by the intact α subunit.

(6) Entries 13–14 (CNBr fragments of β subunit)

As expected, cleavage of the human β chain, which contains a single methionine (Met-55), resulted in two fragments, β (1–55) (entry **14**) and β (56–146) (entry **13**). Both fragments exhibited activity on all target organisms, although the activity of the latter fragment on *E. coli* was much greater than that of the former (MICs 5 versus 100). In contrast to what was observed with the α fragments, the two β-derived fragments (entries **13** and **14**) displayed the same or greater activity than the intact β globin chain (entries **11** and **12**), except that the β (1–55) (entry **14**) fragment showed reduced activity on *E. coli* (MIC 100). In contrast to the results obtained with the α-derived peptides, cleavage of the β subunit by CNBr did not destroy antimicrobial activity, indicating that an active region is found within one or both peptide fragments.

Based on the fact that many antimicrobial peptides are cationic α-helices,[22,23] the C-terminal 90 residues of the β chain possibly carry a smaller helical region that was responsible for antimicrobial activity. We therefore examined the known three-dimensional structure of human hemoglobin[24] for a region within the β chain with these characteristics. Two synthetic peptides, β (116–146) (entry **15**) containing three lysine and four histidine residues, and β (56–72) (entry **16**) containing four lysine and one histidine residues, were prepared (see highlighted regions in Fig. 3), and their antimicrobial activities were assayed.

(7) Entries 15–16 (synthetic β peptides)

Peptide β (116–146) (entry **15**) exhibited potent antimicrobial activity resembling that of the intact β chain fragment against *E. coli, S. aureus*, and *C. albicans* (MICs 2, 50, and 10, respectively). Indeed, in the case of *C. albicans*, the activity of the synthetic peptide (MIC 10) is much greater than the intact β chain (entry **11** — MIC 10,000; entry **12** — MIC 300). From these results, we conclude that at least one determinant of the hemoglobin antimicrobial activity is located in residues 116–146 of the β chain.

It is somewhat surprising that a well-characterized protein such as hemoglobin exhibits a novel biological function such as antimicrobial activity. The presence of certain hemoglobin-derived bioactive peptides in erythrocytes and tissues has been noted previously.[25,26] Although innate immunity to microbial infection is a property common to almost all forms of life, it was quite unexpected that hemoglobin, a well-studied hematopoietic protein, would contribute to innate immunity. Myoglobin, a monomeric protein whose three-dimensional structure is closely related to that of the hemoglobin subunits, but is not found in hematopoietic cells, did not exhibit potent antimicrobial activity on any of the targets used in this study (data not shown). In the three cases represented by *C. albicans* and both gram-positive organisms, intact hemoglobin was less active than isolated chains. The β (116–146) helix is significantly blocked in the intact protein, but its exposure would clearly be greatly enhanced upon subunit dissociation as well as upon protein cleavage (Fig. 3). In vivo digestion of hemoglobin would provide numerous bioactive peptides, each having a unique spectrum of

Table 2. Cyanogen bromide fragments of α and β globins

HPLC fraction	Hb subunit (amino acids)	Theoretical MW (Da)	MALDI-TOF[a] (Da)
I	α (1–32)	3280.7	3281.93343.2[b]
II	α (33–76)	4740.3	4742.94803.8[b]
III	α (1–76)	8050.1	8085.4
IV	α (77–141)	7048.3	7098.3
V	β (56–146)	9822.4	9819.6
VI	β (1–55)	6016.8	6036.46080.0[b]

[a]Matrix-assisted laser desorption ionization-time of flight mass spectrometry: Differences between theoretical and experimental masses are mainly due to peptide formylation during CNBr cleavage in formic acid.
[b]Major peak observed in mass spectrum.

Figure 3. Three-dimensional structure of the hemoglobin tetramer. A diagram of human hemoglobin was produced with InsightII from a Protein Data Bank file (2HHB).[24] The two α subunits are shown in red and the two β subunits are shown in blue and yellow. The positions of the two synthetic peptides, β (56–72) and β (116–146), in both β subunits are indicated in yellow. The activities of the synthetic peptides against the listed microorganisms are provided in parentheses (μg/mL).

action against the various classes of microorganisms. This suggests that hemoglobin-derived peptidic fragments, masked in the intact molecule, provide a defensive function against invading microorganisms.

Experimental

Isolation of hemoglobin

The isolation of hemoglobin from alligator blood was based on a previously published procedure.[18] Red blood cells (RBC) were separated from whole alligator blood by centrifugation. Ice-cold distilled water (2.5 vol) was added to the RBC pellet, and the sample was shaken and kept on ice for 15 min. After centrifugation ($20{,}000\times g$), the supernatant was passed through an ion exchange column (BioRex RG 501-X8, BioRad) and the column was further washed with cold distilled water. The fractions containing hemoglobin were pooled and filtered (0.22 micron nitrocellulose filter, Millipore), and an equal volume of 0.1 M Tris, pH 8.8, was added.

Isolation of globin chains and CNBr fragments

The α and β globin chains (from which the heme was removed)[27] were individually dissolved in 70% formic acid in water (1.1–1.2 mg/mL protein) and cyanogen bromide was added (approximately 6 mg/mg protein). The reaction was flushed with argon and stored in the dark at room temperature for 25–30 h. The solvent was removed in vacuo and a small amount of ethanolamine was added to deformylate protein residues that may have been modified during the cleavage.[28] The samples were sonicated for 45 min and the ethanolamine was removed in vacuo. Phosphate buffer (10 mM, pH 5.5) was added to the residue, the samples were sonicated for 30 min and the insoluble materials were removed by centrifugation (14 000 rpm, 10 min). The extent of subunit cleavage (>90%) was monitored by SDS–PAGE.

Peptides β (116–146) (HHFGKEFTPPVQAAYQKVVAGVANALAHKYH) and β (56–72) (GNPKVKAHGKKVLGAFS) were synthesized by the Columbia University Protein Core Facility.

HPLC separation of hemoglobin fragments

The cyanogen bromide cleaved human hemoglobin fragments were purified by HPLC on a reverse-phase C4-semi-preparative column (YMC-Pack Protein-RP column, 10×150 mm). Fragments from Hb-α were separated using a linear gradient from 0 to 40% solvent B over 40 min. Fragments from Hb-β were isolated with a linear gradient from 40 to 47% solvent B over 36 min (solvent A: 4:1 water:acetonitrile + 0.1% TFA; solvent B: 2:3 water:acetonitrile + 0.1% TFA; flow rate = 3.0 mL/min; detector = 210 nm). The respective fractions were characterized by MALDI-TOF mass spectrometry and by comparison of mass data with the theoretical cleavage pattern of human hemoglobin (see Table 2).

Antimicrobial assay

Bacteria were grown in Trypticase Soy Broth at 37 °C in shake flasks overnight and subcultured until they reached mid-logarithmic phase ($A_{600} = 0.6$). *Candida albicans* was grown in Saboraud's Broth overnight in a shake flask at 37 °C and subcultured until the culture reached a count of $5\text{–}7\times10^6$ cells/mL. The cells were washed twice in 10 mM sodium phosphate, pH 5.5. The washed bacteria were added at a concentration of 1×10^7 CFU/plate and the fungi were added at concentration of 4×10^6/plate in 10 mL of the lower layer (10 mM sodium phosphate, pH 5.5, 1% low electroendosmosis agarose). The layers of *Escherichia coli* also contained 0.02% Triton X-100 and 0.02% BSA. The samples of hemoglobin and related peptides were diluted in 10 mM sodium phosphate, pH 5.5, and were added to wells punched in the agarose layer, allowed to diffuse in the layer, and incubated in a humidified closed container at 37 °C for 3 h. Following this incubation period, 10 mL of 1% Bactoagar containing 6% Trypticase Soy Broth (for bacteria) or 6% Saboraud's medium (for *C. albicans*) was added as a top layer and plates were incubated overnight at 37 °C. Zones of growth inhibition were measured with the aid of a ×7 measuring magnifier (Bausch & Lomb no. 81–34–35). Units correspond to 0.1 mm diameter of clearing. The lines relating units to the protein concentrations in the wells were generated using least squares analysis in Cricket Graph III. The MIC values were obtained as the intercept of the horizontal axis.

Acknowledgements

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