Bacterial Infections*

To gain insight into potential role(s) of bass hepcidin in antimicrobial peptide with iron regulatory properties. Its amino acid sequence is similar to human hepcidin, an antimicrobial peptide with iron regulatory properties. The sequence is similar to human hepcidin, including the presence of an antiparallel β-sheet, a conserved disulfide-bonding pattern, and a rare vicinal disulfide bond. Synthetic bass hepcidin was active in vitro against Gram-negative pathogens and fungi but showed no activity against key Gram-positive pathogens and a single yeast strain tested. Hepcidin was non-hemolytic at microbicidal concentrations and had lower specific activity than moronecidin, which was found to have very potent, broad spectrum antimicrobial activity.

In addition to antimicrobial activities, mammalian hepcidins were found to play an essential role in iron homeostasis. This was first suggested in studies using subtractive cloning approaches in mice subjected to dietary iron overload. Hepcidin gene expression was up-regulated under iron overload conditions, and disruption of the hepcidin gene led to accumulation of iron in the liver and pancreas, as well as iron depletion in resident macrophages. This pattern closely paralleled the iron distribution pattern seen in cases of hereditary hemochromatosis in humans. In another study, overexpression of hepcidin in transgenic mouse pups induced profound anemia and postpartum mortality. These and other observations led to the hypothesis that elevated levels of hepcidin limit dietary iron uptake in duodenal enterocytes and block the release of iron by macrophages, making hepcidin a key regulator/hormone of iron homeostasis in higher vertebrates.

Critical role for hepcidin in human iron regulation was recently corroborated by association of deleterious mutations in the hepcidin gene family of immune-inducible, liver-expressed, and cysteine-rich peptides (1–4). Human hepcidin, also known as liver-expressed antimicrobial peptide, was initially purified as a 25-amino acid peptide from urine and plasma ultrafiltrates during screenings for proteins/peptides with antimicrobial activity (1, 2). Expressed sequence tag data base searches revealed related hepcidin sequences among various species of fish; this observation was confirmed by the characterization of a new hepcidin peptide that was purified from the gill tissues of hybrid striped bass (HBSB); Morone chrysops × Morone saxatilis based on its antimicrobial activity against an Escherichia coli strain (5, 6). Like human hepcidin, the bass hepcidin peptide possesses 8 cysteines involved in 4 disulfide bonds and was predominantly expressed in the liver. Bass hepcidin was copurified from the gill of HSB with another antimicrobial peptide, moronecidin, which was found to have very potent, broad spectrum antimicrobial activity (5).

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Bass hepcidin was purified from the gill of hybrid striped bass (Morone chrysops × Morone saxatilis) based on antimicrobial activity against Escherichia coli. This 21-amino acid peptide has 8 cysteines engaged in 4 disulfide bonds and is very similar to human hepcidin, an antimicrobial peptide with iron regulatory properties. To gain insight into potential role(s) of bass hepcidin in innate immunity in fish, we synthesized the peptide, characterized its antimicrobial activities in vitro, determined its solution structure by NMR, and quantified hepatic gene expression in vivo following infection of bass with the fish pathogens, Streptococcus iniae or Aeromonas salmonicida. Its structure is very similar to that of human hepcidin, including the presence of an antiparallel β-sheet, a conserved disulfide-bonding pattern, and a rare vicinal disulfide bond. Synthetic bass hepcidin was active in vitro against Gram-negative pathogens and fungi but showed no activity against key Gram-positive pathogens and a single yeast strain tested. Hepcidin was non-hemolytic at microbicidal concentrations and had lower specific activity than moronecidin, a broad spectrum, amphipathic, α-helical, antimicrobial peptide constitutively expressed in bass gill tissue. Good synergism between the bacterial killing activities of hepcidin and moronecidin was observed in vitro. Hepcidin gene expression in bass liver increased significantly within hours of infection with Gram-positive (S. iniae) or Gram-negative (A. salmonicida) pathogens and was 4–5 orders of magnitude above base-line 24–48 h post-infection. Our results suggest that hepcidin plays a key role in the antimicrobial defenses of bass and that its functions are potentially conserved between fish and human.

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The atomic coordinates and structure factors (code 1S6W) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The on-line version of this article (available at http://www.jbc.org) contains Figs. A1–S5 and Table S1.

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gene in several consanguine families with severe juvenile hemochromatosis (11, 12) and with demonstration of abnormal hepcidin gene expression levels in patients with other genetic variants of this disease (13).

The association of hepcidin with the innate immune response comes from the observation of a robust up-regulation of hepcidin gene expression following inflammatory stimuli. In bass, experimental infection with the Gram-positive pathogen, *Streptococcus iniae*, strongly up-regulated hepcidin expression 24 h post-infection (6); and in mouse, hepcidin has been shown to be up-regulated by lipopolysaccharide (7), turpentine (14), Freund’s complete adjuvant (15), and adenoviral infections (16). Studies conducted with human primary hepatocytes demonstrated that hepcidin expression responded to addition of IL-6 but not to IL-1α or tumor necrosis factor-α (16). Concordant with this observation, infusion of human volunteers with IL-6 rapidly increased hepcidin production/excretion and was paralleled by a decrease in serum iron and transferrin saturation (17). A strong correlation between hepcidin expression and anemia of inflammation was also found in patients with chronic and inflammatory diseases, including bacterial, fungal, and viral infections (16). These findings, further corroborated in a mouse model, led to the conclusion that induction of hepcidin during inflammation depends on IL-6 and that the hepcidin-IL-6 axis is responsible for the hypoferremic response and subsequent restriction of iron from blood-borne pathogens (17).

Evidence for the essential role of hepcidin in iron homeostasis and hypoferremia of inflammation has come primarily from genetic studies in human and mouse because only two native hepcidin peptides have been purified to date, one from human and the other from bass (1, 2, 6). The structure of human hepcidin shows it to be an amphipathic molecule composed of two distorted antiparallel β-sheets separated by a hairpin loop, containing a vicinal disulfide bond (disulfide bond between adjacent cysteines), and stabilized by three inter-β-sheet disulfide bonds (4). To shed light onto the evolutionary conservation of hepcidin, and its function(s) in innate immunity in bass, we have chemically synthesized and refolded bass hepcidin, and we determined its solution structure and its antibacterial and antifungal activities against a panel of selected human and fish pathogens. We also examined the concept that bass hepcidin acts synergistically with moronecidin, a constitutively expressed antimicrobial peptide, to enhance bass innate immunity.

**Experimental Procedures**

*Synthesis, Refolding, and Purification of Bass Hepcidin—*The Fmoc-amino acid derivatives were obtained from Bachem AG (4416 Bubendorf, Switzerland) and included the following side chain protected derivatives: Arg (2,2,5,7,8-pentamethylchroman-6-sulfonyl), Asn (triphenylmethyl), and Cys (triphenylmethyl). Stepwise assembly was carried out on a Labortechnik SF6000 peptide synthesizer at the 5-mmol scale starting with Fmoc-Phe-resin. Each coupling was monitored for completeness by using the ninhydrin procedure described by Kaiser et al. (19). All couplings were mediated by dicyclohexylcarbodiimide in the presence of 2 eq of 1-hydroxybenzotriazole. Following final removal of the Fmoc group from the peptide resin, 1.0 g of resin-bound peptide was cleaved from the solid support and simultaneously deprotected by using reagent K (19) for 2 h at room temperature. Following cleavage, the peptide was filtered to remove the spent resin beads and precipitated with ice-cold diethyl ether. The crude peptide was collected on a fine filter funnel and washed with ice-cold diethyl ether, yielding 285 mg of crude linear peptide. The peptide solution was subsequently diluted into 1.6 liters of an aqueous buffer containing 2 M guanidinium HCl, 10% isopro pyl alcohol, and 10% dimethyl sulfoxide (Me2SO). The pH of the peptide solution was adjusted to 5.8 with NH4OH and allowed to undergo oxidative folding at room temperature for 18 h. Following oxidation of the disulfide bonds, the peptide solution was acidified to pH 2.5 and poured onto a Vydac C18 column (2.5 × 30 cm). The sample was eluted at a flow rate of 8 ml min⁻¹ with a stepwise gradient from 10, 20, and 40% acetonitrile in H2O containing 0.1% trifluoroacetic acid to the same solvent. Elution of each of the fractions was analyzed by MALDI and RP-HPLC. The 40% fraction containing the refolded peptide was lyophilized resulting in 135 mg of ~40% pure peptide. This fraction was further purified using the same semi-preparative RP-HPLC column and flow rate and a gradient of 10–45% MeCN into 0.1% trifluoroacetic acid in H2O over 120 min. The resulting fractions were analyzed by the two analytical RP-HPLC systems, trifluoroacetic acid and triethylammonium phosphate (20). Pure fractions were pooled and lyophilized. Upon lyophilization, 8.2 mg of bass hepcidin was obtained, representing a yield of 2.9%.

*Amino Acid and MALDI-TOF Mass Spectral Analysis—*Synthetic peptide samples were hydrolyzed in 6 N HCl at 110 °C for 22 h in vacuo. Amino acid analysis was performed on a Beckman 126AA System Gold analyser. MALDI-TOF MS analysis was performed on a Kratos MALDI-TOF mass spectrometer using a-cyano-4-hydroxycinnamic acid as a matrix.

**Sample Preparation and NMR Spectroscopy—**NMR spectra were prepared by dissolving ~4 mg of the peptide in 500 μl of D2O containing 5% H3PO4 and 0.03% NaN3 to give a peptide concentration of ~3.5 μM. The unadjusted pH was 4.7, but in some experiments it was adjusted to pH 7.1 with NaOH. The pH was maintained with NaOH without correction for isotope effects. Spectra were recorded at 278, 298, and 308 K on Bruker Avance 500 (with cryoprobe) and DRX-600 spectrometers. Solvent suppression was accomplished by use of presaturation or the WATERGATE gradient echo sequence (21). Conventional two-dimensional TOCSY, NOESY, DQF-COSY, and E-COSY spectra were obtained using 2048 complex data points in the directly detected dimension and typically 300–400 t1 increments. A TOCSY spin-lock time of 60 ms and NOESY mixing times of 60 and 250 ms were used. In addition, two-dimensional 15N- and 13C-HSQC experiments were performed at natural abundance isotope levels in order to obtain 15N- and 13C resonance assignments, respectively. In these experiments 64 and 256 increments, respectively, were acquired in the indirect dimension and 2048 points in the direct dimension, and decoupling was performed experimentally. A three-dimensional 13C-HSQC-NOESY experiment was performed at natural abundance in order to resolve peak overlap in the H dimension. Spectra were processed using XWINNMR (Bruker AG) or NMR-pipe (22) with 60° phase-shifted, sine-squared window functions applied in both dimensions and were analyzed using XEASY (version 1.3.13) (23) or nmrDraw (22). Spectra were referenced to the H2O signal at 4.77 ppm (298 K) or a small impurity at 0.15 ppm. The pH was adjusted by adding NaOH to the sample to give a peptide concentration of ~3.5 μM.

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Bass Hepcidin Structure and Antimicrobial Activity

CYANA because of peak overlap were included in the weakest class (<5.5 Å). Backbone dihedral angle (φ and ψ) constraints were inferred from \( \frac{Δ_{\text{NN}}}{\Delta_{\text{HNC}}/2} \) coupling constants as described previously (24) or as obtained from TALOS.

CYANA was used for the initial evaluation of upper bound distance restraints. Final structures were generated in X-PLOR-NIH starting from a randomly generated template structure with or without the presence of disulfide bonds and different randomized initial velocity distributions and were subjected to a standard simulated annealing protocol. Fifty structures with the lowest energy were selected from the 100 calculated initially and subjected to further refinement in a water box (29). The best 20 structures based on their stereochemical energies were subjected to a standard simulated annealing protocol. Fifty structures with the lowest energy were selected from the distributions and were subjected to a standard simulated annealing from a randomly generated template structure with or without the exception of the fungal strain, Aspergillus niger, which was provided by Dr. Richard Gallo (Veterans Hospital, San Diego). Logarithmic phase cultures were used in all experiments. Most bacteria and yeast were grown in Luria-Bertani broth (Difco), although streptococcal isolates were grown in Todd Hewitt Broth (THB, Difco). Filamentous fungi were grown in half-strength potato dextrose broth (PDB, Difco) supplemented with 100 μM M6G. The structure of bass hepcidin has been deposited in the Protein Data Bank with accession number 1S6W (32).

**Microbial Isolates—** Aeromonas hydrophila, Aeromonas salmonicida, Edwardsiella tarda, Plesiomonas shigelloides, and S. iniae were laboratory isolates recovered from moribund HSB (Kent SeaTech Corp.). Biochemical analysis and ribosomal DNA (16 S) sequencing were used to confirm their identification. All other bacterial isolates were obtained from the Washington State Culture Collection (WSSCC) with the exception of the fungal strain, Aspergillus niger, which was provided by Dr. Richard Gallo (Veterans Hospital, San Diego). Logarithmic phase cultures were used in all experiments. Most bacteria and yeast were grown in Luria-Bertani broth (Difco), although streptococcal isolates were grown in Todd Hewitt Broth (THB, Difco). Filamentous fungi were grown in half-strength potato dextrose broth (PDB, Difco) supplemented with 100 μM M6G.

**Antimicrobial Assays—** Minimal inhibitory concentration (MIC) for liquid growth inhibition assay and minimal bactericidal concentration (MBC) were determined as described previously (5, 33). Briefly, bacteria, yeast, and filamentous fungi were incubated in the appropriate growth media in the presence of 2-fold serial dilutions of synthetic bass hepcidin (44–5.5 μM final concentrations). Bacterial growth was measured as absorbance at 600 nm. For incubation of cultures, 1 ml of exponential phase bacterial cultures (10^6 CFU/ml) were annealed using standard conditions, was linearized with HindIII (Invitrogen) downstream of the insertion site, and in vitro transcription was performed using a T7 RNA polymerase (DuraScribe™ T7 transcription kit; Epicenter). Competitor RNA was purified, treated with DNase, and resuspended in RNAse-free water following the manufacturer’s instructions (RNeasy™ purification kit; Qiagen). RNA concentrations were measured, and aliquots of 1 μl of serial dilutions were made (10–0.0001 ng/μl) and stored at −80 °C.

**Measurement of Hepcidin Gene Expression—** Hepcidin gene expression in infected and mock-challenged HSB fingerlings was quantified using a competitive RT-PCR assay. A homologous RNA competitor (chep2), which was designed by using a segment of the bass hepcidin prodomain containing a 50-bp deletion spanning two RT-PCR primer binding sites slightly modified from our earlier study (6), 1403F2 (5'-GAGATCGCATGGAATCTGGAAG-3') and 86R2 (5'-GAGGCTGAGCAAGGATTCTCCAG-3'). The amplicon resulting from RT-PCR of the competitor hepcidin mRNA (chep, 99 bp) was designed to be easily amplified in the nanomolar mRNAs. The remaining primers in each group were re-analyzed for proper expression to determine signal expression (A_260), and 50 ng μl^- 1 working aliquots were diluted in RNase-free H2O and stored at −80 °C.

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second, non-competitive RT-PCR was performed to confirm the quality of the RNA samples using PCR primers that amplify a region of 18 S rRNA of HSB (SB18S, SB18Srev). In all cases, RNA was reverse-transcribed and amplified in a single reaction (MasterAmp™; Epicenter) with the primers 1403F2/86R2 or SB18for/SB18rev using the following cycling profile: 1) reverse transcription for 20 min at 60 °C; 2) denaturation for 30 s at 94 °C; 3) annealing for 30 s at 58 °C; 4) extension for 30 s at 72 °C; 5) steps 2–4 were repeated for a total of 20 cycles; and 6) final extension for 2 min at 72 °C. Amplified products were electrophoresed on 2.0% agarose gel stained with ethidium bromide (0.05 µg/ml). For competitive RT-PCR assays, gel images were digitized using an EDAS 120 electrophoresis documentation system (Eastman Kodak), and mean fluorescent intensities of the PCR products (cHEP and bHEP) were calculated densitometrically using NIH Image 1.63 (www.rsb.info.nih.gov/nih-image). Regression curves were generated from each series of six single-tube RT-PCRs by plotting, on double logarithmic scale, the value of the known competitor quantity (0.0001–10 ng) against the fluorescent signal ratio of the resulting RT-PCR amplicons (cHEP:bHEP). The quantity of native hepcidin mRNA for each sample was determined based on the point of signal equivalence (competitor:target = 1) (38).

RESULTS

Synthesis—Synthesis of bass hepcidin was initiated on Fmoc-Phe-Wang resin using a semi-automated protocol where each coupling was monitored using a colorimetric test to ensure the completeness of the reaction and ultimately superior quality of the crude product. Native bass hepcidin possesses 8 cysteines that form 4 disulfide bridges (6). Therefore, after trifluoroacetic acid deprotection and cleavage, the synthetic peptide was allow to refold and the thiol groups to oxidize under slightly acidic conditions in the presence of organic co-solvents and chaotropic agents to keep the peptide from aggregating and precipitating. Me2SO was used as an oxidation accelerator according to the method reported by Tam et al. (39). Aliquots of the reaction were sampled during oxidation and analyzed by RP-HPLC until the HPLC profiles remained unchanged (after 18 h). The purified synthetic product was determined to be homogeneous by analytical RP-HPLC in two different solvent systems (trifluoroacetic acid and triethylammonium phosphate) and co-eluted with a sample of the native hepcidin form (Fig. 1). Amino acid analysis of purified synthetic bass hepcidin showed the following average amino acid ratios: Asx (2) 2.05; Ser (1) 1.00; Pro (1) 0.97; Gly (3) 3.00; Met (1) 0.31, 0.99; Phe (2) 2.18; Val (1) 0.78; Arg (2) 1.82; and Cys (8) 5.46 (both Cys and Met are partially destroyed during the acid hydrolysis method used). MALDI-TOF mass spectral analysis of the purified synthetic bass hepcidin determined a (M + H) of 2256.4 that was consistent with the molecular mass of the native peptide (2255.97 MH+). In addition, the synthetic and native bass hepcidin gave the same dose-response killing curve against E. coli (data not shown). These observations led us to

![Fig. 1. A shows the RP-HPLC oxidation profile from the crude reduced product (bottom chromatogram) to the tetradisulfide-linked peptide (middle chromatogram) following an 18-h oxidation in 10% Me2SO. The top chromatogram shows the purified product obtained following preparative RP-HPLC. Gradient conditions were 5% MeCN in water containing 0.1% trifluoroacetic acid to 40% MeCN in water containing 0.1% trifluoroacetic acid. B, co-elution of a 1:1 mixture of synthetic bass hepcidin with natural material. C, MALDI-TOF mass spectrum of the synthetic bass hepcidin.](image-url)
conclude that the two molecules are identical. Most interestingly, the MALDI-TOF mass profile showed the presence of a non-covalent dimer as a sodium adduct. This species is consistent with observations in other defensin-like peptides that are known to self-associate and form aggregates.

Initial NMR Spectral Analysis—TOCSY and NOESY experiments acquired at different temperatures showed that, at 278 K, there were a number of unusually broad amide resonances (supplemental Fig. S1). This broadening was reduced at higher temperatures, allowing essentially complete H-3 resonances to be made from analyses of DQF-COSY, TOCSY, and NOESY spectra at 298 and 308 K. In addition, the majority of 13C resonances and several backbone amide 15N assignments were assigned from two-dimensional 13C and 15N HSQC spectra, respectively, recorded at natural abundance isotope levels (supplemental Table S1). As noted previously for human hepcidin (4), certain resonances of the 4th and 5th cysteine residues were severely broadened at all temperatures, particularly the NH resonances. The amide resonance of the 5th cysteine was not observed in TOCSY or NOESY experiments at any temperature, and only very weak NH-H-3 cross-peaks in DQF-COSY spectra enabled this assignment. Amide resonances from Cys3, Arg20, and Phe21, the residues closest to the termini of the peptide, were also broadened at all temperatures. The single proline residue was shown to exist in the trans conformation, and there was no evidence of cis-trans isomerization.

Diffusion measurements at 278 and 298 K gave values of 1.4 × 10^{-10} and 2.9 × 10^{-10} m^2 s^{-1}, respectively. Allowing for the difference in water viscosity at these temperatures, these data indicate that the peptide has a similar hydrodynamic radius at both temperatures, thus ruling out temperature-dependent aggregation as the source of line broadening. Amide, H-3, and C-α assignments indicated that the majority of residues were in β-sheet conformation (supplemental Fig. S2). The C-α chemical shifts for each cysteine indicated they were in an oxidized state, as in the native peptide.

Backbone Amides—The temperature dependence of the amide proton chemical shifts was measured, and an amide exchange experiment was carried out to assess potential hydrogen bonding. On the basis of slow solvent exchange and chemical shift temperature dependence, five amide protons were determined to be involved in hydrogen bonds. Based on initial structure calculations, four of these residues, Arg2, Cys3, Gly16, and Cys18, appeared very likely to be involved in two pairs of backbone hydrogen bonds. The first pair was between the amide of Arg2 and carboxyl of Cys18 and the amide of Cys18 and carboxyl of Arg3. The second pair involved the corresponding groups of Cys9 and Gly16. The amide protons of these residues had not fully exchanged with solvent deuterium after 12 h at 298 K and also exhibited temperature coefficients ≤4 ppb/K. The amide proton of Met12 also showed slow exchange with solvent deuterium and a low temperature coefficient. On the basis of initial structure calculations, it seemed likely that the amide of Met12 was hydrogen-bonded to the carboxyl moiety of Cys9, a common arrangement in β-type turns; but as this was slightly ambiguous, it was not included in final structure calculations.

Disulfide Bonding Pattern—Mass spectroscopy data on native bass hepcidin showed previously (5) that all eight cysteines were engaged in disulfide bonds. The synthetic peptide used in this study was shown to be active in a set of antimicrobial assays and was chromatographically equivalent to the native peptide, and thus may be expected to adopt the same structure. A previous study on human hepcidin (4) had determined the disulfide bond linkages for the peptide, including a rare vicinal disulfide bond between the 4th and 5th cysteines, corresponding to Cys8 and Cys9 in bass hepcidin. To determine whether the same pairings existed in bass hepcidin, the NMR data and structures generated in this study were examined carefully. Because of spectral overlap involving the NH and H-3 resonances of Cys8, Cys9, and Cys18, severe broadening of the NH resonances of Cys8 and Cys9, and overlap of the majority of the Cys H-3 resonances, the disulfide bonding pattern of bass hepcidin was not obvious from the NMR data alone. Moreover, several Cys resonances showed NOEs to resonances from more than one other Cys residue. Nonetheless, our data imply that bass hepcidin contains the same disulfide-bonding pattern as found in human hepcidin based on the following observations. An examination of the NOEs observed for each cysteine (supplemental Fig. S3) shows that Cys9 and Cys15 have NOEs only to each other and not to any other cysteines, implying that they are disulfide-linked. Initial structure calculations performed without any disulfide bond constraints showed clearly that Cys2 and Cys19 also form a disulfide bridge. A number of long range NOEs were observed in support of these linkages, in particular a very strong H-3-H-3 NOE between Cys4 and Cys19, as well as weak H-3-H-3 and H-3-H-6 NOEs. The two pairs of hydrogen bonds described above between Arg2 and Cys18 and Cys9 and Gly16 are fully consistent with Cys2–Cys19 and Cys5–Cys18 disulfide links. Strong H-3-H-3 and H-3-H-6 NOEs were also observed between Cys3 and Cys15.

Of the three possible disulfide pairings between the other four cysteines (Cys5, Cys6, Cys9, and Cys18), only one (Cys5–Cys6 and Cys8–Cys18) was not supported by NOE data or structure calculations. All potential hydrogen bonds identified by amide exchange experiments were equally well satisfied by both structures. These two possibilities could not be resolved on the basis of the presence of particular NOEs. The Cys2–Cys4 and Cys3–Cys5–Cys18 pairing, however, would bring several protons from Cys8 and Cys9 into close proximity (<3.5 Å) to protons from Cys18. No NOEs were observed between residues 8 or 9 and 18, although overlap and the broadening of the Cys8 and Cys9 amides could, in part, explain this observation. Further support for a vicinal disulfide link comes from the severe broadening of the amide resonances of both Cys8 and Cys9 compared with all other residues except those at the termini. As all adjacent and nearby residues display sharp linewidths, whatever is causing the broadening is localized to Cys8 and Cys9. The formation of a vicinal disulfide bond would create a closed eight-atom loop that may be conformationally mobile. If the mobility of the loop were on an intermediate (NMR) time scale, this would lead to line broadening for those resonances.

Analysis of structure calculations performed with the two possible disulfide connectivities showed that NMR-derived distance and angular restraints were better satisfied by the Cys5–Cys18 and Cys8–Cys9 pairings, although NOE violations for the other possible pairings were all <0.3 Å. In contrast, the overall energy of the vicinal disulfide-containing structure was slightly higher than the alternative, as the formation of a vicinal disulfide leads to a slightly non-planar peptide bond between the two cysteines (Φ = 175°), as well as unfavorable χ1 angles (χ1 = 86° and −31° for Cys8 and Cys9, respectively) (4). Structure calculations run with no disulfide bond restraints gave an identical structure to calculations performed with the same disulfide pattern as human hepcidin (the backbone r.m.s.d. between the closest-to-average structure of each family over residues 2–19 was 0.56 Å), but not to calculations performed using a starting structure containing the other potential disulfide connectivities (corresponding backbone r.m.s.d. 1.51 Å; Fig. 2). Taken together, these data provide strong evidence that bass hepcidin shares the same disulfide bonding pattern as human hepcidin.

Solution Structure—Parameters and structural statistics
characterizing the final 20 structures of bass hepcidin are summarized in Table I, and stereo views of the structures superimposed over the backbone are shown in Fig. 3. Angular order parameters showed that, apart from the N-terminal glycine, the backbone dihedral angles $\phi$ and $\psi$ were all well ordered, as were the majority of the side chain $\chi^1$ angles (supplemental Fig. S4). The peptide forms a two-stranded, slightly distorted, antiparallel $\beta$-sheet with a flexible hairpin loop from Asn7 to Gly14 that lies above the concave surface of the sheet. Bass hepcidin is well structured over the majority of its length (residues 2–19). The degree of curl of the loop region could not be defined precisely with the available restraints, possibly indicating that it is inherently more flexible than the rest of the molecule. This is supported by the observation that residues 11–13, in the center of the loop, displayed sharp linewidths (supplemental Fig. S1). However, the side chain resonances of Met12 showed several NOEs to protons in the $\beta$-sheet, reflecting some degree of rigidity. Bass hepcidin is positively charged by virtue of the two Arg residues, and it does not contain any acidic residues. The locations of the Phe and Val side chains from the $\beta$-sheet region both lie on the convex surface of the sheet, thus giving the $\beta$-sheet a hydrophobic surface, as seen in human hepcidin. Consecutive disulfide bridges lie on alternate sides of the $\beta$-sheet (Fig. 3), also seen in human hepcidin. The $\beta$-sheet is stabilized by three inter-strand disulfide bonds and four inter-strand hydrogen bonds.

Antimicrobial Spectrum of Activity—Serial dilutions of synthetic hepcidin, beginning at 44 $\mu$M, were tested in vitro in liquid growth inhibition assays against 21 bacterial strains, a filamentous fungus, and a yeast strain (Table II). The peptide was active against a panel of Gram-negative bacteria including three $E$. coli strains, $P$. shigelloides, Klebsiella pneumoniae, Shigella sonnei, Shigella flexneri, and Y. enterocolitica. Hepcidin was not active at 44 $\mu$M against another Klebsiella sp., Klebsiella oxytoca, as well as nine other Gram-negative species tested. The minimum inhibitory concentrations of synthetic hepcidin against Gram-negative bacteria ranged from 5.5 to 44 $\mu$M, and overall, 8/18 (44%) of the Gram-negative species tested were sensitive to bass hepcidin. The MBCs were either equal to or twice the MIC for all bass hepcidin-sensitive strains (Table II). Bass hepcidin showed no activity at 44 $\mu$M against the three Gram-positive bacteria and single yeast strain tested. Hepcidin displayed antifungal activity in vitro against $A$. niger at relatively high concentrations (44 $\mu$M; Table II). Most interestingly, hepcidin was not active against any of the key fish pathogens we tested, including the Gram-positive pathogen, $S$. iniae, and the Gram-negative pathogens, $A$. hydrophila, $A$. salmonicida, and $E$. tarda.

Microbicidal Kinetics—An experiment was conducted to examine the microbicidal kinetics of bass hepcidin and to compare its killing activity to another bass antimicrobial peptide, moronecidin. Moronecidin is a 22-amino acid, linear, amphipathic $\alpha$-helical peptide that was originally co-purified from the gill of HSB with hepcidin (5). These experiments were carried out using $Y$. enterocolitica, where the MIC for hepcidin and moronecidin were measured at 22 and 5 $\mu$M, respectively. We compared the killing kinetics of hepcidin and moronecidin against $Y$. enterocolitica at 30-min intervals over a 3-h time period using 2$\times$ their MIC concentrations for this organism (44 and 10 $\mu$M; Fig. 4). The bactericidal activities of the peptides were assessed by plating cultures and counting CFUs after overnight incubation at 37°C. Bass moronecidin killed $Y$. enterocolitica within minutes of exposure to the bacteria, leading to a 90% decrease in CFU after 30 min, whereas $Y$. enterocolitica cultures were actually growing in the presence of 22 and 44 $\mu$M bass hepcidin at this time. Two and half hours were required for a similar 90% reduction of CFU from the original inoculum with hepcidin at 44 $\mu$M. The microbicidal activity of hepcidin was temperature-dependent as was observed for moronecidin (data not shown) (5).

Fungicidal Activity—A germination assay with spores of the filamentous fungi, $A$. niger, was conducted to test the fungistatic and fungicidal activities of the bass hepcidin and to compare them with those of moronecidin (Fig. 5). No hyphae were observed at a peptide concentration of 44 $\mu$M after 2 days of incubation at 30°C. At lower concentrations, the peptide...
caused delayed growth of hyphae with abnormal morphology (data not shown). After 48 h of exposure to the respective peptides, spores were removed and cultured in fresh medium and examined 48 h later for growth. Bass hepcidin was fungistatic at low concentrations with a lower IC50 concentration (peptide concentration giving 50% growth inhibition) than moronecidin, whereas hepcidin was fungicidal at 44 μM (Fig. 5).

Synergism between Bass Hepcidin and Moronecidin Antimicrobial Activities—Bass hepcidin and moronecidin were originally co-purified from gill tissues of hybrid striped bass (5, 6), thus opening the possibility that these peptides are co-localized in this tissue and may act additively or synergistically to kill invading microorganisms. To test for synergism between the two antimicrobial peptides in vitro, we conducted liquid growth inhibition/killing experiments with a Gram-positive (S. iniae) and a Gram-negative bacterium (Y. enterocolitica) using varying concentrations of the two synthetic peptides. The bacteria were cultured in the presence of synthetic bass hepcidin and moronecidin and plated after 2 h of incubation at 37 °C for determination of CFU (Fig. 6A). We observed (Fig. 6A) that 2-fold decreases in the MIC of each peptide for Y. enterocolitica, when in combination, reduced CFUs by more than 100-fold below that of either moronecidin or hepcidin alone at their MIC concentrations for this bacteria. A 4-fold decrease in the MIC of

<table>
<thead>
<tr>
<th>Microorganisms ATCC no. a</th>
<th>MIC</th>
<th>MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-positive bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis (VRE)b</td>
<td>51,299</td>
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</tr>
<tr>
<td>S. aureus (MRSA)d</td>
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<td>&gt;44</td>
</tr>
<tr>
<td>S. iniae</td>
<td>KSTd</td>
<td>&gt;44</td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aeromonas hydrophilia</td>
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<td>&gt;44</td>
</tr>
<tr>
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<td>11</td>
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<tr>
<td>S. flexneri</td>
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<td>S. sonnei</td>
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<td>44</td>
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<td>Yersinia enterocolitica</td>
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<td>22</td>
</tr>
<tr>
<td>Filamentous fungi</td>
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<td></td>
</tr>
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<tr>
<td>Yeast</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td>66,027</td>
<td>&gt;44</td>
</tr>
</tbody>
</table>

a ATCC indicates American Type Culture Collection number.
b VRE, vancomycin-resistant enterococcus.
c NT, not tested.
d MRSA, methicillin-resistant S. aureus.

* Rent SeaTech isolates from HSB. The highest concentration tested with bacteria and yeast was 44 μM, and 88 μM was used for the fungi A. niger.

Fig. 3. A, stereo view of the backbone atoms of the family of structures generated for bass hepcidin. The two termini are shown, and several of the residues are numbered. B, stereo view of a representative structure of bass hepcidin showing the backbone heavy atoms (gray), side chain heavy atoms (black), and disulfide bonds (orange). C, ribbon diagram of bass hepcidin showing the antiparallel β-sheet (blue) and loop region. D, two views of a surface representation of bass hepcidin. The surface is colored with basic residues in red. The two views are related via a 180° rotation about the vertical axis. A–C of this figure were prepared using MOLMOL (30), and D was prepared using GRASP (69).

Fig. 4. Synthetic bass hepcidin at 22 μM (2× MIC or 1× MBC) and 44 μM and synthetic moronecidin at 10 μM (2× MIC and 2× MBC) or water control was added to freshly diluted (1 × 10^5 CFU ml^-1) in LB) logarithmic phase culture of Y. enterocolitica and incubated at 37 °C. Aliquots of the culture were removed at various time points, diluted in PBS, and plated on THB for colony count. Growth index was calculated as bacterial CFU recovered/initial inoculum. Each point represents an average of three experiments, and the vertical bars show the S.D.
Bass Hepcidin Structure and Antimicrobial Activity

Figure 5. Antimicrobial bass hepcidin (○) and moronecidin (●) were added at different concentrations to 10⁵ spores ml⁻¹ of A. niger. Hyphae density was measured at 600 nm after 48 h at 30 °C and represented as a percentage of absorbance with 100% corresponding to hyphae density without peptide. The IC₅₀ values represent the peptide concentration giving 50% growth inhibition. Results show average of three experiments, and vertical bars show S.D.

Figure 6. Exponential phase cultures of Y. enterocolitica or S. iniae diluted in THB to 10⁵ CFU ml⁻¹ were incubated for 2 h with the indicated concentrations of hepcidin, moronecidin, or water as a control. Aliquots of the bacterial suspensions were plated for enumeration of CFU after overnight incubation. The growth index was calculated as bacterial CFU recovered/CFU before addition of the peptide. The experiment was performed in triplicate for each sample. Vertical bars represent S.D.

Each peptide in combination yielded similar or better killing of Y. enterocolitica than either peptide alone at their MIC (Fig. 6A). Bass hepcidin had no detectable antimicrobial activity against S. iniae after 2 h of incubation with concentrations as high as 88 μM (Fig. 6B). However, at a hepcidin concentration eight times lower (11 μM), in the presence of 1.25 μM moronecidin, strong killing of S. iniae was observed. Under these conditions, a 10-fold reduction in CFU below that of 1.25 μM moronecidin was observed. Although the results shown in Fig. 6, A and B, give a more intuitive visualization of the synergism between the two peptides, the standard measure for synergism is through calculation of the FIC index. We calculated FIC indices against a Gram-positive bacteria (S. iniae) and three Gram-negative bacteria (E. coli, Y. enterocolitica, and S. sonnei) (Table III). An FIC index of 0.5 indicates strong synergy (representing the equivalent of a 4-fold decrease in the MIC of each compound tested), whereas an FIC index of 1.0 indicates that the antimicrobial activity of the two compounds are additive (i.e. a 2-fold decrease in the MIC of each compound tested). The FIC indices calculated for hepcidin and moronecidin were between 0.5 and 0.75, indicating good to moderate antimicrobial synergy between the two peptides.

Hemolytic Activity—The hemolytic activity of bass hepcidin was tested with erythrocytes from HSB. Bass hepcidin displayed essentially no hemolytic activity toward HSB erythrocytes (Table IV). Greater than 98% of the bass erythrocytes exposed to 44 μM hepcidin for 3 h at 37 °C remained intact. This exposure corresponds to a time point when 96% of Y. enterocolitica exposed to 44 μM hepcidin have been killed (see Fig. 6A). After 4 h of incubation with 44 μM hepcidin, more than 97% of the erythrocytes remained intact. No hemolysis was observed after 4 h at hepcidin concentrations of 11 μM and lower.

Temporal Analysis of Bass Hepcidin Gene Expression Following Bacterial Infection—In a previous study by our group, levels of hepcidin gene expression were assessed at 24 h by kinetic RT-PCR between HSB infected by immersion in a live suspension (5 × 10⁷ CFU ml⁻¹) of the virulent fish pathogen S. iniae and mock-challenged controls. Those studies demonstrated that hepatic hepcidin expression in bass was strongly up-regulated (~4,500-fold) following infection with this Gram-positive bacterium (6). However, these experiments only examined a single pathogen and time point post-infection under conditions that did not allow the pathogen dose received by the HSB to be quantified. To extend this study, we examined hepcidin gene expression at intervals over the first 48 h post-challenge following IP injection of a defined dose of A. salmonicida or S. iniae. HSB fingerlings infected with either A. salmonicida or S. iniae exhibited 44 and 78% cumulative mortality, respectively, over the course of 7 days. Both pathogens were recovered from the head kidney (A. salmonicida) and brain/head kidney (S. iniae) of moribund fingerlings, confirming the presence of an active systemic infection. No mortalities occurred in mock-challenged fingerlings, and neither pathogen was recovered from the sacrificed control HSB. Differences in hepcidin expression between experimental HSB fingerlings in-

TABLE III

<table>
<thead>
<tr>
<th>Species ([A]/[B])</th>
<th>MIC Hepcidin</th>
<th>MIC Moronecidin</th>
<th>Lowest FIC index</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. iniae</td>
<td>&gt;88/22</td>
<td>2.5/5</td>
<td>0.56 (1/1.25)</td>
</tr>
<tr>
<td>E. coli</td>
<td>22/5</td>
<td>5/2.5</td>
<td>0.75 (5/2.5)</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td>22/5</td>
<td>5/2.5</td>
<td>0.50 (5/2.5)</td>
</tr>
<tr>
<td>S. sonnei</td>
<td>22/44</td>
<td>5/2.5</td>
<td>0.75/0.5 (11/1.25)</td>
</tr>
</tbody>
</table>

*FIC index = [A]/MICₐ + [B]/MICₐ, where MICₐ and MICₐ are the MICs of peptides A and B alone and [A] and [B] are the MICs of peptides A and B in combination. The MICs for the peptides alone are as given in Table II. The numbers in parentheses are the MICs in combination (hepcidin/moronecidin). Since hepcidin MIC against S. iniae is higher than 88, the highest concentration we tested, we chose this value as the hepcidin MIC in the calculation of the FIC index.
Hemolytic activity of bass hepcidin for bass erythrocytes over time

<table>
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<tr>
<th>Heparin</th>
<th>30 min</th>
<th>90 min</th>
<th>180 min</th>
<th>240 min</th>
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<tbody>
<tr>
<td>µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>0</td>
<td>0.4 ± 0.15°</td>
<td>0.4 ± 0.4°</td>
<td>1.4 ± 0.3°</td>
</tr>
<tr>
<td>44</td>
<td>0</td>
<td>0</td>
<td>2.5 ± 1.2°</td>
<td></td>
</tr>
</tbody>
</table>

*Hemolytic activity is expressed as percent of controls ± S.D.*

Bass Hepcidin Structure and Antimicrobial Activity

- Hepcidin mRNA copy number was low in the livers of healthy control HSB fingerlings at time 0 and at 24 h, comprising ~6 × 10^-5% of total RNA in liver (4.37–4.93 × 10^8 copies ng^-1 RNA). Resting levels of hepcidin mRNA in bass liver were ~5–7 fg ng^-1 total RNA (data not shown). Hepcidin gene expression in bass fingerlings was rapidly up-regulated following IP challenge with *S. iniae* and *A. salmonicida*, Gram-positive and Gram-negative organisms, respectively. For both fish pathogens, hepcidin expression increased roughly 3 orders of magnitude between 4 and 8 h, 4 orders of magnitude by 16 h, and nearly 5 orders of magnitude by 48 h (Table V; Fig. S5B). Hepcidin hepatic gene expression levels reached >50% and >60% of their 48 h peak levels by 16 and 24 h, respectively, and continued to increase through the end of the experiment at 48 h. Hepcidin mRNA comprised ~3% of the total liver RNA at 48 h post-infection (2.3–2.4 × 10^8 copies ng^-1 total RNA). The rate of increase and overall levels of bass hepcidin mRNA was strikingly similar in HSB fingerlings challenged with similar inocula of either Gram-negative (*A. salmonicida*; 2.3 × 10^8 copies ng^-1 RNA at 48 h) or Gram-positive (*S. iniae*; 2.4 × 10^8 copies ng^-1 RNA at 48 h) fish pathogens (Table V).

**DISCUSSION**

This is the first report characterizing the structure and antimicrobial activities of a non-mammalian hepcidin peptide. Our findings indicate that the bass and human hepcidin peptides adopt a very similar three-dimensional structure and have the same disulfide-bonding pattern. They also both contain a rare vicinal disulfide bond, indicating that key structural attributes for the hepcidin are conserved between human and fish. Our previous work and this study show that many of the defining characteristics of both the genes and the mature peptides are remarkably similar between bass and human.

**Structure**—The structures of bass and human hepcidin show significant similarity (4). The amino acid sequences of the two molecules share 52% homology and 62% similarity. Both molecules consist of a slightly twisted, two-stranded, antiparallel β-sheet connected by a more flexible hairpin loop that curls over the concave surface of the β-sheet. The β-sheets of the two hepcidins display similar hydrogen bonding patterns and similar close contacts across the sheet. They also share the same disulfide-bridging pattern. The major difference is that the N- and C-terminal arms of bass hepcidin cross over one another to a much greater degree than in human hepcidin. Human hepcidin consists of a hydrophobic surface on the convex face of the molecule and a positively charged concave surface. Whereas bass hepcidin shows a similar hydrophobic convex surface, the clustering of charged residues is most apparent at one end of the molecule. This region contains the N and C termini and the two positively charged Arg guanidino groups, whereas the rest of the molecule is uncharged (Fig. 3D). It is interesting to note that bass hepcidin shares many structural characteristics with other antimicrobial peptides (42). Bass hepcidin is as follows: (i) cationic with an overall charge of +1.7 at neutral pH, (ii) adopts an amphipathic structure (42), and (iii) contains a β-hairpin structure cross-linked with disulfide bridges as has been observed in antimicrobial peptides purified from arthropods (tachypleus, thanatin, and gomisin) (43–45) and mammals (protegrin, θ-defensin-like, and lactoferricin B) (2, 46, 47).

**Vicinal Disulfide in Hepcidins**—Vicinal disulfides distort the local backbone structure significantly, resulting in an energetically unfavorable non-planar peptide bond as well as unfavorable fixed side chain χ^1^ angles. The backbone torsion angles around the vicinal disulfide and the disulfide torsion angles observed in bass hepcidin are in good agreement with those of human hepcidin (4), as well as other proteins containing vicinal disulfide bonds (40, 48, 49). The amide resonances for the two Cys residues forming the vicinal disulfide bond displayed severely broadened line widths, as observed in human hepcidin, but in contrast to all surrounding residues. One explanation for this is that the eight-membered closed loop formed by the cystine could be undergoing conformational exchange on an intermediate NMR time scale. Most interestingly, the amide protons displayed the broadest line widths, whereas the H^N^ and H^α^ protons were not significantly broadened. Such conformational exchange does not indicate that the structure is unstable or reactive but it may be functionally significant. A recent review (40) has highlighted that vicinal disulfide bonds share a number of common stereochemical features, including a non-planar peptide bond in the trans conformation, and they are often located in the center of a type VIII β-turn in the peptide backbone. Bass hepcidin also contains a non-planar peptide bond between the cysteines and the φ/ψ angles of the two residues (Cys^8^-70°/−40°, and Cys^9^-150°/156°) are very close to those of a model type VIII turn ( residue (i + 1) −60°/−30° and residue (i + 2) −120/120°).

The vicinal disulfide bond is known to be functionally significant in a number of different proteins. A spider toxin retains the same fold but loses its neurotoxic activity following reduction of the vicinal disulfide (48). Other proteins such as methanol dehydrogenase (50) and the acetylcholine receptor (51) lose activity upon reduction of their vicinal disulfide bonds. In addition, the vicinal disulfide bond in mercuric ion reductase is believed to be important in mercuric ion binding (52). The different local conformation associated with a reduced or oxidized vicinal disulfide bond has led to speculation that such an entity may form a “redox-activated conformational switch” (40). The vicinal disulfide bond in bass and human hepcidin is located in the loop region of the molecule that is the most flexible part of the structure and shows the highest degree of sequence diversity between species. The combination of flexibility, sequence diversity, and the significance of the vicinal disulfide bond leads us to speculate that the loop region of hepcidin is, functionally, the most important part of the molecule.

**Antimicrobial Activities**—In this study we examined the spectrum for bass hepcidin of antimicrobial activity against 21 species of bacteria including strains tested previously with human hepcidin. Consistent with studies of human hepcidin, bass hepcidin was active against *E. coli* but had little or no detectable activities against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, or *Candida albicans* (2). Bass hepcidin and human hepcidin were also both active against *A. niger* in spore germination assays. Our results indicate that bass hepcidin and human hepcidin were both active in a similar range of concentrations against Gram-negative bacteria (2). The anti-
microbial potency of bass hepcidin contrasted sharply with another bass AMP, moronecidin, that was also purified from HSB gill tissue (5). Moronecidin is a 22-amino acid, linear, cationic peptide with an amphiphatic, α-helical structure that exhibits a more potent, broader spectrum of bactericidal activity than hepcidin (5). Peptides like moronecidin are thought to aggregate and interact with negatively charged bacterial membrane components and to disrupt them by forming pores or solubilizing the membrane via a “detergent” effect (53–55). This direct membrane disruption is believed to kill the bacterium by creating osmotic imbalance and loss of cytoplasm. Bass hepcidin is cationic and adopts an amphiphatic structure in solution and thus has the potential to interact with and disrupt bacterial membranes like linear, α-helical peptides. Our studies with Y. enterocolitica demonstrate that in vitro hepcidin kills this bacterium much more slowly than does moronecidin. This may reflect inherent biophysical differences between the two peptides (56). Hepcidin is less cationic and amphiphatic than moronecidin. Both of these parameters have been shown to be important structural attributes for potent antimicrobial activity (57). Alternatively, Y. enterocolitica is known to have an efflux pump/potassium antipporter to combat the antimicrobial activities of host cationic peptides. Thus, the differences in bactericidal activity between the two peptides may reflect different susceptibilities of the peptides to this antibiotic resistance mechanism (58). Finally, hepcidin may kill bacteria by a mechanism independent of membrane permeabilization (e.g. inhibiting a key metabolic process), which may require prolonged contact with the bacteria to exert microbicidal activity. Further investigation is required to elucidate the mode of action for hepcidin.

**Hepcidin Expression in Vivo Following Infection**—Our results show that infection of bass with either a Gram-positive (S. iniae) or Gram-negative (A. hydrophila) fish pathogen induces hepcidin gene expression in the liver with very similar kinetics. The first hepcidin transcripts were detected within hours following experimental infections, and expression was maximal at 48 h post-infection. The rapidity and remarkable amplitude of this expression profile are consistent with the acute phase response to infections observed in mammals. Human and mice hepcidin expression both require the inflammatory cytokine IL-6, thus defining hepcidin as a type II acute phase response protein (17). To date, quantitative hepcidin expression in mice has not been quantified following bacterial infections to allow comparison to our fish model. However, mice do show a 4-fold increase in hepcidin expression in response to inflammatory stimulators (14). Other studies in human patients with anemia of inflammation show up to 100-fold greater concentrations of hepcidin in their urine (16).

Despite the limited spectrum and potency of hepcidin antimicrobial activity observed in vitro, there are several possible mechanisms by which hepcidin could be effective in vivo as an antimicrobial compound (1, 3, 59). It is possible that tissue concentrations of hepcidin reach higher levels than we tested in vitro, compensating for the levels of specific activity observed in vitro. The dramatic up-regulation of hepcidin expression in liver and other tissues upon experimental infection of HSB with fish pathogens supports this hypothesis (6). In this study, bass liver hepcidin expression increased 3 orders of magnitude within 16 h of infection, 4 orders of magnitude within 24 h, and was nearly 5 orders of magnitude above baseline by 48 h post-infection. The magnitude and duration of the up-regulation of hepcidin expression in the liver following infection suggests that high concentrations of hepcidin are important to the innate immune response against these pathogens. Another mechanism by which hepcidin may potentially exert strong antimicrobial effects *in vivo* is through synergistic interactions with other inducible acute phase response proteins and/or constitutively expressed antimicrobial compounds in the tissues. There are a number of examples of co-localization of antimicrobial compounds in various tissues and cell types, as well as specific evidence of synergistic activity when AMPs are combined *in vitro* (60–62). Our previous studies have demonstrated that both the hepcidin and moronecidin genes are expressed in the gill tissue of bass and that their mature peptides reside in this tissue (5, 6, 63). Thus, our demonstration of synergism between hepcidin and moronecidin antimicrobial activities *in vitro* against both Gram-positive and Gram-negative bacteria may reflect an elegant addition to innate immune systems of teleosts. A model where the inducible hepcidin peptide acted synergistically with constitutively expressed AMPs such as moronecidin would predict an increased broad spectrum antimicrobial defense during the early stages of an infection.

**Hepcidin, Inflammation, and Hypoferremia**—In mammals, hepcidin plays a key role in the hypoferremic response during inflammation, and given the similarity of the two structures and activities, there is potential for a similar role for hepcidin in bass and other teleosts (3, 16, 59). Bacterial pathogens require iron for growth, and most have evolved sophisticated mechanisms for obtaining iron from their hosts to support their proliferation (64). In this regard, hepcidin has been proposed to help combat infection by restricting iron availability to invading pathogens through a strong hypoferremic response, and thus limiting their proliferation (see reviews by Ganz (3) and Ashrafian (65)). The potential for hepcidin-induced hypoferremia in fish is consistent with studies in trout and salmon, where lower free iron in plasma was observed 24–48 h after injection of lipopolysaccharide (66, 67). In addition, symptoms of anemia have been observed in bass infected with *S. iniae* or *A. salmonicida*, both of which we have shown to be potent inducers of hepcidin expression (6, 68).

Strong conservation of the structure and rare vicinal disulfide between bacterial and human suggests that the hepcidins are functionally constrained from sequence divergence. Because bass hepcidin does not contain any acidic residues, it is not surprising that we found no evidence of direct binding of bass hepcidin to ferric iron by NMR (data not shown). Our data suggest that hepcidin has the potential to exert its effects on the innate immune response of teleosts through a combination of...
of activities. The bass model employed in these studies should provide a powerful approach to further elucidate hepcidin function(s), including it potential role in hypoferremia in teleosts.

Acknowledgments—We thank Steve Taylor for generous support in the early stages of this project. We thank Yann Lefievre, Daniel Nugent, Ilya Khaytin, and Kim Nguyen for assistance in synthesis and refolding of bass hepcidin, and Shenggen Yao for assistance in collecting NMR spectra.

REFERENCES