Albumin Binding of Short Cationic Antimicrobial Micropeptides and Its Influence on the *in Vitro* Bactericidal Effect

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The interactions between a range of small cationic antibacterial tripeptides and bovine and human serum albumin in a buffered aqueous solution at 25 °C have been studied using isothermal titration calorimetry. Results from the binding study indicate a single binding site on albumin with a dissociation constant between 4.3 and 22.2 μM for the different peptides. In a theoretical mouse model, a dissociation constant in this range corresponds to 95% albumin binding. The effect of this albumin interaction on the antibacterial capacity of the peptides against *Staphylococcus aureus*, strain ATCC 25923 was studied by including albumin in the assays at a 0.55 mM concentration. Presence of albumin induced a 10-fold increase of the minimal inhibitory concentration for the bulk of the peptides. Albumin itself has no effect on the bacterial growth and this increase is entirely ascribed to a strong competing protein binding. Collectively these results indicate that these antibacterial peptides do bind to albumin and that this binding strongly reduces the effective concentration of peptides available to combat bacteria.

Introduction

Albumin, a 66 kDa monomer forming three helical domains, is the most abundant protein in plasma. The protein is present in humans at around 0.6 mM, and it plays an important role as it binds and transports a range of endogenous molecules such as fatty acids, hemin, and bilirubin.[1] The multiple binding sites can also bind exogenous compounds displaying structural features similar in size and polarity, and therefore it also binds hydrophobic drugs like Propofol, Diazepam, and Ibuprofen to mention but a few.[2−4] While a protein binding may aid in the solubilization of hydrophobic drugs it may also, at high binding affinities, prevent the drugs from reaching their target in a sufficient concentration to exert their efficacy.[5] Determination of the binding to albumin is thus important for the development of any new drug.

Antimicrobial peptides represent a versatile class of promising molecules to combat the growing problem of antibiotic resistance.[6] Recent decades have seen a boom in the cases of antibiotic resistance among different bacteria which unfortunately has not been followed by a similar rise in new classes of antibiotics to combat them. Our group, has over the years worked toward simplifying these complex molecules in an attempt to bring antimicrobial peptides closer to clinical use.[7−10] One step has been the identification of the minimum antibacterial motif in cationic antibacterial peptides (CAP)[a] using derivatives of lactoferricin.[7] These minimal peptides can be surprisingly small, and a pronounced antibacterial activity has been shown in a series of dipeptides displaying adequate lipophilic bulk and cationic charge required by the pharmacophore. Generally, two units of bulk, each similar to a phenyl group in size and polarity and two cationic charges are sufficient, even though there exist differences among the motifs between different target bacteria.[8] Incorporation of bulky, nongenetically coded amino acids can in some instances be beneficial for the activity while simultaneously allowing for a wider range of possible structural modifications.[9] Initial studies on the ability of CAPs to destroy bacteria (both antibiotic sensitive and multi resistant strains) *in vitro* have been most promising and suggest further efforts. CAPs are believed to exert their antibacterial action by interacting with the negatively charged bacterial surface.[11] Once close enough, the CAPs are able to insert their hydrophobic section into the cytoplasmic membrane, resulting in permeabilization, leakage, and cell death.[12] The specific mechanisms behind the cell death is however both bacteria and peptide dependent.

Strong binding to plasma proteins like albumin could decrease the effective concentration of CAPs at the bacterial surface, and therefore it is of interest to evaluate what effect possible protein binding would have on the bactericidal potency. In this paper we describe the use of isothermal titration calorimetry (ITC) to determine the binding of a range of CAPs to albumin (bovine and human) and the influence of this binding on the antibacterial activity of the peptides. The SAR of the CAP library with regard to antibacterial activity, as well as protein binding, is also discussed.

Results and Discussion

All the peptides used in this study are based on the same "scaffold" consisting of a nongenetically coded biphenyl alanine (Bip) moiety flanked by two arginines. Bip has previously been successfully used in the preparation of CAPs, and it offers a convenient way of increasing the hydrophobic bulk of a small peptide.[8,9] Boc-Bip is commercially available and easily incorporated into the growing peptide chain. A range of other, similarly bulky and hydrophobic, derivatives are available, but Bip was used throughout the study for simplicity and to aid the interpretation. Diversity is instead obtained by modifying the

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[a] Abbreviations: Cationic antibacterial peptide, CAP; isothermal titration calorimetry, ITC; bovine serum albumin, BSA; human serum albumin, HSA; diisopropylethylamine, DIEA; 1-hydroxybenzotriazole, 1-HOBt; chlorotripyrrolidinophosphonium hexafluorophosphate, PyCloP; *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HBTU; reversed phase HPLC, RP-HPLC; biphenyl alanine, Bip; minimal inhibitory concentration, MIC.

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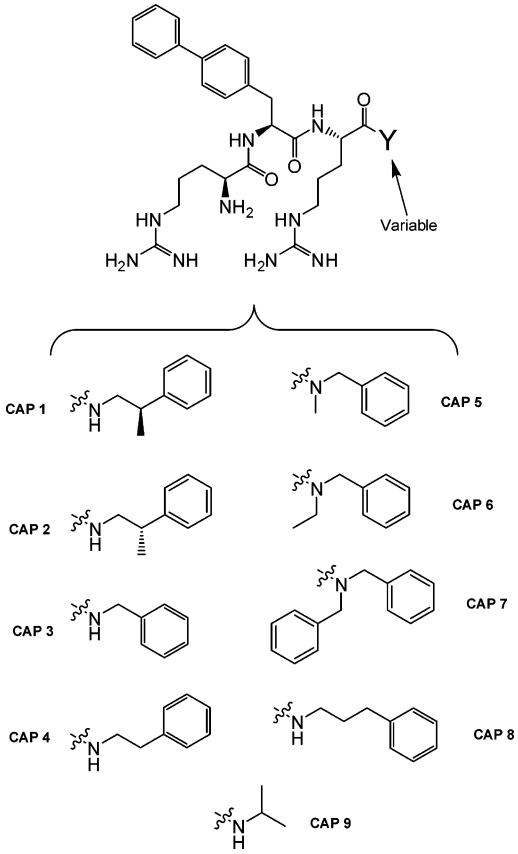

Figure 1. General structure of the peptide scaffold (Arg-Bip-Arg-Y) used in the binding studies. "Y" indicates the different C-terminal modifications.

C-terminus in various ways, as shown above in Figure 1, yielding a library of secondary and tertiary amides differing in both bulk, polarity, and stereochemistry and known to have antibacterial effect while displaying variation in stability toward degradation (unpublished experiments; manuscript to be submitted to *Biochemistry*) which is an important property for these compounds. It should be noted that the Y-group diversity has been limited to small alterations in order to maintain a high antibacterial activity.

ITC allows for the determination of the heat generated during a binding event, and data collected during analysis can give detailed information about the forces involved in the interaction.[13−15] Stepwise additions of ligand to a stirred protein solution generate heat effects based on both dilution and ligand−protein interactions. Thermal power, i.e., heating or cooling, is applied to the reaction cell to keep it at the same constant temperature as a reference cell containing only water, and this thermal power is proportional to the heat of reaction. The technique, which is simple, rapid, and versatile, has previously been used in a range of studies of the interactions between protein and drugs.[16] Thermodynamic descriptors such as the association constant, enthalpy, and the stoichiometry of a given interaction can be determined with high accuracy. Initial binding studies were performed solely with bovine serum albumin (BSA, ≥99%). The repeated injections of CAP to the stirred BSA solution resulted in an exothermic release of energy represented by the peaks shown in Figure 2. The size of the peaks decreased with the number of additions, as less binding sites became available on the BSA molecules upon increased CAP concentrations.

Data from the ITC studies suggest the formation of a 1:1 complex between the CAPs and BSA. The peak size becomes

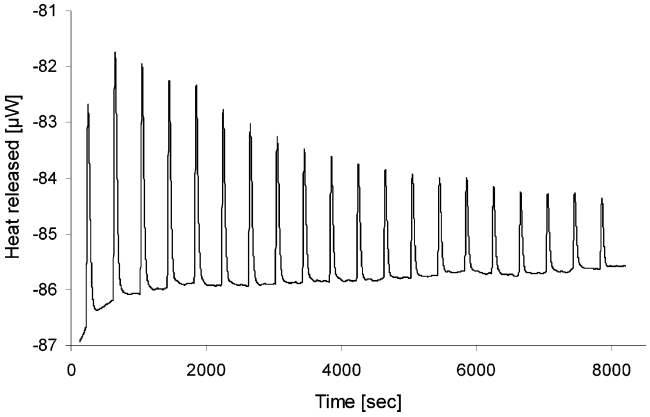

Figure 2. Experimental data from the ITC runs, in this case 20 sequential additions of **CAP 3** to BSA at 25 °C. Each peak represents the generated heat upon addition. The first peak is small due to slow syringe leakage during the initial equilibration step.

close to that of the heats of dilution after ∼11 injections (same amount of peptide and BSA in the reaction cell), representing a 1:1 complex even though the stoichiometry generated from the binding isotherm, as can be seen in Table 1, is slightly lower, averaging 0.8:1 (CAP:BSA). The data were, after subtraction of the heats of dilution, fitted to a one-site binding model developed using the BindWorks software. A correlation between the model and the data is shown in Figure 3. The dissociation constant (K_D, the inverse association constant ($1/K_a$)) for the interactions was determined to be between 4.3 and 22.2 μM (Table 1) using the binding isotherm for each peptide (Figure 3). Table 1 also presents the thermodynamic parameters for binding of the peptides to BSA and confirms a similar binding mechanism for the peptides. The free energy of binding (ΔG) varies from −26.7 to −30.6 kJ/mol with both favorable enthalpic and entropic contributions (Table 1).

While albumin preferably binds anionic drugs, there are examples of binding of a range of cationic drugs to albumin with similar binding affinities as reported here.[17,18] As a range of peptides, including the arginine-containing nonapeptide vasopressin, also bind to albumin,[19] it is not entirely surprising that the different CAPs associate with BSA to such an extent as in our experiments. The similarity in binding strength among the different peptides suggests a binding mode independent of the variable Y-groups used (Table 1). It remains unclear, however, whether the binding occurs in any of the two major binding sites, site I and site II, responsible for most exogeneous compound binding described in the early work by Sudlow,[3] but as the ligands for these sites usually bear one or two negative charges, such a binding seems less likely. Many other ligands bind to albumin in areas other than the well studied site I and II,[18] and we expect this to be the case for the library of Arg-Bip-Arg amides used in this study. Whether the binding is mainly governed by hydrophobic interactions via the Bip moiety or electrostatic interactions between the two Arg and acidic functionalities on BSA is still under investigation. A likely binding mode could be through a combination of both, as the albumins offer both deep hydrophobic binding pockets and an anionic surface.[4] This is further supported by ITC data (Table 1) which suggest that the binding is both enthalpy and entropy driven, indicating favorable electrostatic as well as hydrophobic interactions in the complexes. The isoelectric point (Ip) of BSA is between 4.7 and 4.9, yielding a net negative charge under the experimental conditions employed, well suited for ionic interactions with the two Arg groups.[1] The saturation at stoichiometric amounts and the excellent fit to the theoretical

Table 1. Data from the ITC Runs and the Theoretical Mouse Model

peptide	molecular mass calcd (obsd)	K_D (μM)[a]	n[a,b]	ΔG (kJ/ mol)[a,c]	ΔH (kJ/mol)[a,c]	$T\Delta S$ (kJ/mol)[a]	fraction protein bound (%)[d]
CAP 1	670.9 (670.5)	12.2	0.9	−28.1	−14.6	13.5	96.5
CAP 2	670.9 (671.5)	12.4	0.8	−28.0	−12.5	15.5	96.4
CAP 3	642.8 (642.5)	4.3	0.9	−30.6	−10.1	20.5	98.6
CAP 4	656.8 (657.4)	11.1	0.7	−28.3	−16.3	12.0	96.7
CAP 5	656.8 (657.4)	6.7	0.9	−29.6	−13.7	15.9	97.9
CAP 6	670.9 (670.5)	17.3	0.7	−27.2	−15.1	12.1	95.2
CAP 7	732.9 (732.8)	10.0	0.8	−28.6	−12.5	16.1	97.3
CAP 8	670.9 (670.5)	22.2	0.7	−26.7	−15.2	11.5	93.8
CAP 9	594.8 (594.8)	14.1	0.8	−27.7	−8.7	19.0	95.6

[a] Data from the ITC experiments and the binding isotherms. [b] Stoichiometry of the interaction, experimental error ±0.1. [c] Fitting error ±15%. [d] Calculated using eqs 1−3.

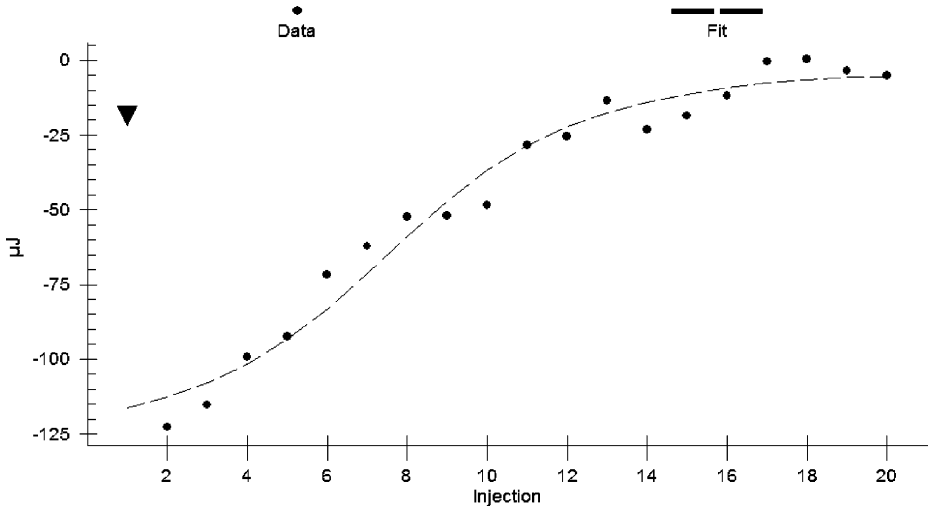

Figure 3. Binding isotherm for **CAP 7** and fit to the theoretical 1:1 binding model. The data from the first injection (▼) is not included due to slow peptide diffusion from the syringe during the equilibration step prior to the first injection. STD: 6.94.

Table 2. Data from the Antibacterial Activity Assay[a]

peptide	MIC (μg/mL)	MIC with BSA (μg/mL)[b]
CAP 1	4 ± 0	31 ± 15
CAP 2	4 ± 0	31 ± 15
CAP 3	5 ± 1	47 ± 3
CAP 4	4 ± 0	47 ± 3
CAP 5	7 ± 1	38 ± 13
CAP 6	5 ± 1	38 ± 13
CAP 7	2 ± 0	13 ± 7
CAP 8	2 ± 0	25 ± 0
CAP 9	47 ± 3	110 ± 10

[a] Generated using standardized microdilution techniques. Experiments performed in triplicate. [b] Present at a concentration of 0.55 mM.

model point toward a single binding site with affinity for the peptides. The fact, that the peak size does not equal that of the heats of dilution upon saturation is merely an indication of the presence of weak nonspecific interactions in addition to this binding site, which is only to be expected, considering the size of albumin and the many potential interaction points on its surface.

A 20 g mouse with 1.67 mL of blood injected with 0.28 mg of CAP (14 mg/kg, resulting in a 2.3-fold excess of BSA compared to CAP) in a total volume of 200 μL was used as theoretical model to calculate the amount of BSA bound CAP. The sequences for bovine, human, and murine serum albumin align with 70−76% identity (see Supporting Information for sequence alignment analysis), making comparisons between these systems feasible. The extent of complexation was calculated using eq 3, derived from eqs 1 and 2, where [M], [P], and [MP] represent the concentrations of macromolecule ([M], BSA in this case), peptide [P], and complex [MP], respectively. $[M_{tot}]$ and $[P_{tot}]$ represent the total concentrations of BSA (0.55 mM in this model due to dilution from the large injection volume) and peptide. K_a is the association constant obtained from ITC analysis.

$$K_a = \frac{[MP]}{[M]\cdot[P]} \tag{1}$$

$$K_a = \frac{[MP]}{(([M_{tot}] - [MP])\cdot([P_{tot}] - [MP]))} \tag{2}$$

$$[MP]^2 - \left([M_{tot}] + [P_{tot}] + \frac{1}{K_a}\right)\cdot[MP] + [M_{tot}]\cdot[P_{tot}] = 0 \tag{3}$$

As is shown in Table 1, the theoretical binding to albumin was very similar, roughly ranging from 94 to 98%. It is clear that the small difference in dissociation constant within the library here plays little role for the extent of protein binding.

Staphylococcus aureus (antibiotic resistant strains as well as sensitive ones) are highly sensitive to this class of molecules, and the minimal inhibitory concentrations (MIC) for the different CAPs against strain ATCC 25923, determined through a standard micro dilution technique,[20] are shown in Table 2.

While the hydrophobicity offered by the Bip side chain together with the two positively charged arginines is sufficient for some bactericidal activity, it is obvious that incorporation of additional bulky hydrophobic elements will further improve this activity. Incorporation of an extra phenyl group on the C-terminus is enough to generate a tenfold decrease in MIC. While the different Y-groups hardly seemed to influence

Table 3. Binding and Antibacterial Data for the Peptides on Different Albumin of Different Purity

peptide	albumin[a]	K_D (μM)[b]	n [b,c]	ΔG (kJ/mol)[b,d]	ΔH (kJ/mol)[b,d]	$T\Delta S$ (kJ/ mol)[b]	MIC with albumin (μg/ mL)[e]
CAP 3	HSA I	15.7	0.8	−27.4	−24.2	3.4	40 ± 9
	HSA II	15.5	0.8	−27.4	−25.1	2.3	80 ± 20
	BSA I	4.3	0.9	−30.6	−10.1	20.5	47 ± 3
	BSA II	13.0	1.1	−27.9	−11.4	16.5	30 ± 10
CAP 7	HSA I	10.4	0.7	−28.4	−25.8	2.6	6 ± 1
	HSA II	15.9	0.7	−27.4	−20.2	7.2	11 ± 8
	BSA I	10.0	0.8	−28.6	−12.5	16.1	13 ± 7
	BSA II	12.3	0.8	−28.0	−12.9	15.1	6 ± 0
CAP 9	HSA I	10.8	0.8	−28.3	−16.4	11.9	113 ± 10
	HSA II	18.2	0.7	−27.1	−7.1	20.0	100 ± 9
	BSA I	14.1	0.8	−27.7	−8.7	19.0	110 ± 10
	BSA II	14.5	0.8	−27.6	−15.7	11.9	80 ± 11

[a] Albumin purities: HSA I ∼99%, HSA II ≥96%, BSA I ≥99%, BSA II ≥96%. [b] Data from the ITC experiments and the binding isotherms. [c] Stoichiometry of the interaction, experimental error ±0.1. [d] Fitting error ±15%. [e] Generated using standardized microdilution techniques. Experiments performed in duplicate. Albumin is present at a concentration of 0.55 mM.

albumin binding, its role in killing bacteria is obvious, as **CAP 9**, lacking any bulky hydrophobic moieties, displays the highest MIC value, while **CAP 7** with its two phenyl groups displays a 25 times lower MIC value (Table 2). It has previously been shown that a balance between charge and hydrophobic bulk exists for this group of molecules, and a clear distinction is seen here for the peptides with aromatic Y-groups even though size, orientation, and stereochemistry seem to play little role in this case.[8] Their BSA complexation is similar, and one can assume a similar behavior for the binding to bacteria even though the albumin lacks the anionic lipopolysaccarides of the bacteria responsible for the initial binding.[11,12] This indicates that binding only is not enough for killing bacteria, adding further support to the theory that insertion of a hydrophobic element is a crucial step for cytoplasmic membrane permeabilization.

A 10-fold increase in MIC is seen when BSA is included in the bactericidal assay at physiological concentration as can be seen in Table 2. This corresponds well with the roughly ∼95% fraction that is bound to BSA in the theoretical model under similar conditions, thereby reducing the effective concentration of free CAP ten times. The concentration of the CAPs at MIC are some three times lower than in the theoretical model (data not shown) so a slightly higher protein binding could be expected in those cases. Albumin binding of **CAP 9** with a Y-group differing in both size and hydrophobicity from the rest of the peptides did not increase the MIC to the same extent, suggesting a slightly different behavior in the presence of bacteria. It should be noted that the experimental determination of K_a used to calculate the theoretical bound fraction, for practical reasons, is performed in the absence of competing bacteria. Keeping a constant concentration of bacteria during the course of the binding study would indeed be very difficult. The increased osmolality caused by BSA itself has no effect on the growth of *S. aureus*, and control experiments without CAPs (with and without BSA) showed comparable growth rates (data not shown).

To establish if these observations were solely associated with BSA of certain purity, complementary experiments were performed on HSA as well, with three peptides diverse in structure and antimicrobial activity (**CAP 3**, **CAP 7**, **CAP 9**). Four albumins, two human, and two bovine of different purities were selected for this study. The purities were different (see the Experimental Section for details about the different albumins) to allow investigation of the potential role of associated fatty acids on peptide binding. The results from these experiments are summarized below in Table 3.

The binding of the three peptides to the different albumins was generally very similar to that presented in Table 1 (corresponding to BSA I in Table 3) with dissociation constants ranging from 4.3 to 18.2 μM. All peptides displayed nearly identical ΔG values. The pure, essentially fatty acid free albumins BSA I and HSA I associated with the peptides to the same extent as those of less purity (≥96%), BSA II and HSA II. The binding contribution to albumin would be difficult to evaluate with a protein sample of much lower purity. This similarity in binding implies that the role of associated endogenous fatty acids is of little significance for the association, at least within the frame of these experiments. It further suggests that the peptide binding site is different from the fatty acid binding sites on the albumin surface. Of more interest is perhaps the difference in $T\Delta S$ seen for HSA compared to BSA for **CAP 3** and **CAP 7**. These peptides both have bulky, lipophilic Y-groups, and the contribution to the overall binding for these seems to differ slightly between the different classes of albumins although they bind to the same extent. **CAP 9** with its C-terminal isopropyl modification does not display this difference. Further studies are underway to investigate the detailed mode of binding for this class of molecules, using peptides displaying more diverse structural features which may impair the antibacterial effect. The extent of the increase in MIC was similar for the different albumins. Binding of **CAP 3** induces a larger increase than does **CAP 7** and **CAP 9** for both HSA and BSA.

It may be perceived that the interactions between the peptides and the anionic bacterial surface may be strong enough to cause the peptides to dissociate from albumin, preventing the protein from inhibiting the bactericidal effect. However, since albumin itself is anionic at physiological pH, the bacterial surface needs to offer a superior binding environment to displace the bound peptide. For the peptides with a bulky Y-group, no evidence of such competitive displacement is detected though, suggesting that the charged guanidine groups, responsible for the initial bacterial interaction, become unavailable upon binding to the binding site. **CAP 9**, lacking a bulky Y-group, is however easier to displace by the bacteria, and the increase in MIC is lower for this peptide. Unfortunately, this peptide is the least efficient bactericidal compound in the library.

As the protein binding seems to lower the effective concentration of CAPs, it will be important to take it into consideration for future studies in blood and for *in vivo* testing of these and similar molecules. Rational drug design could be a way of approaching the problem with albumin binding and lowered *in vivo* efficacy, but that requires detailed information about the albumin/peptide complex.[18,21] The lowered effect of binding of **CAP 9** illustrates that it should be feasible to rationally design peptides of this class that lower the unwanted increase in MIC caused by albumin complexation and thus still maintain a high antibacterial activity. It is also important to bear in mind that

the parts of the molecule responsible for protein binding seem, to a large extent, to be the same as those needed for bactericidal effect, leaving little room for large molecular alterations. Co-administration of a displacer compound is also a potential strategy to increase the effect of the bound peptides which, just like the previously mentioned drug design approach, requires a more detailed knowledge about the chemistry of the binding site.[18] One way to minimize the influence of protein binding could be dosage modification. It is however difficult to discuss dosage issues before proper knowledge is obtained about how these compounds behave in such a complex matrix as blood. More studies are thus needed and are underway.

Conclusion

To conclude, ITC has been used to study the interactions between a range of active antibacterial peptides and bovine and human serum albumin. It was shown that the different peptides bind to albumins to a similar extent, with a dissociation constant of around 10 μM. The mode of binding is unclear at the moment, but all the peptides seem to bind to a single binding site. In a theoretical mouse model this corresponds to ~95% protein binding. Possessing a net negative charge at physiological pH, it is likely the albumins compete with the bacterial surface for peptide binding. The bound peptides do not seem to be able to participate in destroying bacteria, as a tenfold increase in MIC is seen when albumin is included in the antibacterial assay in physiological concentrations. Collectively the results indicate that these antibacterial peptides bind to albumin and that this binding effectively lowers the effective concentration of peptides available to combat bacteria. This drop in activity upon protein binding is not desired, but can hopefully be circumvented by proper dosage allowing the peptides to effectively continue to destroy bacteria. While lowering the effective concentration of CAPs *in vitro*, a high protein binding may not necessarily be a problem considering the complexity *in vivo* with regards to the pharmacokinetics of the compounds, i.e., volume of distribution, mode of clearance and half-life.

Experimental Section

Chemicals. Essentially fatty acid and globulin free bovine serum albumin ≥99% (BSA I) (A0281), bovine serum albumin ≥96% (BSA II) (A9085), essentially fatty acid free human serum albumin ~99% (HSA I) (A3782), and human serum albumin ≥96% (HSA, HSA II) (A4327) were supplied by Sigma-Aldrich. Boc-Arg-OH and Boc-*p*-phenyl-Phe-OH were supplied by Bachem. Benzylamine, isopropylamine, 2-phenylethylamine, 3-phenylpropylamine, (*R*)-2-phenylpropylamine, (*S*)-2-phenylpropylamine, *N*,*N*-methylbenzylamine, *N*,*N*-ethylbenzylamine, and *N*,*N*-dibenzylamine providing the C-terminal diversity were purchased from Fluka. Diisopropylethylamine (DIEA), 1-hydroxybenzotriazole (1-HOBt), chlorotripyrrolidinophosphonium hexafluorophosphate (PyCloP), and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′ tetramethyluronium hexafluorophosphate (HBTU) were purchased from Fluka. Other reagents and solvents were supplied by Sigma-Aldrich and were used without further purification with the exception of DCM, which was filtered through alumina.

General Procedure for Solution-Phase Peptide Coupling Using HBTU. The peptides were prepared in solution by stepwise amino acid coupling using Boc protecting strategy according to the following general procedure.[7] The C-terminal peptide part with a free amino group (1 equiv) and the Boc protected amino acid (1.05 equiv) and 1-HOBt (1.8 equiv) were dissolved in DMF (2−4 mL/mmol of amino component) before addition of DIEA (4.8 equiv). The mixture was cooled on ice, and HBTU (1.2 equiv) was added in portions before it was shaken at ambient temperature for 1−2 h on a rocking table. The reaction mixture was diluted with ethyl acetate and washed with citric acid, sodium bicarbonate, and brine before the organic solvent was removed under vacuum. Complete removal of the Boc protecting group of the resulting peptide was achieved through the use of 95% TFA or acetyl chloride in anhydrous methanol. This approach was also used for incorporation of the different amines forming the C-terminal amides (apart from the capping with *N*,*N*-dibenzylamine which was prepared according to the procedure below) yielding the Y-group diversity.

Procedure for Solution-Phase Amide Formation Using PyCloP.[22] Boc-Arg-OH (1 equiv), *N*,*N*-dibenzylamine (1.1 equiv), and PyCloP (1 equiv) were dissolved in a mixture of dry DCM and DMF (2:1 v/v). The solution was cooled on ice, and DIEA (2 equiv) was added under stirring. The solution was stirred for 1 h at room temperature. The reaction mixture was evaporated, redissolved in ethyl acetate, and washed with citric acid, sodium bicarbonate, and brine. The solvent was removed under vacuum and the Boc protecting group removed in the dark using 95% TFA.

CAP 3: ^{1}H NMR (D_2O), δ 0.96 (d, J = 6.9 Hz, 3H), 1.13−1.28 (m, 2H), 1.34−1.49 (m, 2H), 1.52−1.64 (m, 2H), 1.81−1.94 (m, 2H), 2.61−2.70 (m, 1H), 2.92−3.05 (m, 5H), 3.15−3.20 (m, 3H), 3.96 (t, J = 7.1 Hz, 1H), 4.02 (t, J = 6.5 Hz, 1H), 4.61 (dd, J = 9.7, 6.7 Hz, 1H), 7.06 (d, J = 7.2 Hz, 2H), 7.22 (dd, J = 8.5, 6.1 Hz, 1H), 7.30 (dd, J = 16.9, 7.9 Hz, 4H), 7.42 (t, J = 7.4 Hz, 1H), 7.52 (t, J = 7.7 Hz, 2H), 7.72 (dd, J = 18.3, 7.8 Hz, 4H); ESMS: calcd for $C_{36}H_{50}N_{10}O_3$: 670.9, found 670.5; Purity determined by HPLC: Retention time: 13.80 min, purity 99.7%. See Supporting Information for data on remaining compounds.

Peptide Purification and Analysis. The peptides were purified using reversed phase HPLC (RP-HPLC) on a Delta-Pak (Waters) C_{18} column (100 Å, 15 μm, 25 × 100 mm) employing a mixture of water and acetonitrile (both containing 0.1% TFA) as the eluent. The peptides were further analyzed by RP-HPLC using an analytical Delta-Pak (Waters) C_{18} column (100 Å, 5 μm, 3.9 × 150 mm). Positive ion electrospray mass spectrometry on a VG Quattro quadrupole mass spectrometer (VG Instruments Inc., Altringham, UK) ensured isolation and identification of the correct products.

ITC Studies. Heats of interaction were determined using a CSC 5300 NanoIsothermal Titration Calorimeter III with a 1 mL cell volume (Calorimetry Sciences Corporation, Lindon, UT). In a typical titration experiment, the peptide (2.1 mM) was added in 20 aliquots (5 μL) to a stirred (200 rpm) solution of albumin (0.11 mM) in an aqueous buffer at 25 °C. The buffer used was a 50 mM Tris-HCl, 10 mM $CaCl_2$ at pH 8.2. An interval of 400 s between the injections was allowed to reach equilibrium between the interacting species. The heats of dilution were determined in a similar fashion where the peptides were added to a stirred buffer solution without albumin. Subtraction of the dilution heat yielded the heat of interaction, and a binding isotherm from which the association constant and complex stoichiometry was calculated using BindWorks analysis software.

Microbiological Studies. MIC determinations and growth studies, with and without albumins, on *S. aureus*, strain ATCC 25923, were performed by Toslab AS using standard methods.[20]

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Supporting Information Available: Experimental and ^{1}H NMR spectroscopic data for **CAP 1−9** in conjunction with LC traces and structures; sequence alignment analysis for human, bovine, and murine serum albumin. This material is available free of charge via the Internet at http://pubs.acs.org.

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