Investigating the nucleic acid interactions and antimicrobial mechanism of buforin II

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Abstract Buforin II (BF2) is an antimicrobial peptide that is hypothesized to kill bacteria by entering cells and binding nucleic acids. To further investigate this proposed mechanism, we used computer modeling and experimental measurements to consider the interactions between BF2 and DNA. Computational and experimental results imply that the peptide forms specific interactions with DNA. Moreover, we observe a general correlation between DNA affinity and antimicrobial activity for a series of BF2 variants. Thus, our results support the proposed mechanism for BF2 and provide a useful approach for evaluating the nucleic acid interactions of other antimicrobial peptides.

Keywords: Histone-derived antimicrobial peptide; Buforin II; Parasin; Hipposin; Peptide–DNA interaction; Molecular dynamics

1. Introduction

Although the majority of antimicrobial peptides kill cells through membrane disruption [1], a few peptides, such as pyrrhocoricin [2] and indolicidin [3], act by entering bacterial cells and interfering with some critical physiological process. Buforin II (BF2) has also been hypothesized to target intracellular processes in bacteria. BF2 is a 21-amino acid long peptide active against a wide range of bacteria [4]. It shares complete sequence identity with a portion of histone H2A that directly interacts with nucleic acids. In fact, researchers proposed that BF2 kills bacteria by entering cells and interacting with nucleic acids [5]. In addition to homology with histone H2A, this proposed mechanism was based on two observations. First, BF2 can bind DNA and RNA in vitro [4,5] and lipid vesicles in vivo [6] without causing significant membrane permeabilization. Second, BF2 can bind DNA and RNA in vitro [5]. Although the proposed mechanism is quite intriguing, this data does not directly connect nucleic acid binding to antimicrobial activity. Moreover, it is unclear whether there are specific interactions between BF2 and nucleic acids or whether they only bind because of their opposite net charges. Although previous studies have considered the structure of BF2 in solution and bound to membranes [6–8], none have characterized how BF2 binds nucleic acids on the molecular-level. In this paper, we used computational and experimental techniques to characterize the nucleic acid binding of BF2 and investigate how this binding relates to antimicrobial activity.

2. Materials and methods

2.1. Molecular modeling

Since the BF2 sequence is identical to a portion of histone H2A, we developed an initial model of BF2 bound to DNA based on a Xenopus laevis histone·DNA complex crystal structure (1AOI) [9]. Specifically, we extracted residues 16–36 from chain G of this structure along with the adjacent section of DNA (bases 107–127 of chain I and bases 166–186 of chain J). The residue of the extracted peptide corresponding to Phe 10 of BF2 was mutated to Trp for consistency with experimental measurements described below. This F10W mutation does not significantly affect the antimicrobial activity or membrane interactions of BF2 [6]. The complex was refined using molecular dynamics (MD) simulations in AMBER 8.0 [10] with the ff03 force field. For simulations, the complex was solvated with 8282 TIP3P waters, and Na⁺ ions were added to neutralize the overall system charge. All Arg and Lys residues and the N- and C-termini were ionized, while the single His sidechain was left uncharged. Before MD, the system was subjected to 100 steps of steepest descents minimization. The minimized system was heated to 300 K over 20 ps, and the trajectory was extended to a total length of 10 ns with constant temperature (300 K) and isotropic constant pressure (1 bar). A timestep of 2 fs was used, and bonds to hydrogen were constrained using SHAKE [11]. Short-range interactions were calculated with a cutoff of 8 Å, and long-range electrostatics were calculated using PME [12]. A control simulation (data not shown) with a shorter DNA strand (13 bp) yielded similar properties.

MD analyses were performed using tools in the AMBER suite. Interaction energies were calculated with the molecular mechanics-Generalized Born surface area (MM-GBSA) approach [13], averaging snapshots saved every 10 ps from the last 5 ns of the MD simulation. Calculated interaction energies showed the same trends when averaged over the last 2 ns of the simulation. The solvation component of interaction energy was calculated using a GB/SA approach [14], with a salt concentration of 100 mM and a dielectric constant of 1 and 80 for the solute and solvent, respectively.

2.2. Fluorescent intercalator displacement assay

Chemically synthesized wild type and mutant BF2 at >95% purity was obtained from EZ-Biolab (Westfield, IN). All peptides had unblocked termini and included the F10W mutation described above. DNA oligos with the same sequence as the portion of DNA directly bound by BF2 in the MD simulation (AAATACACTTTTGGT and its complement) were obtained from IDT (Coralville, IA). DNA binding was measured using a fluorescence intercalator displacement (FID) assay [15,16]. Briefly, a quartz cuvette was loaded with thiazole orange (0.55 μM) in STE buffer (10 mM Tris, 50 mM sodium acetate, 100 mM NaCl, pH 7.4). The cuvette was then placed in a photometer equipped with a 495 nm excitation filter and a 520 nm emission filter. An increasing concentration of buffer, sample, or DNA was added, and the fluorescence was measured.

Abbreviations: BF2, buforin II; MD, molecular dynamics; FID, fluorescent intercalator displacement; MM-GBSA, molecular mechanics-Generalized Born surface area
NaCl, 1 mM EDTA, pH 8.0). The fluorescence of this solution was measured (excitation 509 nm, emission 527 nm) and normalized to 0% relative fluorescence. DNA in STE buffer was added to the cuvette (1.1 µM bp final concentration). After equilibrating for 5 min the fluorescence of this solution was measured and normalized to 100% relative fluorescence. Aliquots of a peptide solution were then added with mixing, allowing 5 min of incubation after each addition before measuring fluorescence. C50 (concentration of peptide required to reduce fluorescence to 50% of the initial emission) was determined by a curve fit to data; all fits for data included had $R^2 > 0.95$. Relative DNA binding constants were obtained by comparing the inverse of C50 [15].

2.3. Radial diffusion assay

The antibacterial activity of BF2 variants was measured using the radial diffusion assay of Lehrer and co-workers [17]. For the assay, a culture of *Escherichia coli* in LB media grown overnight (14–16 h) at 37 °C was diluted 1:1000 into 50 ml of TSB and incubated with shaking for 2.5 h at 37 °C. This culture was then centrifuged at $\approx 900 \times g$ for 10 min at 4 °C. The resulting pellet was washed with 10 ml of sodium phosphate buffer (10 mM, pH 7.4), pelleted again, and resuspended in 5 ml of buffer. $4 \cdot 10^6$ CFU were mixed with 10 ml of molten underlay agar (1:10 TSB, 1% agarose (w/v), 10 mM sodium phosphate, pH 7.4) at 42 °C with vortexing for 15 s. This agar was poured into a Petri dish and allowed to gel. Wells were formed in the solid media using a pipette attached to a bleach trap, and 2.5 µl of sterile peptide solutions (4 × 10⁻³ M) were added to wells. After incubating for 3 h at 37 °C, the plates were covered with an additional layer of overlay agar (6% (w/v) TSB, 1% (w/v) agarose) and incubated overnight at 37 °C. After overnight incubation, the diameter of the zone without bacterial growth around each well was measured. Measurements from 48 different wells on plates from at least two separate experiments were averaged for each peptide.

3. Results and discussion

3.1. Molecular modeling of BF2 - DNA interactions

To create an initial model of BF2 bound to nucleic acids, we extracted the histone residues identical to BF2 and the region of DNA bound by these residues from a crystal structure of the *X. laevis* histone - DNA complex [9]. However, the protein - DNA interactions in the large histone complex could differ from those that would occur for the short BF2 peptide. Therefore, we refined the extracted peptide - DNA complex using a 10 ns MD simulation. In this simulation, the peptide structure rapidly equilibrated to an overall structure similar to the initial model (Fig. 1A), with BF2 bound in the major groove of DNA (Fig. 1B). The final peptide structure had a Ca RMS deviation of 1.6 Å from the initial histone conformation. However, some adjustments did occur in specific residue positions, such as the Arg 5 sidechain discussed below.

The MM-GBSA method of Kollman et al. [13] was used to calculate the interaction energy between each individual BF2 sidechain and DNA. As expected, the strongest predicted interactions between BF2 and DNA involved basic sidechains (Fig. 2A). All H-bonding interactions between basic BF2 sidechains and DNA involved phosphate groups in the nucleic acid backbone. Since these groups would be identical for all nucleic acid bases, BF2 likely targets nucleic acids in a non-sequence specific manner.

Interestingly, the interaction energies are significantly weaker for some specific basic sidechains (e.g. Arg 5 and Lys 21) compared to others (e.g. Arg 2 and Arg 20) (Fig. 2A and B). This observation implies that BF2 does not only bind DNA
through the non-specific electrostatic attraction between a cationic peptide and nucleic acids. The weaker nucleic acid interaction of Lys 21 is not surprising since the adjacent negative charge of the peptide C-terminus repels that entire residue from the nucleic acid backbone. However, the weaker interaction of Arg 5 arises because this sidechain, which is in direct DNA backbone contact in the histone structure, moves away from DNA in the MD simulation. A control simulation with a shorter DNA strand showed similar structural properties for BF2 and a similar trend in interaction energies for many basic residues (e.g. Arg 2, Arg 20, Arg 5, and Lys 21), although the relative interaction energies of Arg 14 and Arg 17 were more equivalent in this simulation.

3.2. Experimental measurements of BF2 · DNA interactions

To verify that specific interactions occur between BF2 sidechains and DNA, we experimentally compared the DNA binding affinity of BF2 mutants. Four mutants were chosen based on the MM-GBSA analysis (Fig. 2), two to residues with stronger predicted interaction energies (R2A and R20A) and two to residues with weaker predicted interaction energies (R5A and K21A). These residues were chosen for mutations since they showed consistent interaction energy trends in both our MD simulations.

The relative DNA binding constants for these mutants were measured using a FID assay [15,16], which has been used to measure the binding of other compounds to the major and minor grooves of DNA [16,18]. The relative binding constants from these measurements follow a trend consistent with the predicted interaction energies of the peptides, as R2A and R20A mutations affect DNA binding more than R5A and K21A mutations (Fig. 3). However, the R5A and K21A mutations clearly decrease the DNA affinity of the peptide. One likely reason for the decreased affinity of R5A and K21A is that neutralizing any positively charged sidechain would decrease the non-specific electrostatic attraction between BF2 and DNA. However, it is also possible that BF2 can bind DNA in other conformations that have more direct interactions of these sidechains with DNA, although these conformations were not sampled in our simulations. Nonetheless, the clear differences in binding for different charge neutralization mutants confirm that specific BF2 sidechains do have stronger interactions with DNA.

3.3. Correlation of DNA affinity and antimicrobial activity

We also used our series of BF2 variants to determine whether DNA binding affinity is related to the peptide’s antimicrobial activity. To this end, we measured the antibacterial activity of the peptides using a radial diffusion assay [17,19], which has been used in previous studies of BF2 and other histone-derived antimicrobial peptides [20–22]. In this assay, a peptide with greater antimicrobial activity causes a larger region of restricted bacterial growth on the plates. These studies also included one double-mutant, R2A/R5A, for which we also measured the DNA binding affinity using the FID assay.

In general, antimicrobial activity correlated with the relative DNA binding affinity of the peptides (Fig. 4). This relationship supports the assertion that BF2 kills bacteria through its interactions with nucleic acids, since peptides with reduced DNA interactions showed decreased antibacterial activity. One variant, R20A, deviated from the overall correlation between antibacterial activity and DNA binding (Fig. 4), and it would be interesting for future studies to consider why this deviation arises. For example, the R20A modification might affect membrane interactions, altering the peptide’s membrane translocation or permeabilization properties.

4. Summary

The computational and experimental results described in this paper provide the first molecular-level insights into BF2 · DNA interactions. Interestingly, these results imply that BF2 forms specific interactions with DNA. Moreover, disrupting BF2 · DNA interactions generally decreased the antibacterial activity of the peptide. This observation supports the proposed antimicrobial mechanism for BF2 [5], although our data does not preclude BF2 having another yet unidentified intracellular target.

Recent studies have identified other histone-derived antimicrobial peptides [20–25]. As well, other peptides are proposed to target bacteria through interactions with nucleic acids [26,27]. An approach similar to the one used to consider BF2 in this study could be quite useful for future investigations.
probing the nucleic acid interactions and mechanism of these other peptides.

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References