Membrane-Binding Peptides

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RE Coil: An Antimicrobial Peptide Regulator

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Antimicrobial peptides (AMPs) are universal effector molecules of the innate immune system.[1] The peptides provide local antimicrobial responses that target microbial membranes. They kill microorganisms by attacking various components at cellular surfaces and in the cytoplasm through self-promoted uptake.[1] The peptides can also exhibit immunomodulatory activity and inhibit and "monitor" bacterial growth.[1] However, the specific mechanisms that enable such control over microbial invasion remain unknown. With a growing demand for new treatments that would circumvent bacterial cross-resistance, the elucidation of such mechanisms at the molecular level is of considerable interest in applied chemistry. Herein we propose a molecular rationale for the regulation of antimicrobial intervention on the basis of an α-helical antimicrobial peptide regulator.

The antimicrobial activity of AMPs is attributed to their ability to fold upon contact with microbial surfaces.[1] The adopted conformations, α helices or β sheets, appear to play an auxiliary role in affording amphipathic structures—three-dimensional shapes with hydrophobic and cationic amino acid residues that form separate clusters.[2] This behavior enables AMPs to assemble within anionic microbial membranes into membrane-disrupting pores or channels.[1,2] What has yet to be shown is whether the ability of AMPs to assemble and form such structures may be associated with other more specific functions. We hypothesize that AMPs form these structures to regulate antimicrobial activity.

To investigate this hypothesis, we designed an antimicrobial regulator in the form of an α-helical system comprising two individually unfolded complementary peptides: an antimicrobial sequence rich in arginine residues (R coil) and its binding partner based on oppositely charged glutamate residues (E coil; Figure 1). The antimicrobial component binds to, and disrupts, microbial membranes, whereas the complementary sequence cannot bind membranes and exhibits no antibacterial effects. Together the two peptides fold into an α-helical coiled coil (RE coil), whereby the antimicrobial component becomes inactivated (Figure 1). To construct the regulator, we adapted original parameters introduced by others for the design of amphipathic and membrane-active sequences[3] and built upon our own experience in engineering coiled-coil systems.[4]

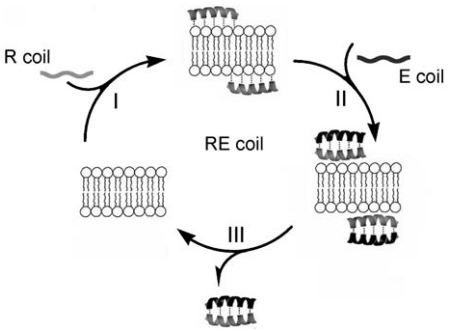

Figure 1. Idealized representation of the RE-coil mechanism. I) R coil in a random coil conformation folds into helices upon interacting with membranes. II) E coil is a random coil until it binds R coil with the formation of a coiled coil. III) R coil bound by E coil loses its membrane activity and is removed from the cycle.

The peptide sequences are composed of heptad repeats typical of α helices, PHPPHPP, in which P is polar or small (e.g. alanine) and H is hydrophobic.[5] In both peptides, isoleucine and leucine residues were used in alternate hydrophobic positions to provide the stoichiometric pairing of R coil with E coil.[4] In R coil, the polar sites were created from basic arginine residues and neutral glutamine and alanine residues to give an RI(Q/A)RLR(Q/A) repeat. E coil was constructed from EI(Q/A)(A/Q)LEE repeats to mirror R coil electrostatically, with polar faces formed by acidic glutamate residues and neutral glutamine and alanine residues. Upon binding microbial membranes, R coil folds into an amphipathic α helix (Figure 2a; see also Figure S1 in the Supporting Information). When mixed with E coil, R coil is converted into the membrane-inactive RE coil formed through the interfacial burial of hydrophobic residues and bridging electrostatic interactions (Figure 1, Figure 2b; see also the Supporting Information).

To probe the regulator, we first monitored its folding cycle in solution by circular dichroism (CD) spectroscopy. Neither of the individual peptides folded in aqueous buffers at micromolar concentrations (see Figure S2a in the Supporting Information). In the presence of anionic phospholipid vesicles, the composition of which mimics that of bacterial membranes, R coil underwent a coil–helix transition. No

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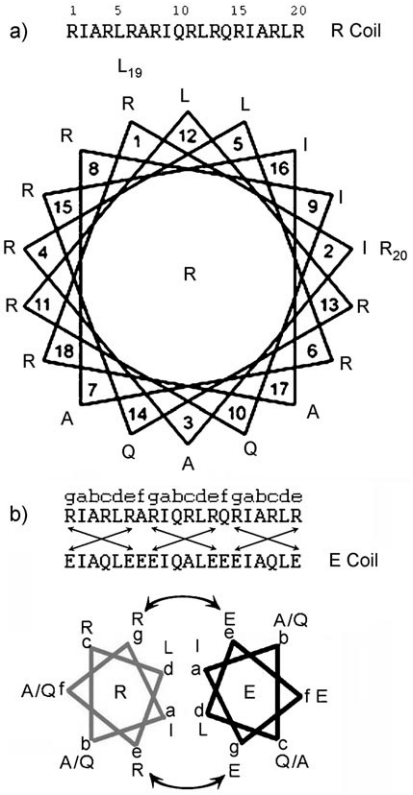

Figure 2. RE-coil design. a) R-coil sequence, linear and configured onto a helical wheel with 3.6 residues per turn. b) RE coil, linear sequences (R and E) and on coiled-coil helical wheels (3.5 residues per turn) with electrostatic interactions shown as double-headed arrows. PHPPHPP heptads are designated g–f.

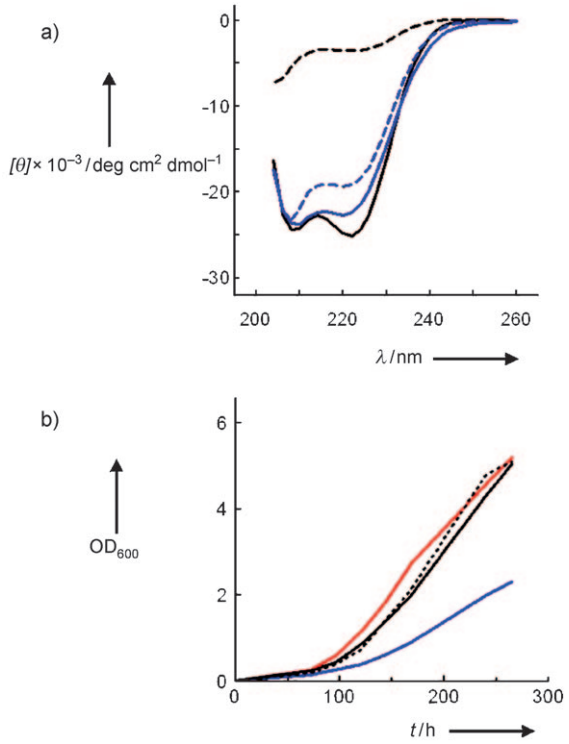

Figure 3. Probing of the RE-coil regulator. a) CD spectra for the peptides (30 μM) in the presence (black dashed line: E coil, blue dashed line: R coil, blue solid line: R + E coil) and absence (black solid line: RE coil) of anionic membranes. b) Growth of *M. tuberculosis* (H37Rv) with (red solid line: E coil, blue solid line: R coil, black solid line: RE coil) and without (dotted line: blank) regulator components (100 μM). OD_{600} = optical density at 600 nm.

appreciable structuring was detected for E coil under the same conditions (Figure 3a). In contrast, the addition of E coil to the R coil/membrane preparation (R + E coil) led to a sharp increase in helicity nearing that recorded for the equimolar mixture of the peptides (RE coil) in lipid-free buffers (Figure 3a). Subsequent additions of R coil and E coil produced corresponding additive changes in helicity that suggested a mixed type of binding: membrane-bound R coil and assembled RE coil (see Figure S2b in the Supporting Information). The observations are consistent with Figure 1 in that R coil alone efficiently binds membranes and is converted into RE coil by E coil.

To confirm RE coil as a membrane-inactivated form of R coil we performed a dye-release assay with anionic model membranes loaded with carboxyfluorescein (see the Supporting Information).[6] Gratifyingly, only R coil was able to cause the release of the dye to a notable extent (see Figure S3 in the Supporting Information).

The effect was also assessed in structurally more heterogeneous biological membranes. Whereas E coil was inactive against Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus* and *Micrococcus luteus*), R coil showed strong antibacterial activity against all of the tested strains. RE coil also showed antibacterial activity. Notably, RE coil was as active an antimicrobial agent as R coil against *M. luteus*. This result implies that RE coil may retain the membrane activity of R coil, which is sufficiently toxic for some bacteria. Because the effect was not fully consistent across the tested bacteria we sought additional evidence.

As mentioned above, AMPs rapidly assemble into peptide–lipid transmembrane channels in microbial membranes.[1,2] The lifetime of such channels is very short; their collapse within tens of seconds leads to membrane disintegration.[1g,2f] In physiological terms, the rapid assembly and collapse of these channels complements the necessity of the enzymatically unstable AMPs to respond to microbial challenge within similar time limits.[1,2] Indeed, in our case R coil induced the maximum level of membrane leakage at given lipid–peptide ratios within the first 5 min (see Figure S3 in the Supporting Information). Therefore, in technical terms, the continuous or direct monitoring of RE-coil dissociation from the membrane is equivalent to monitoring of the competition between the binding of R coil by E coil and the structuring of R coil into transmembrane assemblies that subsequently collapse.

We assessed the regulatory mechanism of RE coil by using two series of dye-release competition experiments. In one, E coil was added to membranes before R coil, so that R coil could both bind to membranes and be inactivated by E coil. Consistent with the design, only the residual activity of R coil was detected in four RE-coil cycles (see Figure S4a in the

Supporting Information). Importantly, no accumulative effects were observed between the cycles. Furthermore, as revealed in the other series, E coil inhibited the membrane activity of R coil in a stoichiometric manner: The peptides were added to membranes sequentially, first R coil and then E coil in different ratios. The activity of R coil decreased as a function of the increasing ratio of E coil to R coil (see Figure S4b in the Supporting Information). The results of these two assays indicate that E coil effectively competes for R coil and inactivates it by disrupting its association with membranes.

Until this point, only mature or preformed membranes with low recovery potential were used. As a consequence, the impact of the intermolecular dynamics of R coil and RE coil on membrane binding may not be readily distinguished; instead a slower and more dynamic membrane environment was required. Arguably, such an environment can be best provided by *Mycobacterium tuberculosis*, a bacterium with membranes characterized by profound self-healing capacities.[7] Mycobacterial envelopes are highly adaptable architectures, and their maturation is an extremely slow and uneven process. As a result of these properties, the bacterium has become resistant to all but a very small number of known antibiotics.[7b] This quality made *M. tuberculosis* an excellent test model for the delineation of the growth-inhibitory mechanisms of R coil and RE coil. Indeed, it was possible to monitor the inhibition of *M. tuberculosis* growth by R coil; inhibition by R coil proved to be consistent over the period of membrane formation (ca. 300 h).

Fluorescence microscopy revealed that R coil efficiently binds to mycobacterial envelopes (an estimated 5–10% of the total number of cells in 15 min); this result supports a membrane-mediated mechanism of inhibition (Figure 4). Further incubation with R coil (>1 h) resulted in more-efficient binding of the peptide (ca. 80%) and the irreversible morphological alteration of the cells, which tended to clump (Figure 4c). This R-coil-specific effect on cell morphology provides clear evidence of the modification of mycobacterial envelopes. By marked contrast, both E coil and RE coil had no effect on growth or cell morphology, and did not bind to the bacterium (Figure 3b and Figure 4a).

Finally, in hemolytic tests that complemented the bacterial tests, all peptides were found to be weakly lytic towards erythrocytes (Table 1) and did not permeabilize neutral model membranes designed to mimic mammalian systems (see Figure S3 in the Supporting Information). Collectively, the results indicate that R coil 1) preferentially targets microbial membranes in a manner characteristic for naturally occurring AMPs (such as magainin), but not for hemolytic peptides (such as melittin; Table 1), and 2) is inactivated by association with E coil.

In summary, by using a de novo designed system, we have demonstrated a molecular rationale for the regulation of antimicrobial intervention by antimicrobial peptides. The findings are also in strong accord with most recent reports that suggest a role for coiled-coil assembly in the activity of membrane-binding peptides.[8] Application of the control of peptide association with microbial membranes demonstrated herein to physiological antimicrobial systems may lead to the development of a new class of therapeutically relevant antimicrobial modulators, biosensors, and dosage regulators.

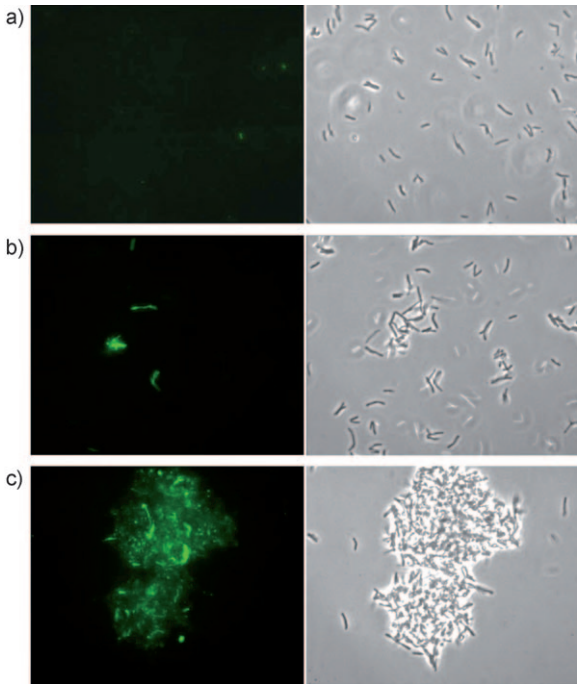

Figure 4. Binding of the peptides to the surface of *Mycobacterium bovis* (BCG) cells. False-color (left) and corresponding phase-contrast images (right) of cells treated with a) carboxyfluorescein-labeled RE coil for 1 h, b) carboxyfluorescein-labeled R coil for 15 min, and c) carboxyfluorescein-labeled R coil for 1 h.

Table 1: Biological activity of the peptides used in the study.

Peptide	Minimum inhibitory concentration (MIC) [μM]				HE[a] [μM]
	E. coli	*P. aeruginosa*	*S. aureus*	*M. luteus*	
R coil	9	38	10	0.3	>300
E coil	>100	>100	>100	>100	>500
RE coil	>50	>50	>50	0.6	>300
magainin 2	4	21	14	n/d[b]	>300
melittin	1	10	3	n/d	<10

[a] Horse erythrocytes: 50% lysis. [b] Not determined.

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