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Reversible Self-Assembly: A Key Feature for a New Class of Autodelivering Therapeutic Peptides

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Received January 23, 2009
Revised Manuscript Received April 3, 2009
Accepted April 14, 2009

Abstract: Effective delivery is a critical issue in the use of conventional free drugs. Studies on the structure−function relationship of a therapeutic antibody-derived candidacidal decapeptide (killer peptide, KP) revealed its ability to spontaneously and reversibly self-assemble in an organized network of fibril-like structures. This process is catalyzed by 1,3-β-glucans. While the self-assembled state may provide protection against proteases and the slow kinetic of dissociation assures a release of the active dimeric form over time, the β-glucan affinity is responsible for targeted delivery. Thus, KP represents a novel paradigm of targeted autodelivering drugs.

Keywords: Killer peptide; peptide self-assembly; drug delivery; laminarin

Selective delivery to the proper biological targets and persistence in the bloodstream are major issues especially in the formulation of drugs of peptide or protein origin.[1] Recently, the hypothesis has emerged to use amyloids in the formulation of long-lasting drugs, taking advantage of their resistance to biochemical degradation.[2] In addition, since polypeptide self-assembly can also produce aggregates identified as hydrogels, these biomaterials have been employed as a drug delivery system or in tissue engineering.[3−6]

The synthetic decapeptide KP, AKVTMTCSAS, was derived from the variable region of the light chain of a recombinant antiidiotypic antibody that mimics the activity of a wide-spectrum antimicrobial *Pichia anomala* killer toxin targeting β-glucan cell wall receptors.[7−9] KP was reported to be microbicidal *in vitro* against a number of pathogenic microorganisms, to display an inhibitory activity against HIV-1 and influenza A virus, and to modulate immune cell function by different mechanisms of action.[9−17]Moreover, parenteral administration of KP proved to exert a therapeutic effect in experimental models of vaginal and systemic

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10.1021/mp900024z CCC: $40.75
Published on Web 04/14/2009

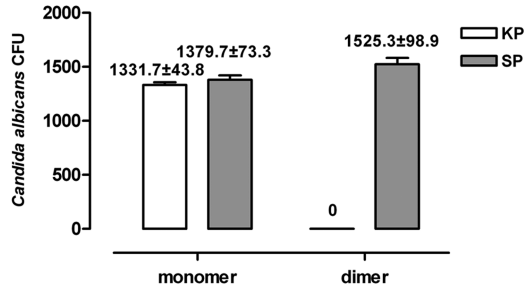

Figure 1. Differential candidacidal activity of freshly solubilized monomer and dimer KP. A colony forming unit (CFU) assay was carried out as previously described[9] by using peptides soon after solubilization at the concentration of 10 μg/mL. Monomer and dimer scramble peptides (SP) were used as growth control.

candidiasis, disseminated cryptococcosis, and paracoccidioidomycosis.[9,11,17]

In nonreducing conditions, solubilized KP molecules easily dimerize due to the formation of disulfide bridges. Dimers turn out to be the functional unit of KP as confirmed by the observation that a stable synthetic dimer-KP showed an instant and total candidacidal effect (Figure 1) and that the C7S substitution (AKVTMTSSAS, SKP) led to an inactive peptide (data not shown).

The circular dichroism (CD) spectrum of a freshly prepared aqueous solution of either KP monomers or synthetic dimer-KPs (Figure 2a) presented a negative band at 200 nm indicative that the peptides are in random coil conformation. However, with time, as a consequence of KP dimer self-assembly, a progressive structural evolution to an antiparallel β-sheet conformation was observed (Figure 2a). The alternation of hydrophobic and hydrogen-bond donor residues provides the chemical complementarity and structural compatibility propitious to β-structured self-assembly. Consistent with this model, we observed that the nonmicrobicidal scramble form of KP, MSTAVSKCAT (SP), that lacks the favorable residues pattern, maintained a random coil conformation even when kept for 6 months in the same conditions used for native KP. A similar result has been obtained with the C7S mutated decapeptide SKP (data not shown).

Dilution of a 1 month old KP stock solution produced, with time, a progressive change of the CD spectra profile (Figure 2b), indicative of a loss of secondary structure. After 6 h, the CD spectrum revealed that the peptide, still in its active form, exhibited a random coil conformation. The same behavior was observed if the sample was heated at 35 °C for 1 h (data not shown). Recognizing that water molecules are strong hydrogen bond competitors, the observed concentration- and temperature-dependent dissociation processes suggest that the dimeric peptides aggregate as the result of a network of hydrogen bonds rather than of hydrophobic interactions.

KP self-assembly could be visualized as gross aggregates, by transmission electron microscopy, in samples stored at 4 °C for several months and stained as previously described[18] (Figure 2c).

The ability of laminarin, a soluble 1,3-β-glucan, to determine the loss of candidacidal activity in a dose-dependent manner,[9] indicated that the binding to β-glucans present in the yeast cell wall is a critical step for the antifungal activity of KP.

Addition of laminarin to a freshly prepared peptide solution induced an immediate conformational conversion of KP from random coil to antiparallel β-sheet (Figure 2d). Moreover, addition of laminarin to incipient self-assembled peptides enhanced the CD signal that reached the intensity of a KP mature aggregate in a few minutes (Figure 2d). These findings suggest an increase in aggregate content and/or an increased order of the existing antiparallel β-sheet aggregates resulting in a higher dichroic activity. Summarizing, we suggest that laminarin has a catalytic role in KP self-assembly due to its ability to act as a template on which the peptides can bind and more easily assemble.

Interestingly enough, differently from self-assembled KP, the laminarin-catalyzed KP aggregates proved to dissociate only partially (Figure 2e). Electron micrographs of the negatively stained material, obtained by adding laminarin soon after peptide solubilization, showed the presence of an organized network of KP aggregates (Figure 2f). Noteworthy, though in this case the aggregates appear as nonbranched fibril-like structures, thioflavin T binding assay discarded the possibility that they are amyloid fibrils (data not shown).

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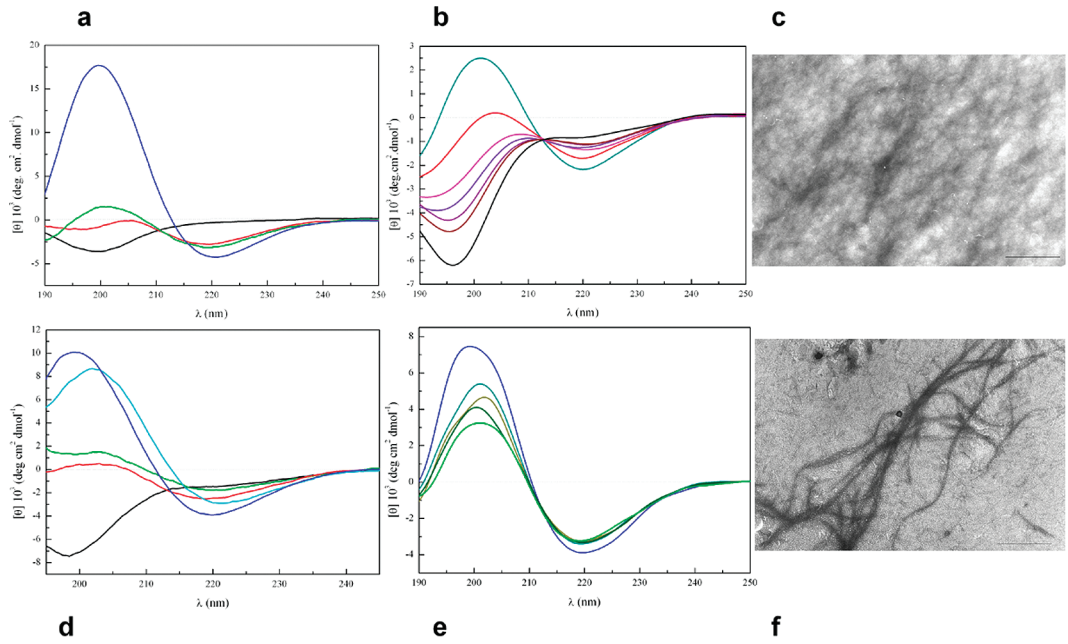

Figure 2. (a–c) Reversible self-assembly of KP. (a) CD spectra of 100 μM KP acquired at 20 °C using samples prepared from the stock solution kept at 4 °C for 1 h (black line), 3 days (red line), 1 month (light green line), and 6 months (blue line). (b) CD spectra of 100 μM KP prepared from the 1 month old stock solution as a function of time: (dark green line) 0 min, (red line) 20 min, (magenta line) 40 min, (violet line) 60 min, (dark purple line) 80 min, (maroon line) 100 min, and (black line) 330 min. (c) Electron micrograph of 1 mM KP prepared from the stock solution stored 24 months at 4 °C. Bar: 1 μm. (d–f) Catalytic effect of laminarin upon KP assembly and reversibility. (d) CD spectra of 100 μM KP prepared from the stock solution at different times: (black line) KP fresh solution, (red line) KP fresh solution + 500 μM laminarin, (light green line) KP 15 days old solution, (light blue line) KP 15 days old solution + 500 μM laminarin, (dark blue line) KP 5 months old solution. (e) CD spectra of 100 μM KP prepared from the 15 days old solution + 500 μM laminarin as a function of time: (dark blue line) 0 min, (dark green line) 60 min, (gold line) 120 min, (green line) 180 min, and (light green line) 240 min. (f) Electron micrograph of 1 mM KP from the stock solution soon after solubilization + 5 mM laminarin. Bar: 1 μm. All KP samples were prepared from a 2 mM stock aqueous solution stored as indicated above. CD measurements were carried out on a Jasco 715 spectropolarimeter (JASCO International Co. Ltd.) coupled to a Peltier PTC-348WI system for temperature control. CD far-UV spectra were recorded from 190 to 250 nm and averaged over four scans, using a 1 mm cell path. Following baseline correction, the measured ellipticity, θ (mdeg), was converted to the molar mean residue ellipticity $[\theta]$ (deg·cm^2·dmol^{-1}). For the electron microscopy studies, grids were prepared as previously described[18] and examined with a Philips EM 208S transmission electron microscope.

Addition of *C. albicans* cells to the freshly prepared peptide solution led to a CD spectral profile similar to the ones observed for KP aggregates self-assembled or catalyzed by soluble laminarin (Figure 3a). Moreover, after direct incubation with yeast cells, again a network of KP aggregates was observed by electron microscopy (Figure 3b and c). Noteworthy, the aggregates appeared to originate from both the surface of the bud (Figure 3b) and the axial pole of the fungus (Figure 3c) and to propagate as self-assembled fibril-like structures. This finding is in agreement with the recognized preferential exposure of β-glucans during *C. albicans* budding as well as at the site of permanent scars generated after cell separation.[19]

When KP was mixed with laminarin and then *C. albicans* cells were added to the solution, no aggregates were observed at the yeast surface (Figure 3d). The fact that preferential binding to soluble 1,3-β-glucan prevented KP localization on the yeast cell is consistent with the previous observation that addition of laminarin could abolish KP candidacidal activity.[9]

Overall, this study revealed that KP molecules, after dimerization via the formation of disulfide bridges, self-assemble in antiparallel β-sheet structures that resemble physical hydrogels.[20] In addition, since the aggregates' extent and stability are concentration- and temperature-dependent, the peptides are allowed to solubilize back with time to their free active form upon dilution or temperature increase.

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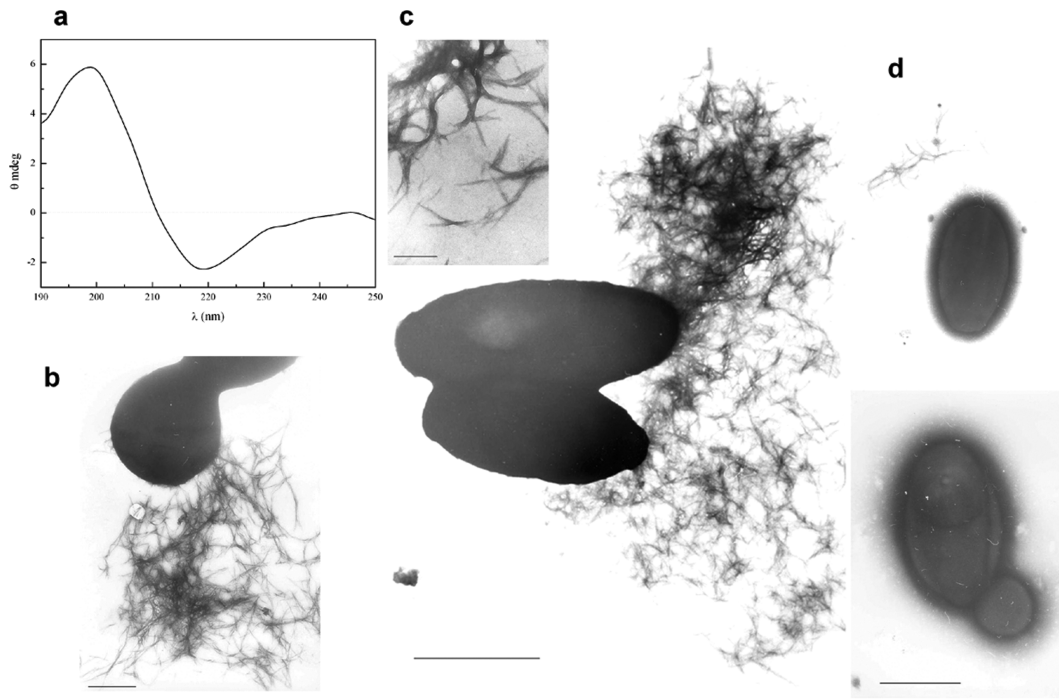

Figure 3. KP interaction with *Candida albicans.* (a) CD spectra of 1 mM KP from the stock solution soon after solubilization incubated with *C. albicans* ($\sim 5 \times 10^7$ cells/mL) for 150 min (cell path 0.1 mm). (b) Electron micrograph from a sample of 1 mM KP from the stock solution soon after solubilization added with a suspension of *C. albicans* ($\sim 5 \times 10^7$ cells/mL). Bar: 1 μm. (c) Same sample as in (b). Bar: 3 μm. Inset: Details of fibril-like structures at higher magnification (bar: 300 nm). (d) Electron micrographs from a sample of 1 mM KP soon after solution + 5 mM laminarin added with a suspension of *C. albicans* ($\sim 5 \times 10^7$ cells/mL). Bar: 3 μm.

Based on this model, it is feasible to envisage that the KP ordered aggregates, observed on the surface of yeast cells where β-glucans are exposed, represent a pool of peptides that, due to their self-assembled state, are expected to be protected *in vivo* from protease degradation while they can regulate the release of the active form into the medium, thus extending over time the candidacidal effect of the drug.

To our knowledge, KP is the prototype of short peptides sharing antibiotic and self-assembly properties. Reversible self-assembly, therefore, may represent a new paradigm of autodelivering therapeutic peptides, while the affinity for β-glucans assures a target selectivity.

Abbreviations Used

KP, killer peptide; SKP, serine substituted KP; SP, scramble peptide; CD, circular dichroism.

Acknowledgment. The authors deeply thank Giorgio Medici for his technical assistance in electron microscopy studies. This work was supported by grants from Italian Institute of Health (Research Project "Coinfezioni, infezioni opportunistiche e tumori associati all'AIDS", Grant No. 50G.30).

MP900024Z