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Stabilization of Helix by Side-Chain Interactions in Histatin-Derived Peptides: Role in Candidacidal Activity

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Candida albicans is an opportunistic pathogen prevalent in AIDS patients and oral candidiasis. Azole-based drugs are currently used in the treatment of candidiasis. Histidine-rich peptides (histatins), are the natural inhibitors of candida species present in human salivary secretions. Sequence comparison of histatins revealed the common motif –KRKFHE– in active peptide fragments. Molecular modeling analysis showed structural similarity between this segment of histatins and azole-based drugs. The helical conformation adopted by histatin-5 may be stabilized by two side chain–side chain interactions (Phe . . . His and Arg . . . Glu). Based on sequence comparison of histatin peptides and molecular modeling, a synthetic 10-residue peptide derived from histatin-5 was helical and possessed significant anti candida activity. This peptide may be used as a template to develop histatin-based drugs for treating oral candidiasis.

Candida species are common part of the normal flora in the oral cavity of over 50% healthy individuals (1–2). In HIV-infected patients, the initial manifestation of oral lesions may be associated with several opportunistic pathogens including *Candida. albicans* which leads to oral candidiasis (3). The susceptibility of AIDS patients to oral candidiasis results from the deregulation of the immune system. It is possible that commensal strains *of C. albicans* become pathogenic in the transition from asymptotic HIV-positive state to AIDS. *C. albicans* is sensitive to many antifungal drugs such as nystatin, azole antimicrobials such as fluconazole, miconazole etc. Treatment usually requires multiple daily topical application of gels, lozenges, chewing gums and mouth washes (4). However, with the increasingly common use of fluconazole up to 400 mg/day over long periods of time in patients with relatively advanced disease state, mucosal candidiasis resistant to fluconazole is increasingly recognized (5–7).

Histatins are the natural inhibitors of candida in saliva and are present as a family of basic, histidine-rich polypeptides in the human parotid and submandibular-sublingual secretions (8–9). At physiological concentrations, histatins are highly effective candidacidal molecules *in vitro* (10). Histatins exert their function as a result of altering the permeability of the bacterial membranes for which a helical conformation may be necessary (11). Histatin-5 is one of the basic histidine-rich peptides also present in saliva with fungicidal activity. An earlier study from our laboratory on synthetic peptides derived from this peptide has shown that the C-terminal 16-residue peptide, **C16**, has activity comparable to the native molecule (11).

Currently available therapies do not always adequately relieve oral candidiasis in immune-

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Abbreviations used: HIV, human immunodeficiency virus; Fmoc, fluorenylmethoxycarbonyl; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; aa, amino acid; TFE, trifluoroethanol; ED_{50}, effective concentration required to kill 50% of candida cells.

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TABLE I
Candida Active Histatins and Peptide Fragments

Peptide	Sequence	Activity
Histatin-1 (H1)	DSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN	Yes
Histatin-3 (H3)	DSHAKRHHGYKRKFHEKHHSHRGYR SNYLYDN	Yes
Histatin-5 (H5)	DSHAKRHHGYKRKFHEKHHSHRGY	Yes
H1 fragment	RKFHEKHHSHR	Yes
H3 fragment	RKFHEKHHSHRGYR	Yes
H5 fragment (C16)	GYKRKFHEKHHSHRGY	Yes

suppressed patients due to various factors such as inadequate concentration of antimicrobial drugs in saliva or poor absorption of drugs from the gastrointestinal tract and/or emergence of resistant strains to classical antibiotics such as fluconazole (6). Also, in HIV-infected patients, decreased production of histatins has been observed (12). The objective of this study is to develop small bioactive histatin peptide fragments which would form the template for peptidomimetic design and drug development for oral candidiasis in normal as well as immune-suppressed individuals. In this regard, sequence comparison of the histatin peptides in salivary secretions revealed that there is a common motif (−KRKFHE−) in all active peptide fragments (Table I). Molecular modeling of **C16** sequence and miconazole suggest possible similarity between the dipeptide segments Phe-His and/or His-His with azole moiety. Also, two specific side chain interactions (Phe . . . His and Arg . . . Glu) were identified in the helical conformation adopted by **C16**. Based on this, we have selected a 10-residue segment (KRKFHEKHHS; **H10**) containing the common motif and report here the synthesis, conformational analysis and candidacidal activity of **H10** and its comparison with other histatin peptides of similar length. This segment is helical and possessed significant anticandida activity.

MATERIALS AND METHODS

Molecular modeling. The software package SYBYL 6.3 (Tripos Associates, St. Louis, MO) was used to model miconazole and to carry out a full molecular mechanics optimization. Molecular dynamics analysis (100 ps) for **C16** (helix as starting conformation) was carried out. The miconazole structure was superimposed onto **C16** by aligning first the active nitrogen of the imidazole group of the azole with possible imidazole groups of the peptide. The carbon backbones were next aligned and finally ring structures were superimposed.

Peptide synthesis. The peptide KRKFHEKHHS was synthesized as a C-terminal amide (**H10-1**) and N- and C-terminal protected peptide (**H10-2**) by solid phase peptide synthesis using Fmoc chemistry. The synthetic peptides were purified using reverse phase HPLC using a linear gradient of 0−40 % acetonitrile and water (both containing 0.1% TFA). The peptides were characterized by amino acid analysis, mass spectra and peptide sequencing.

Conformational analysis. Circular dichroism (CD) measurements were used to assess the tendency for these peptides to adopt an ordered conformation in aqueous buffer and trifluoroethanol (TFE). CD spectra were measured on a JASCO J-600 spectropolarimeter which was calibrated using the ammonium salt of (+)-10-camphorsulfonic acid. Spectra were recorded between 250 and 190 nm at 0.2 nm intervals with a time constant of 2 sec at 25 °C. Data were collected from 5 separate scans and averaged. A cylindrical quartz cell of path length 0.1 cm was used for the spectra range with a sample concentration in the range 0.1−0.25 mM. The molar ellipticity is expressed as $deg.cm^2.dmol^{-1}$.

Candidacidal assay. A microassay system (11) was used for measuring the candidacidal activity of the synthetic peptides. The ED_{50} values were calculated using the procedure PROBIT of SPSS program package on a Vax computer based on four separate, duplicate activity assays.

RESULTS AND DISCUSSION

Salivary histatins (24−38 aa long) possess a relatively high number of histidines. Bioactive histatin-derived peptides range in size from 12 to 16 residues and occur mostly at the C-terminal end of histatin-5 (Table I). For this study, we have chosen to model **C16** as a helix

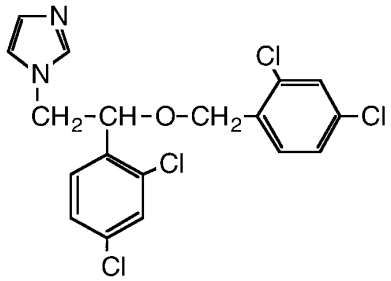

FIG. 1. Chemical structure of miconazole.

based on the recent NMR study which showed that **C16** adopts a helical conformation in non-aqueous solvents (13). Azole antimycotics are small molecules which contain an imidazole group and at least two aromatic groups which are linearly arranged along a flexible carbon chain containing three to five bonds (Fig. 1). Miconazole was chosen as a representative azole for comparing the structural similarities or mimicry with **C16** since both histatins and azole mycotics cause similar end effects on fungal cells (14). The optimized structure of miconazole and **C16** can be superimposed with Phe and His at positions 14 and 15 or with His-His at positions 18 and 19. The imidazole and other triazole moiety of azole drugs fit well with the Phe-His peptide segment (Fig. 2) in which the halogen containing aromatic ring and Phe side chain of **C16** can occupy the same space. Fluconazole which has an imidazole moiety and a triazole moiety also fits with this peptide segment (data not shown). Thus, the molecular fitting analysis suggests that the azole drugs partially mimic the region Phe-His-Xxx-Xxx-His-His of histatins. This analysis explains why salivary histatins which initially exert their effects at

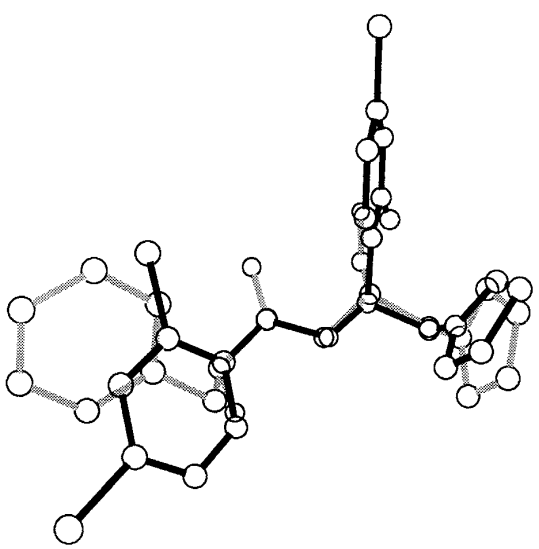

FIG. 2. Molecular fit of the helical segment Phe-His of histatin (shaded structure) and miconazole (filled structure). The two imidazole moieties are similarly juxtaposed while the space occupied by Phe of histatin is covered to a significant extent by the dichlorophenyl group of miconazole. Note also good fit between the peptide and the organic backbone.

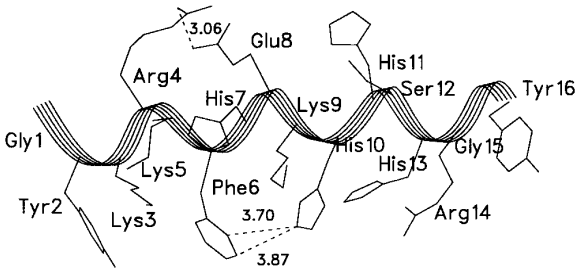

FIG. 3. Energy-minimized molecular model of **C16**. The interaction between Arg-4 and Glu-8 (~3.1 Å) and between Phe-6 and His-10 (~4.0 Å) is shown. Interestingly, several Arg-Glu salt bridge interactions do occur in helical regions of protein structures (21). For example, such a salt bridge exists in the crystal structure of salivary amylase (22). The interaction between Phe and His is also observed in the structure of ribonuclease A in the N-terminus between residues Phe-8 and His-12 (23).

the yeast plasma membrane are similar to many azole antimicrobials such as fluconazole and miconazole in their fungicidal activity.

In an earlier study, a peptide derived from histatin-5, HHGYKRKFHE (**M10**), containing both His-His and Phe-His segments was shown to be significantly less active (10% candidacidal activity) at 500 μM, the highest concentration tested for histatin-5 (11). This peptide did not exhibit any tendency to adopt helical conformation. Interestingly, in a separate study, Armstrong and Baldwin (15) have shown that His at the N-terminus (position 3) is destabilizing compared to His at C-terminus. In fact, **M10** which contains His-His residues N-terminal of Phe-His adopts a random coil in aqueous buffers or methanol or trifluoroethanol (TFE). This strongly suggests that His residues C-terminal to Phe-His is important for helix formation. Also, the inability of **M10** to adopt helical conformation may be explained due to unfavorable interaction between end group charges at the termini and the helix dipole (16–17). We reasoned that removal of such unfavorable specific interactions may be necessary to adopt helical conformation.

Inter-residue interactions may also play a significant role in the formation and maintenance of the helix. His residue also plays an important role in the pH-dependent stability of helix formed by the isolated C-peptide (residues 1–13) of ribonuclease A (18). Amino acid replacement studies have shown that His-12 interacts with Phe-8 (ring to ring interaction) in maintaining the helix. We therefore, hypothesized that in histatin-derived peptides such specific side chain to side chain interaction may play a role in the stabilization of helix. In order to test this hypothesis, we analyzed the conformational dynamics of C16 for intramolecular side chain to side chain interactions that may help maintain the helical conformation. Two interactions were identified and are shown in Fig. 3. These are the Arg(4) . . . Glu(8) and the Phe(6) . . . His(10) side-chain side-chain interactions in **C16**.

Many membrane permeabilizing peptides of insect as well as animal origin have successive basic residues located either at the N- or C-terminus. These residues have been shown to play a significant role in the initial interaction with membrane phospholipid heads groups (19). Therefore, in our selection of the peptide for this study, we retained the N-terminal highly basic tripeptide sequence, Lys-Arg-Lys, to provide maximal interaction with the negatively charged phospholipid head-groups of the *C. albicans* membrane. We have, therefore, selected a 10-residue segment, KRKFHEKHHS (**H10**) for this study based on the following criteria: 1) The superposition of the miconazole and C16 suggested possible structural similarity between Phe-His or His-His to miconazole; 2) Most histatin-derived peptides possessing high candidacidal activity contain the dipeptide segments, Phe-His and/or His-His; 3) In the **C16**

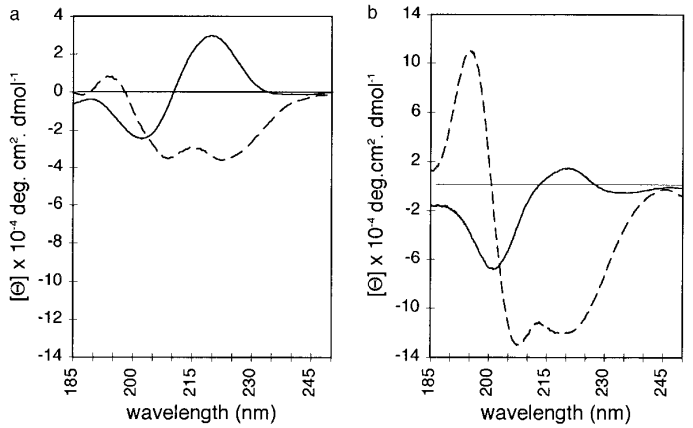

FIG. 4. CD spectra of **H10-1** (a) and **H10-2** (b). In aqueous medium (—), the peptides exhibit random coil structures as evidenced from the slight positive bands at about 220 nm and a strong negative band at about 200 nm. In contrast, note the characteristic negative extrema in TFE (---) for the peptides characteristic of helical structures. The helicity exhibited by **H10-2** is 5-fold higher than **H10-1** and comparable to **C16** peptide (11).

sequence, His occurs next to an aromatic amino acid (as found in miconazole) only at positions 14 and 15; 4) His-His segment is present C-terminal to the Phe-His segment; 5) Arg(4) . . . Glu(8) and the Phe(6) . . . His(10) side-chain side-chain interactions are present as in **C16**; 6) basic residues Lys-Arg-Lys are needed. These were maintained in N-terminus since it is present near the N-terminus in **C16**.

We tested these hypotheses for helix stability as well as for bioactivity by synthesizing **H10** and measuring its circular dichroism and candidacidal activity. Since the importance of interaction between terminal charges to dipole in helix stabilization or destabilization has been very well established, we chose to protect the terminal groups (16). We therefore synthesized two peptides, **H10-1** with C-terminal protection as amide (KRKFHEKHHS-NH_2) and **H10-2** with N- and C-terminal protection as octanoyl and C-terminal protection as amide [CH_3-$(CH_2)_6$-CO-KRKFHEKHHS-NH_2)].

The CD spectra (Fig. 4) clearly shows that the peptides, **H10-1** and **H10-2** exhibit a clear transition from random coil structures in aqueous buffers to helical structures in TFE. In contrast, **M10** peptide has very little ordered structure either in TFE or water (11). The helix stabilization observed in **H10** peptides compared to **M10** strongly suggests that two factors mentioned above, namely, the side-chain side chain interactions and the removal of the charge-dipole interaction between the COO^- and the helix dipole contribute to this transition. In this regard, conformational studies on other synthetic peptides have shown that removal of the C-terminal negative charges by amidation increases the helix formation (20). The doubly protected peptide **H10-2** has a much higher helicity compared to **H10-1**. There is almost 5-fold increase in helicity as a result of the removal of the N-terminal alkylation. The molar ellipticity values for **H10-2** is comparable to the C-terminal active peptides **C16** and **C14** (Table II; 11). The protection at the N-terminus abolishes the unfavorable electrostatic interaction between the positive charge on the α-amino group and the helix dipole. Clearly, for **H10-2**, the removal of the N-terminal charge by alkylation plays a role in the helix stabilization. In this regard, self-association by the strongly hydrophobic alkyl group may influence the helix formation (17). Thus, there are three mechanisms by which **H10** peptides increase their tendency to adopt helical structures. First, by the reduction in the charge repulsions between the charged

TABLE II
Helicity and ED_{50} Values for Histatin-5 Peptides

Peptide	Sequence	Helicity[a]	ED_{50}[b]	Reference
H10-1	KRKFHEKHHS-NH_2	18	57	This study
H10-2	Octanoyl-KRKFHEKHHS-NH_2	60	32	This study
C16	GYKRKFHEKHHSHRGY	100	12	11, 13
C10	HEKHHSHRGY	—	>200	11
M10	HHGYKRKFHE	—	>200	11
Histatin-5	DSHAKRHHGYKRKFHEKHHSHRGY	N/A	5	11

[a] Helicity is defined as the % ratio $[\Theta]_{Mobs}/[\Theta]_{M100}$, where $[\Theta]_{Mobs}$ is the observed molar ellipticity at 222 nm for the peptides and $[\Theta]_{M100}$ is corresponding value for C16 expressed in deg. $cm^2.dmol^{-1}$.

[b] ED_{50} is defined as the molar concentration of the peptide required to kill half of maximal *C. albicans* cells. The loss of viability used in the calculation of ED_{50} values is expressed as [1 − (cell survival after peptide incubation)/(cell survival in buffer alone) × 100.

ends of the helix macrodipole and the terminal charges; second, induction and maintenance of the specific side chain to side chain interactions; third, by hydrophobic association of the N-terminal protecting groups. These observations strongly support our surmise that even smaller histatin-derived peptides can adopt helical structures.

The fungicidal activity of **H10-1** and **H10-2** is given in Table II. Even though **H10-2** is only 10-aa long, its ED_{50} is only 3-fold lower than **C16**, the parent peptide from which it was derived. In comparison, **H10-1** exhibits only 2-fold lower activity which can ascribed to the effect of unblocked N-terminus. A similar length peptide, **M10**, is neither helical nor has significant candidacidal activity ($ED_{50} > 200\ \mu M$). Thus, helix dipole and end group charges play a significant role in the tendency for helix formation of smaller histatin-derived peptides which is reflected in their candidacidal activity. The availability of short α-helical peptides derived from histatins would provide useful frameworks for the design of small molecules which have desirable properties such as biocompatibility and stability. Additionally, one could incorporate other bioactive templates into the design to develop peptide drugs for the treatment of oral candidiasis in normal as well as immunosuppressed individuals.

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