FEBS Letters 491 (2001) 76–80 FEBS 24606

Zinc and copper bind to unique sites of histatin 5

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Received 16 December 2000; accepted 19 January 2001

First published online 30 January 2001

Edited by Pierre Jolles

Abstract Metal binding has been suggested to be relevant in the antifungal and antibacterial mechanism of histatin 5, a human salivary protein. Proton nuclear magnetic resonance (NMR) spectra were obtained to investigate the specificity of metal binding to the seven histidyl, one aspartyl and one glutamyl amino acid side-chains of histatin 5 in aqueous solutions. Three $C^{\varepsilon 1}$–H histidyl and the C^{γ}–H glutamyl resonances of histatin 5 were selectively altered in spectra of solutions containing three equivalents of zinc. Copper binding to histatin 5 resulted in a reduced intensity of C^{β}–H aspartyl resonances, while no evidence for calcium binding was found. These results indicate that zinc binding to histatin 5 involves His-15 present within the –H–E–X–X–H– zinc binding motif, and copper binding occurs within the N-terminal D–S–H–, ATCUN motif.

Key words: Histatin; Zinc; Copper; Antimicrobial peptide; Nuclear magnetic resonance; Protein binding; Saliva

1. Introduction

The histatins are a family of antimicrobial proteins produced in the salivary glands of humans and higher primates [1]. Histatins and histatin variants exhibit a broad spectrum of direct antimicrobial activity against fungal and bacterial oral pathogens and have been shown to be inhibitors of matrix metalloproteinases, gingipains and clostripain [2,3]. These proteins may therefore contribute to innate resistance to oral infections. Histatins have a high content of basic amino acids [4]. For instance, the histatin 5 peptide (D–S–H–A–K–R–H–H–G–Y–K–R–K–F–H–E–K–H–H–S–H–R–G–Y) contains seven histidine residues and seven lysine and arginine residues among its 24 amino acids. This composition results in a net positive charge of the peptide in neutral and acidic solutions. Because of their weak amphipathic character, histatins have been suggested to act on microbial targets by a different mechanism from that of other basic antimicrobial peptides [5].

Interactions of histatins with specific proteins, lipids and metal ions have been reported. Anionic mucins bind histatin 5 in saliva based on interactions with mucin sialic acid residues [6]. ATP efflux from cells and specific interactions with plasma membrane transport proteins has been proposed to explain the killing of fungal cells by histatins [7], and other interactions with mitochondrial proteins or lipid components have been suggested to explain the inhibitory effect of histatins on *Candida albicans* respiration. Association of histatin with zinc, copper and nickel has been demonstrated by mass spectrometry indicating unique binding stoichiometries [8]. High affinity binding constants have been determined for zinc- and copper-histatins using isothermal titration calorimetry to be 1×10^5 M^{-1} and 3×10^7 M^{-1}, respectively [9]. The potential role of these complexes is suggested by the unique properties of zinc–histatin 5, which catalyzes vesicular fusion [10]. The multiple histidines of histatins provide many potential sites for the interaction of metals, and a zinc binding motif, –H–E–X–X–H–, occurs once in the protein sequence. Peptide fragments of the intact histatin 5 protein containing this metal binding motif have been identified to possess the enzyme inhibitory function of histatins toward matrix metalloproteinases 2 and 9, suggesting that metal-chelation may participate in this functional capacity of the protein [2]. However, in contrast to the consensus zinc binding motif of metalloproteinases [11], the histatin sequence contains many neighboring basic residues. A copper binding ATCUN motif is also located in the N-terminal region of histatins 3 and 5 [12].

In organic solvents the structure of histatin 5 is predominantly α-helical [13]. In aqueous solutions, unordered structures have been observed for histatins 3 and 5 by nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy [14,13]. Helix-to-coil transitions may be a feature of histatins which enable the proteins to interact with and translocate across membrane bilayers. A C-terminal 16 residue fragment of histatin 5 has been demonstrated to bind to dimyristoylphosphatidylcholine vesicles by FTIR and CD spectroscopy [15]. This binding interaction imparts helicity to the protein fragment, and may be mechanistically important in the binding of histatins to cellular targets. Metal ions also induce structural changes as suggested by CD spectra of histatin 5 in solutions containing zinc, copper and nickel [8,10]. The function of histatins and other intrinsically unstructured proteins may depend upon their conformational propensities when associated with target molecules [16].

It is currently unknown how metal ions are associated with histatins, and if these associations are specific for particular amino acid side-chains. Histidine is known to be involved in protein metal-chelation, either by itself, or more often in as-

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PII: S0014-5793(01)02157-3

sociation with other amino acid side-chains [17]. However, several other amino acids in the histatin 5 sequence are potential ligands for divalent metal ion coordination. Structural insight into metal–histatin complexes may provide mechanistic information with respect to the antibacterial or enzyme inhibitory functions of histatins. However, crystallization of these complexes has not been achieved. We have investigated metal binding to histatin 5 by proton NMR spectroscopy with the aim of identifying protein ligands involved in metal complexation. The interactions of zinc and copper with histatin 5 are shown to involve the participation of specific amino acid side-chains, while no evidence for calcium binding was obtained.

2. Materials and methods

2.1. NMR sample preparation

Histatin 5 was synthesized by American Peptide Co. (Sunnyvale, CA, USA). The peptide was repurified by reversed phase HPLC using a published method [4]. D_2O and 3-(trimethylsilyl) tetradeutero sodium propionate (TMSP) were from Aldrich Chemical Co. (Milwaukee, WI, USA). NMR samples were prepared by adding weighed amounts of histatin 5 to solutions containing 10% D_2O and TMSP. The protein concentration of samples were typically 4.5 mM. The absorption at 276 nm was used to estimate protein concentration using tyrosyl extinction coefficients [18]. Protein-containing solutions were stored over 0.01 g of Chelex resin equilibrated at pH 7 in the sodium form. Metal–histatin solutions were typically prepared at metal to protein ratios of 3:1, which are expected to saturate the high affinity metal binding capacity of histatin 5 [14]. All glassware, NMR tubes, and pipettes were soaked in 5% nitric acid and extensively rinsed with metal-free water before use.

2.2. NMR spectroscopy

Spectra were recorded on a Bruker DMX 500 spectrometer. 128 scans were used routinely to obtain spectra with a spectral width of 6000 Hz with 4096 points for a final spectral resolution of 1.47 Hz point^{-1}. Water suppression was achieved using the WATERGATE pulse sequence [19]. All spectra were recorded at 25°C. Titrations of histatin 5 solutions were made by adjustment of pH with NaOH or HCl. After each NMR spectrum the pH of the solution was recorded. This pH was taken to be that of the previously recorded NMR spectrum. No adjustments to pH readings were made to correct for the 10% deuterium content of the solvent. All spectra were calibrated to the TMSP signal at 0 ppm.

2.3. Estimation of pK_a values

Titration curves of individual resonances were generated by plotting the resonance positions in ppm as a function of pH using Kaleidagraph curve-fitting algorithms (Synergy Software, Reading, PA). The pK_a and Hill coefficients were determined by the method of Markley and Finkenstadt [20] using a Levenberg–Marquardt algorithm for curve-fitting. Starting values for curve-fitting parameters were estimated from the resonance positions at the extremes of the pK_a range of titrating groups. Hill coefficients of 1 were used to initiate the iterative fits. Initial values of pK_as for histidine were set to 6, and those of Asp and Glu were set to 4. Curve fits to the experimental data with R values greater than 0.999, and χ^2 values of less than 0.002 were obtained.

3. Results

Proton NMR spectra of histatin 5 contained sharp resonance signals in the aromatic region representing histidyl C$^{\varepsilon 1}$–H protons, labeled A–D, and resonances assigned to the Tyr-24 N–H proton, labeled E (Fig. 1). The two resonances associated with peak B and the three superimposed resonances in peak C have similar chemical shift positions at pH 6.3, indicating very similar magnetic environments under these conditions. The resonance labeled A is assigned to His-15 based on previous NMR studies of histatin 5 in aqueous solutions [13,14]. The peak labeled D was tentatively identified as His-7 based on the expected influence of a neighboring arginine residue on its titration behavior (see below).

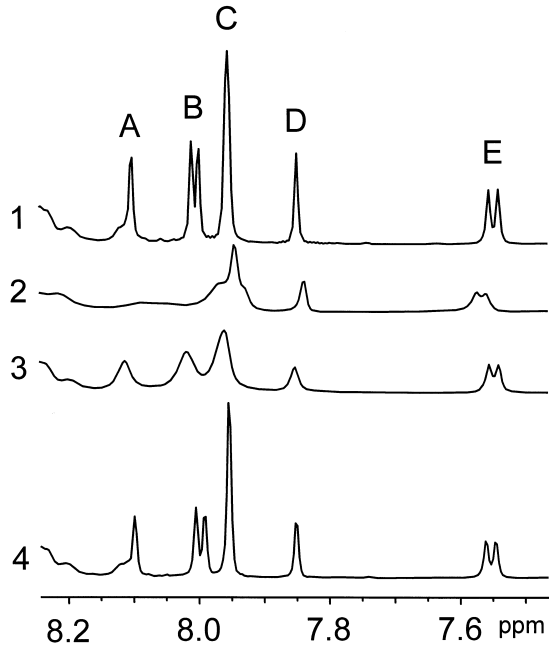

Fig. 1. ^{1}H-NMR spectrum of histatin 5, histidyl C$^{\varepsilon 1}$–H region. Spectrum 1: 4.5 mM histatin 5, pH 6.38; spectrum 2: 3.95 mM histatin 5, 13.4 mM zinc chloride, pH 6.38; spectrum 3: 3.92 mM histatin 5, 11.6 mM copper chloride, pH 6.29; spectrum 4: 4.54 mM histatin 5, 20.7 mM calcium chloride, pH 6.29. All spectra were recorded at 25°C with protein solutions containing 10% D_2O and TMSP reference.

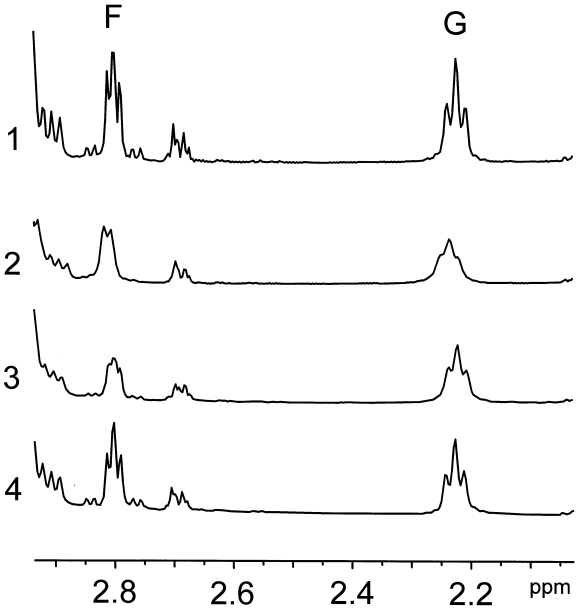

Fig. 2. ^{1}H-NMR spectrum of histatin 5, Asp-1 C$^{\beta}$–H and Glu-14 C$^{\gamma}$–H region. Solution conditions of spectra are as given in the legend of Fig. 1. Spectrum 1: no metal histatin 5; spectrum 2: histatin 5 and zinc; spectrum 3: histatin 5 and copper; spectrum 4: histatin 5 and calcium.

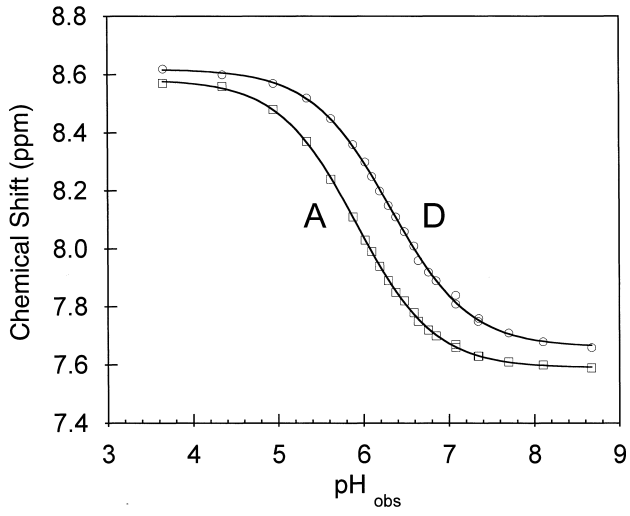

Fig. 3. pH-dependent ^{1}H-NMR chemical shifts in the histidyl $C^{\varepsilon 1}$–H region. The labels A and D denote the histidyl resonances of His-15 and His-7, respectively, identified in the spectra of Fig. 1. pH adjustments were made on a 4.5 mM histatin 5 solution containing 10% D_2O at 25°C. Curves represent best-fits of the data points to the Henderson–Hasselbalch equation independently fitting the pK_a, Hill coefficient, and chemical shift titration end-points.

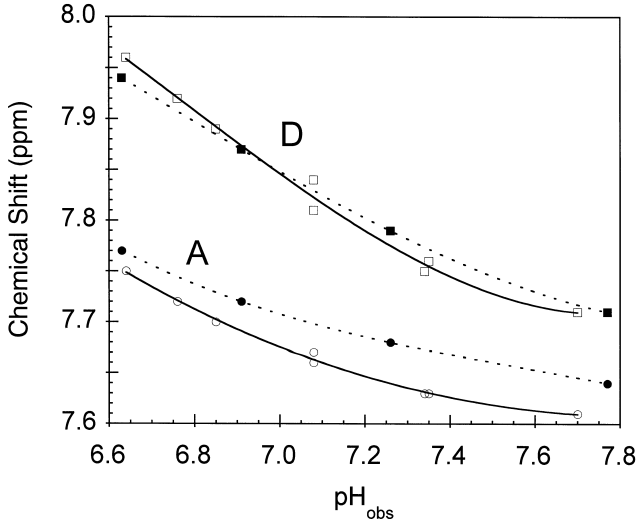

Fig. 4. Zinc-dependent ^{1}H-NMR chemical shift of His-15 and His-7 $C^{\varepsilon 1}$–H resonances. The labels A and D correspond to those identified in Fig. 1 for solutions conditions given for spectra 1 and 2 (see legend). Resonances of histatin 5 in the absence (open symbols) and presence (solid symbols) of zinc are shown. The curves shown are polynomial fits given to facilitate readability. Adjustment of the zinc–histatin solution above pH 8 resulted in precipitation, while below pH 6.3 the resonance assigned to His-15 was absent.

Addition of divalent metals to histatin 5 solutions resulted in changes in the histidyl resonances in the presence of zinc and copper, but not calcium (Fig. 1). Addition of three molar equivalents of zinc to histatin 5 resulted in a selective change in the resonance intensity of the A and B resonances (Fig. 1, spectrum 2). However, the spectrum of zinc–histatin 5 showed only minor changes in the C, D, and E resonances. Spectra of copper–histatin complexes showed no such selectivity, but all $C^{\varepsilon 1}$–H resonances were observed to be broader in line-width compared with metal-free spectra (Fig. 1, spectrum 3). Addition of calcium to histatin 5 solutions resulted in spectra which were unchanged from that of the metal-free protein (Fig. 1, spectrum 4).

Selective changes in zinc and copper spectra were also observed in the region from 2 to 3 ppm (Fig. 2). The corresponding region of the metal-free histatin 5 spectrum is shown in Fig. 2, spectrum 1. The resonances labeled F and G correspond to the C^{β}-protons of the single aspartic acid and the C^{γ}-protons of the single glutamic acid residue, respectively. The Glu-16 C^{γ}-proton resonances were observed to broaden in the zinc–histatin 5 spectrum (Fig. 2, spectrum 2), while the Asp-1 C^{β}-proton resonance multiplet was also affected by zinc binding. Copper–histatin spectra at similar ratios of metal to protein (3:1) show less broadening of resonances in the Glu-16 C^{γ}-proton resonances (Fig. 2, spectrum 3). Calcium addition to histatin 5 solutions at similar stoichiometric ratios showed no changes compared with the metal-free spectra (Fig. 2, spectra 4 and 1, respectively).

The pH titration of metal-free histatin 5 solutions allowed for the investigation of titration parameters of the ionizable amino acid side-chains of histatin 5. A plot of the titration curves for the A and D resonances of Fig. 1 is shown in Fig. 3. The resonances labeled B and C of Fig. 1 titrated in a narrow range between these two curves. A summary of the titration parameters for the histidyl $C^{\varepsilon 1}$–H resonances as well as those of other ionizable groups of histatin 5 are given in Table 1. Titration of zinc–histatin solutions prepared at similar pH and concentration resulted in spectra showing a selective shifting of the His-15 resonance compared with the his-

Table 1
Histatin 5 titration parameters[a]

Observed proton(s)[b]	Titrating group (figure label)	pK_a
His-15 $C^{\varepsilon 1}$–H	imidazole (A)	6.3
His-(3,8,18,19,21) $C^{\varepsilon 1}$–H	imidazole (B, C)	6.1–6.2
His-7 $C^{\varepsilon 1}$–H	imidazole (D)	5.9
Asp-1 C^{β}–H	carboxyl (F)	2.8
	primary amine (F)	7.2
Glu-16 C^{γ}–H	carboxyl (G)	3.2
Tyr-10 C^{ε}-H	phenol	9.8
Tyr-24 C^{ε}-H	phenol	10.3
Tyr-24 N–H	carboxyl (E)	3.0

[a]pK_a values were determined by fitting of data to the model of Markley and Finkenstadt [20]. See Section 2 for details.
[b]Titration parameters are given for assigned resonances, or resonances identified based on nearest neighbors in the histatin 5 amino acid sequence: His-7, Tyr-10 and Tyr-24. The Asp-1 titration curve was biphasic with the largest chemical shift inflection (0.3 ppm) observed for the N-terminal amino group.

tidyl resonance attributed to His-7 (Fig. 4, solid symbols, labeled A and D, respectively).

4. Discussion

Knowledge of the antimicrobial and enzyme inhibitory functions of saliva is of importance in the development of therapies which control oral disease. Alterations of membranes have been proposed to account for the anti-candidal activity of histatins both at the level of disruption of mitochondrial [21] or plasma membrane functions [7]. The catalytic properties of zinc–histatin complexes affecting fusion of small unilamellar vesicles in vitro suggests a mechanism for histatin activity based on metal association [10]. The NMR evidence presented in this study indicates specificity with respect to the histidyl and acidic amino acid side-chains participating in metal binding interactions, and extends previous findings demonstrating unique stoichiometries and binding affinities for zinc– and copper–histatin 5 complexes [8,9].

The specificity of zinc binding by histatin 5 was most clearly evident from the loss of the resonance assigned to His-15. This histidine is within a zinc binding motif found in several metalloproteinases, but the zinc binding sequence of histatin 5 includes basic residues which are rare in non-conserved residue positions within and surrounding the consensus sequence [11]. Other histidyl $C^{\varepsilon 1}$–H resonance changes also suggest site-specific binding of zinc to histatin 5. In addition, the relative chemical shift position of the His-15 $C^{\varepsilon 1}$–H resonance changed in the presence of zinc, providing further evidence for the selectivity of this metal binding site. Shifts of histidyl resonances have previously provided evidence for selective association of zinc to histidyl residues of RNase A [22]. A histidyl resonance partially assigned to His-7 was insensitive to zinc binding, demonstrating the selective association of zinc to elements of the histatin 5 sequence. The theoretical importance of nearby charges on the ionization of myoglobin histidyl resonances has been characterized [23], and nearest neighbor information has been used previously to indicate partial assignments of the multiple histidyl resonances of dihydrofolate reductase [24]. Other possible zinc binding residues include the carboxylic acid groups of histatin 5. Glu-16, which follows His-15 in the primary sequence, has a broadened C^{γ}-proton signal in the presence of zinc. This involvement of the Glu-16 side-chain could indicate a direct interaction in zinc coordination, or a hydrogen bonding interaction with an associated water molecule [25]. The capacity of histatins for zinc binding is sensitive to pH, with higher metal-to-protein stoichiometries observed at higher pH values [8]. Methods which investigate zinc-exchange between different binding configurations may be useful to apply in the investigation of dynamic features of zinc bound to histatins [26].

Copper binding has been shown to occur at a single high affinity site of histatin 5 [9]. Visible spectral changes indicate that this high affinity site is the N-terminal D–S–H–, ATCUN motif. Although no specific changes in histidyl resonances were observed for copper–histatin complexes, the reduced intensity of the C^{β}-proton resonances of Asp-1, relative to that of Glu-16, indicate that copper is bound near the N-terminal sequence of histatin 5. The general broadening observed for histidyl $C^{\varepsilon 1}$–H resonances may represent the association of additional copper ions by imidazole side-chains. Cu (II) has a longitudinal relaxation time associated with its electron spin, τ_s, which results in broadening of signals from protons within a radius of about 1 nm [27]. Recent evidence suggests that copper is bound initially by the ATCUN sequence motif of histatin 5, but additional copper ions may subsequently be bound with low affinity [9].

5. Conclusion

The selective changes in the line shape and intensity of the His-15 and Glu-14 resonances in histatin 5 ^{1}H-NMR spectra provide evidence for zinc binding by the –H–E–X–X–H– motif in the physiological pH range. The specificity of metal binding is also indicated by chemical shift differences observed for the His-15 resonance in spectra of zinc–histatin 5 and metal-free protein solutions. Copper binding to histatin 5 is indicated by changes in histidyl and aspartyl proton resonances. However, the site of copper binding is not limited to the D–S–H–, ATCUN motif as indicated by the general broadening effect on all histidyl resonances for copper–histatin complexes at a 3:1 ratio. While there is strong support for the binding of zinc and copper to histatin 5, the present investigation provides clear evidence for the specificity of metal binding to different regions within the histatin 5 sequence. Sequence-specific association of divalent metal ions may pertain to the unresolved functional activities of salivary histatins.

Acknowledgements: The authors would like to thank Dr. Heloisa Gusman for comments on the manuscript. This work was supported in part by NIH/NIDCR Grants DE05672 and DE07652.

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