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Nature of the pH-induced conformational changes and exposure of the C-terminal region of chromogranin A

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Chromogranin A is known to undergo pH induced conformational changes, and the difference in conformation is supposed to be responsible for the difference in Ca^{2+} binding property. To gain insight regarding the overall structure and the nature of pH-induced conformational changes of chromogranin A, limited trypsin digestions were carried out at pH 5.5 and pH 7.5. The resulting fragments were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the amino acid sequences of the tryptic fragments were determined. From these analyses it was shown that the chromogranin A structure consists of an N-terminal compact core region and a rather loosely organized C-terminal region and that the change of pH from 7.5 to 5.5 loosened the overall structure of chromogranin A, exposing the C-terminal region. Since the conserved C-terminal region (residues 407–431) was shown to exist in monomer-dimer and monomer-tetramer equilibria at pH 7.5 and 5.5, respectively, the conformational changes of the region at pH 7.5 and 5.5 were studied by circular dichroism spectroscopy using a synthetic peptide representing the conserved C-terminal region. When the pH was changed from 7.5 to 5.5, the coil structure of the C-terminal peptide decreased with an accompanying increase of α-helicity.

Chromogranin A; Conformation; pH; C-terminal region

1. INTRODUCTION

Chromogranin A (CGA) is the major soluble protein of adrenal medullary chromaffin cells and is found in virtually all neurons and endocrine cells [1,2]. Chromogranin A is thus considered to be a marker protein of neuroendocrine cells [3]. Chromogranin A has recently been identified as a low affinity, high capacity Ca^{2+} binding protein [4], and has been suggested to be responsible for the Ca^{2+} storage function of the secretory vesicle which is a major inositol 1,4,5-trisphosphate-sensitive intracellular Ca^{2+} store of adrenal medullary chromaffin cells [5]. Besides the Ca^{2+} storage function, CGA is suggested to be a precursor of hormones [6–8], and is also known to undergo pH- and Ca^{2+}-induced major conformational changes [9].

Chromogranin A contains two highly conserved regions: one in the near N-terminal region and the other in the C-terminal region. The conserved C-terminal region (residues 407–431) was shown to exist in the monomer-dimer and monomer-tetramer equilibria at pH 7.5 and 5.5, respectively [10]. It was therefore postulated that the conserved C-terminal region might be responsible for the oligomerization of CGA [10]. The oligomerization property suggested that the conserved C-terminal region goes through pH-dependent conformational changes, thereby changing the binding property of the region.

Although it has been known that CGA goes through pH-dependent conformational changes [4,9], there is no information regarding the structure-function relationship of chromogranin A. Therefore, to obtain the structural information of CGA and to address the nature of pH-induced conformational changes, chromogranin A was subjected to limited tryptic digestion and the amino acid sequences of the tryptic fragments were determined. In addition, to gain insight with regard to the potential conformational changes of the conserved C-terminal region and the oligomerization mechanism, a CGA peptide representing the C-terminal region was synthesized and its secondary structure was studied using circular dichroism (CD) spectroscopy.

2. MATERIALS AND METHODS

2.1. *Materials*

Trypsin, soy bean trypsin inhibitor, 3-[cyclohexylamino]-1-propanesulfonic aicd (CAPS) were obtained from Sigma. Polyvinylidene fluoride (PVDF) membranes (Pro Blott) were from Applied Biosystems.

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Abbreviations: CGA, chromogranin A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CD, circular dichroism; CAPS, 3-[cyclohexylamino]-1-propanesulfonic aicd; PVDF, polyvinylidene fluoride.

2.2. *Limited tryptic digestion of chromogranin A*

Chromogranin A from bovine adrenal medulla was purified according to Yoo and Albanesi [9], and used for limited trypsin digestion. In this procedure, purified chromogranin A (120 μg) either in 20 mM sodium acetate, pH 5.5, 0.1 M KCl or in 20 mM Tris-HCl, pH 7.5, 0.1 M KCl was mixed with trypsin at a substrate/enzyme ratio of 1,000:1 (w/w) in a total volume of 0.35 ml. The reaction mixture was incubated at 37°C for a specific length of time, and 50 μl aliquots were transferred to a tube containing soybean trypsin inhibitor at a inhibitor/trypsin ratio of 2:1 (w/w). The 50 μl aliquot was then mixed with SDS-PAGE dye mixture, boiled for 2 min, and subjected to 12–20% gradient SDS-PAGE according to Laemmli [11].

2.3. *Electro blot and amino acid sequencing*

The tryptic fragments separated on a 12–20% gradient SDS-PAGE gel were transferred onto a PVDF membrane by electroblotting for 1–2 h at 400 mA, 60 V in CAPS buffer [12]. The electroblotted protein fragments were then visualized by staining in a 0.5% Coomassie blue solution for 2 min, followed by destaining. The destained PVDF membrane was air dried, after which the appropriate protein bands were cut out for amino acid sequencing. Amino acid sequences were determined by the Edman degradation method using an Applied Biosystems 470A protein sequencer and a 120A PTH analyzer. The first 7–8 amino acids from the N-terminus of each fragment were determined.

2.4. *Chromogranin A peptide synthesis*

A peptide ((W)ELESLSAIEAELEKVAHQLQALRRG), representing residues 407–431 of bovine chromogranin A, was synthesized and purified by high-performance liquid chromatography. The integrity of the peptide was ensured through analysis by fast atom bombardment mass spectrometry and by amino acid composition analysis. A tryptophan residue was added to the N-terminus of the peptide to facilitate analysis. The purity of the peptide was higher than 98%.

2.5. *Circular dichroism spectroscopy*

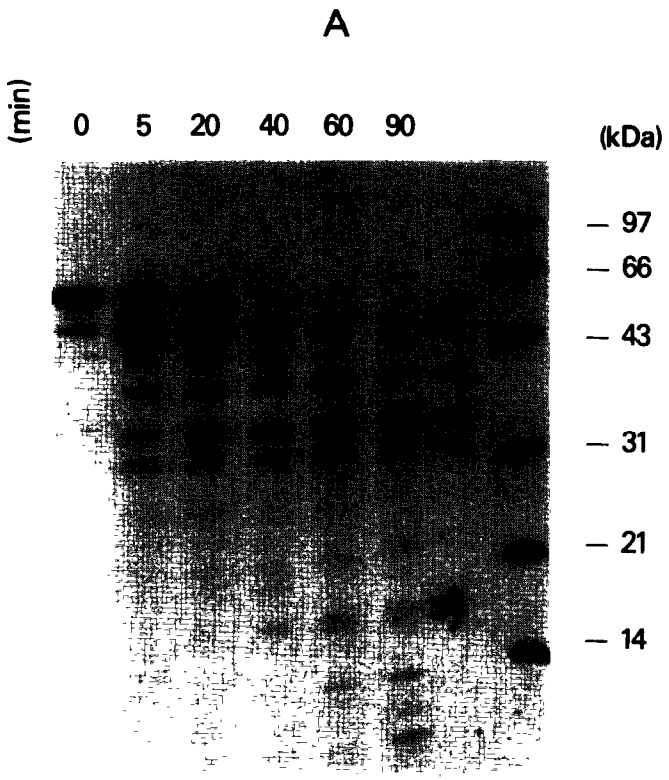
Circular dichroism (CD) spectra were recorded using a Jasco J-600 spectropolarimeter using a cell with a path length of 0.02 cm and a peptide concentration of 50 μM in either 15 mM sodium acetate, pH 5.5, or 15 mM Tris-HCl, pH 7.5. All spectra were taken at 22°C and were the average of at least two scans. Analysis of the spectra was carried out using an algorithm obtained from Jasco (Easton, Maryland), which is based on the spectra of five globular proteins (myoglobin, lysozyme, ribonuclease, papain, and cytochrome *c*) [13,14].

3. RESULTS

Chromogranin A has previously been shown to be proteolyzed differently by trypsin at pH 5.5 and 7.5 [4], reflecting structural differences at these two pH levels. To gain further insight with regard to the nature of structural differences at the two pH levels and to obtain overall structural information, chromogranin A was subjected to limited trypsin digestion at the two pH levels and the resulting chromogranin A fragments were analyzed by SDS-PAGE, followed by amino acid sequence analysis of each fragment (Figs. 1 and 2). As shown in Fig. 1A, chromogranin A was proteolyzed into small fragments by trypsin at pH 5.5. The amino acid sequence analyses of these fragments, as shown in Fig. 1B, indicate that the C-terminus of chromogranin A was proteolyzed first by the protease. The larger fragments 1–5 retained the N-terminal side of chromogranin A while the smaller fragments 6 and 7 were the peptides either proteolyzed from both the N- and C-terminal sides of the protein (fragment 6) or the remaining N-terminal fragment (7). Although the presence of fragment 6 with both of its N- and C-terminal sides proteolyzed, coupled with the unsequenced faint bands at ~ 45 kDa, implies a possibility of initial proteolysis at the N-terminal region, the proteolytic pattern of more distinct bands clearly indicates that the N-terminal region of the molecule is better protected than the C-terminal region.

As shown in Fig. 2A, trypsin digestion of chromogranin A at pH 7.5 produced a relatively well protected ~ 60 kDa fragment (fragment 1) plus several smaller fragments. Amino acid sequence analyses indicated that the 60 kDa fragment is the N-terminal portion of chromogranin A without the ~ 15 kDa C-terminal fragment (Fig. 2B). Fragments 2 and 3 were also N-terminal fragments, demonstrating the fact that the C-terminal region is proteolyzed by trypsin before the N-terminal region. One distinctive difference between the fragments proteolyzed with trypsin at pH 5.5 and 7.5 is that a core segment of chromogranin A with a size of ~ 60 kDa remained relatively intact at pH 7.5 whereas the same fragment was proteolyzed continuously by trypsin at pH 5.5, suggesting that the N-terminal core structure is more compact and better protected at pH 7.5.

Since the results in Figs. 1 and 2 indicated that the C-terminal region of CGA is more exposed than the N-terminal region, and a C-terminal peptide was shown to dimerize at pH 7.5 and tetrameriz at pH 5.5 [10], it was of interest to determine whether any pH-dependent structural change of the C-terminal region is involved. To determine the conformational changes of the C-terminal region, the secondary structural change of a synthetic bovine CGA peptide ((W)ELESLSAIEAELEKVAHQLQALRRG), representing the conserved C-terminal residues 407–431, was determined by CD spectrometry at pH 5.5 and 7.5 (Fig. 3). The CD spectra showed that the magnitude of molar ellipticity at 222 nm increased as the pH was changed from 7.5 to 5.5. The increase in the magnitude of the molar ellipticity in the region from ~ 207 to ~ 225 nm indicates an increase of α-helicity at pH 5.5, while the decrease in the magnitude of molar ellipticity at ~ 200 nm indicates a decrease of coil structure as the pH changed from 7.5 to 5.5 [13–15]. Estimation of the secondary structure based on the composite CD spectra of five globular proteins (myoglobin, lysozyme, ribonuclease, papain, cytochrome *c*) [13,16] suggested ~ 52% α-helix at pH 5.5 and ~ 15% α-helix at pH 7.5, with root mean square deviations of 3–4%. The presence of 15 mM Ca^{2+} at pH 5.5 did not result in any detectable structural changes. The same concentration of Ca^{2+} at pH 7.5 also did not appear to change the conformation of the peptide (not shown). Since the presence of Ca^{2+} does not appear to cause detectable structural changes of the C-terminal peptide, it appears that the different pH values are the determining factor for the conformation of the peptide.



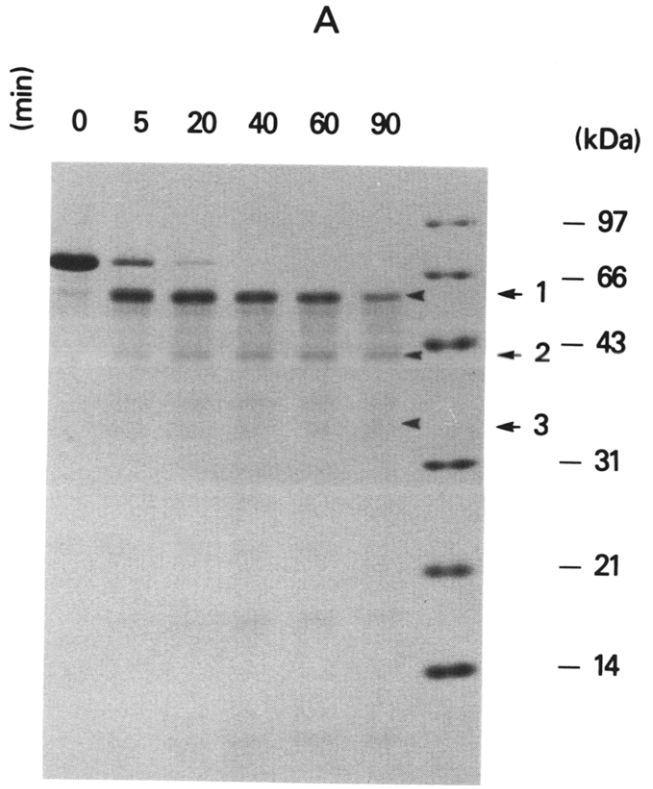
B

Tryptic Fragments	Amino Acid Sequence	Size (kDa)	Location in Chromogranin A
1	LPVNSPMN	60	1 N—C
2	LPVNSPMN	51.5	2 N—C
3	LPVNSPMN	42	3 N—C
4	LPVNSPMN	35	4 N—C
5	LPVNSPMN	31	5 N—C
6	AAPGWPE	17	6 N—248—C
7	LPVNSPM	16	7 N—C

Fig. 1. Limited trypsin digestion pattern of chromogranin A and the amino acid sequence of the tryptic fragments. (A) Chromogranin A in 20 mM sodium acetate, pH 5.5, 0.1 M KCl was subjected to limited trypsin digestion and analyzed by 12–20% SDS-PAGE. Each lane has 17 μg of chromogranin A. Length of incubation at 37°C is expressed in minutes at the top of each lane. The numbers with arrows on the side indicate the fragments whose amino acid sequences were determined (cf. Fig. 1B). (B) The amino acid sequences of the tryptic fragments from A were determined and the relative location of each fragment on chromogranin A is shown as a bar on the right. The estimated size of each fragment was determined based on the migration pattern on the gel.

4. DISCUSSION

The trypsin digestion pattern of chromogranin A at pH 7.5 and pH 5.5 and the amino acid sequence data of the isolated fragments indicate that the N-terminal region of chromogranin A forms a compact core structure which is relatively resistant to digestion by trypsin (Figs. 1 and 2). By contrast, the C-terminal region of the molecule was cleaved first by trypsin at both pH levels, indicating that this region is more exposed for ready contact with other molecules. The difference in the trypsin digestion pattern at pH 7.5 and 5.5 as revealed by SDS-PAGE reflects the pH-induced conformational changes of chromogranin A. Moreover, the trypsin digestion of chromogranin A at pH 5.5 produced smaller fragments more readily than at pH 7.5, indicating that the fragments cleaved additionally at pH 5.5 are those that had previously been buried at pH 7.5. In addition, the 60 kDa N-terminal core structure which had been protected at pH 7.5 also began to be cleaved by trypsin



B

Tryptic Fragments	Amino Acid Sequence	Size (kDa)	Location in Chromogranin A
1	LPVNSPMN	60	1 N—C
2	LPVNSPMN	43	2 N—C
3	LPVNSPMN	35	3 N—C

Fig. 2. Limited trypsin digestion pattern of chromogranin A and the amino acid sequence of the tryptic fragments. (A) Chromogranin A in 20 mM Tris-HCl, pH 7.5, 0.1 M KCl was subjected to limited trypsin digestion and analyzed by 12–20% SDS-PAGE. Each lane has 17 μg of chromogranin A. Length of incubation at 37°C is expressed in minutes at the top of each lane. The numbers with arrows on the side indicate the fragments whose amino acid sequences were determined (cf. Fig. 2B) and the molecular size markers on the right lane are the same as Fig. 1. (B) The amino acid sequences of the tryptic fragments from A were determined and the relative location of each fragment on chromogranin A is shown as a bar on the right. The estimated size of each fragment was determined based on the migration pattern on the gel.

at pH 5.5, suggesting a further structural relaxation of even the compact N-terminal core structure. Since there are only two cysteine residues in the entire CGA molecule and these two cysteines are located in the 17th and 38th positions from the N-terminus [17,18], formation of the compact N-terminal core structure may owe its existence to a disulfide bond formed between the two cysteines [19]. In our previous studies, it was shown that chromogranin A bound more Ca^{2+} at pH 5.5 than at pH 7.5, i.e. 55 mol of Ca^{2+} bound/ mol of protein with a dissociation constant (K_d) of 4 mM at pH 5.5 versus 32 mol of Ca^{2+}/mol of protein with a K_d of 2.7 mM at pH 7.5 [4]. One of the interpretations of these results is that there is a structural relaxation of chromogranin A at pH 5.5, promoting more Ca^{2+} binding. Therefore, it appears that the lower Ca^{2+} binding capacity of chromogranin A at pH 7.5 is at least partially due to the relatively compact structure at this pH, making it more difficult for Ca^{2+} to bind the protein. The overall loosening of CGA at pH 5.5 appears to enhance the probability of Ca^{2+} coming in contact with the protein, but with lower affinity, possibly due to the increased freedom of movement by the ligands.

The C-terminal region of CGA appears to be struc-

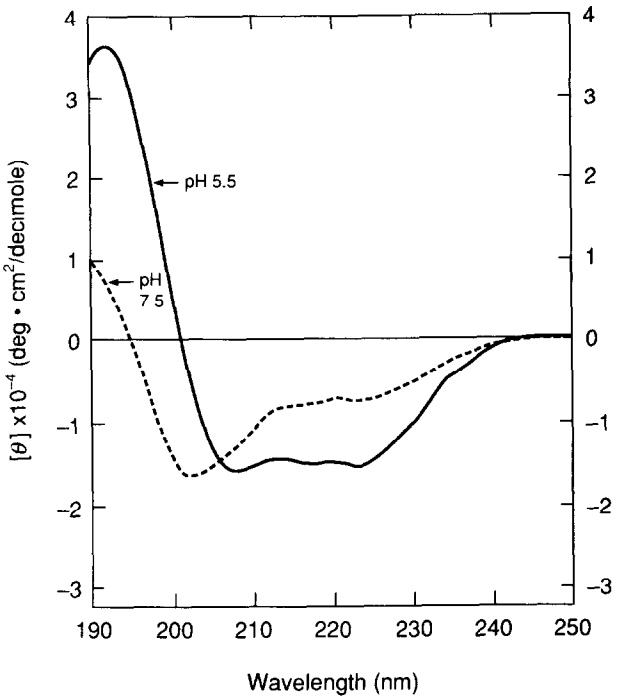


Fig. 3. CD spectra of the synthetic C-terminal peptide (residues 407–431) of chromogranin A. CD spectra of the conserved C-terminal region of bovine chromogranin A at the peptide concentration of 50 μM either in 15 mM sodium acetate, pH 5.5 or in 15 mM Tris-HCl, pH 7.5.

turally flexible, i.e. exposed at pH 5.5 and buried at pH 7.5, as evidenced by the preferential cleavage of the C-terminal region at pH 5.5. The fact that the 15 kDa C-terminal protein is among the first cleaved CGA fragment at pH 5.5 and the N-terminal 60 kDa core protein is better protected than the 15 kDa C-terminal region at pH 7.5 indicates that the 15 kDa C-terminal domain has a greater flexibility to interact with other molecules. It is apparent that the 15 kDa C-terminal domain which consisted of the last ~ 80 amino acid residues of CGA is located at the surface of CGA and undergoes major conformational changes as the pH level changes. This was confirmed by the circular dichroism study of the C-terminal peptide. As shown in Fig. 3, the C-terminal peptide existed in a highly helical (52% α-helix) state with a reduced coil structure at pH 5.5, but the helical state was significantly reduced (15% α-helix) as the pH was raised to 7.5. In this regard, it is noteworthy that the α-helicity of intact CGA also increased at pH 5.5 compared to the helicity at pH 7.5; CGA had 40% α-helical structure at pH 5.5 compared to 25% at pH 7.5 [9]. Given that CGA exists in a dimeric state at pH 7.5 and in a tetrameric state at pH 5.5 [20], the structural flexibility appears to be directly linked to each oligomeric state of CGA. It appears therefore that the C-terminal region may not only protrude out, but also assume a more helical structure at pH 5.5, thereby enabling it to interact with other C-terminal peptides or with the C-terminal regions of other CGA to form tetramer. Although this interpretation is consistent with the notion of flexible movement of the 15 kDa C-terminal domain of CGA, and the reduced α-helicity and the increased coil structure at pH 7.5 may be accompanied by withdrawal or burial of the C-terminal region, it is not currently clear whether the conserved C-terminal region is directly responsible for the pH-dependent oligomerization of CGA. Nevertheless, in light of the fact that the 26 residue C-terminal CGA peptide formed dimer at pH 7.5 and tetramer at pH 5.5 [10], and the ΔG° values of dimerization and tetramerization accounted for most (>80%) of the ΔG° values for dimerization and tetramerization of intact CGA [10], it appears likely that the increased α-helical structure of the C-terminal region at pH 5.5 is primarily responsible for the tetramerization of both the C-terminal peptide and intact CGA.

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