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Effects of calcium on recombinant bovine chromogranin A

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Abstract

Bovine chromogranin A, the acidic calcium-binding protein characteristic of endocrine secretory vesicles, has been expressed in *Escherichia coli* using the pET3a vector system under T7 polymerase control. The expressed protein is located in the bacterial cytosol and can be purified from bacterial proteins by a heat treatment step, followed by gel filtration, anion-exchange, and reversed-phase chromatography. The purified recombinant chromogranin A has an apparent M_r of ca. 72,000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, in spite of its 432-amino acid polypeptide chain, consistent with observations on natural chromogranin A. The primary structure has been confirmed by mass spectral analysis of tryptic peptides, by Edman degradation of the intact protein, and by immunoreactivity with sequence-specific antibodies. Analysis by circular dichroism spectroscopy shows pH- and concentration-dependent spectra. The spectra are Ca^{2+}-dependent from 5 to 40 μM.

Keywords: calcium binding; circular dichroism; chromogranin; recombinant protein

CGA is a highly acidic protein (pI, 4.8) found in the peptidergic secretory granules of most endocrine cells and many neurons (Banks & Helle, 1965; Helle, 1966; Blaschko et al., 1967; Smith & Kirshner, 1967; Smith & Winkler, 1967; O'Connor et al., 1983; Winkler & Fischer-Colbrie, 1992). CGA is a precursor for several peptides recently reported to have biological activity: pancreastatin, an amidated peptide that suppresses glucose-stimulated insulin secretion in pancreatic islets (Tatemoto et al., 1986; Eiden, 1987; Huttner & Benedum, 1987); chromostatin, a peptide reported to arrest catecholamine secretion from adrenal chromaffin cells (Galindo et al., 1991, 1992); and vasostatin, the amino-terminal peptide that inhibits the contraction of venous segments stimulated by K^+, noradrenalin, or endothelin (Aardal et al., 1992), and which has also been reported to inhibit parathyroid hormone secretion stimulated by low calcium (Drees et al., 1991). CGA also binds calcium and catecholamines and self associates in the presence of calcium at high protein concentration (Cohn et al., 1981; Reiffen & Gratzl, 1986a,b; Leiser & Sherwood, 1989; Yoo & Albanesi, 1990; Videen et al., 1992). It is difficult to obtain sufficient CGA of a single well-characterized molecular species for either structural or functional studies, because the protein is extensively proteolytically processed (Wohlfarter et al., 1988) and occurs in phosphorylated (Settleman et al., 1985a), glycosylated (Smith & Winkler, 1967; Geissler et al., 1977; Fischer-Colbrie et al., 1982), and proteoglycan forms (Falkensammer et al., 1985; Rosa et al., 1985; Gowda et al., 1990) as well. In order to pursue further studies with a defined CGA polypeptide, we have expressed the cDNA for bovine CGA in the pET3a vector system devised by Studier (Studier & Moffat, 1986; Studier et al., 1990). The recombinant CGA isolated from the bacterial cytosol can be phosphorylated and binds calcium. The pure protein exhibits a pH-, concentration-, and calcium-dependent CD spectra.

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Abbreviations: CD, circular dichroism; CGA, chromogranin A; HPLC, high pressure liquid chromatography; *m/z*, mass-to-charge ratio; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TIC, total ion current.

Results

Solubility and apparent molecular weight of recombinant CGA

Before beginning the purification of recombinant CGA, we determined that solubilization of the proteins in the *Escherichia coli* periplasmic space by incubation with lysozyme did not result in the release of CGA immunoreactivity. However, sonication of the cells resulted in the release of CGA in the cytosolic fraction, with no evidence for the formation of an inclusion body. Only the soluble proteins showed an immunoreactive band of apparent M_r 72,000, as revealed by immunoblot analysis with sequence-specific antibodies to many regions of CGA (see below). The 24,000-Da discrepancy in molecular weight from the value of 48,464 predicted by the cDNA sequence is consistent with previous reports. Benedum et al. (1986) and Iacangelo et al. (1986) showed that translation of mRNA hybrid selected on the CGA cDNA yielded a polypeptide that migrated on SDS-PAGE as a protein with M_r 72,000. Therefore, the cause of this anomalous behavior must lie solely within the nature of the primary structure. It is possible that the high percentage of acidic residues in CGA, ca. 30%, alters the binding of sodium dodecyl sulfate to the polypeptide chain.

Purification of recombinant CGA

Figure 1 shows a summary of the purification procedure as analyzed by SDS-PAGE. The gels shown in panels A and C were stained with Coomassie Blue G250 to visualize all proteins; the gels in panel B and D were treated with Stains-All (Campbell et al., 1983), an anionic dye demonstrated to have an affinity for calcium-binding proteins. This feature was particularly useful, since there is no bioassay for intact CGA. The proteins in lanes 1 and 2 (panel B) do not exhibit a strong staining with the Stains-All, possibly because of interference from other proteins. However, there is a clear, strong enhancement in the Stains-All-positive CGA band, corresponding to the protein band that cross-reacts with the sequence-specific CGA antibodies.

The bacterial cytosolic proteins were processed by a heat-treatment step, followed by gel filtration on a column of Superose 12, chromatography on a DEAE ion-exchange column, and C-8 reversed-phase HPLC. CGA has only two cysteinyl residues, residues 17 and 38, which form an amino-terminal disulfide loop (Benedum et al., 1986, 1987; Iacangelo et al., 1986). Denatured, unreduced recombinant CGA did not react with sulfhydryl-specific reagents. In each preparation there is a small amount of protein in which the disulfide bond is not formed. This protein is easily removed from the correct product by the final HPLC purification step.

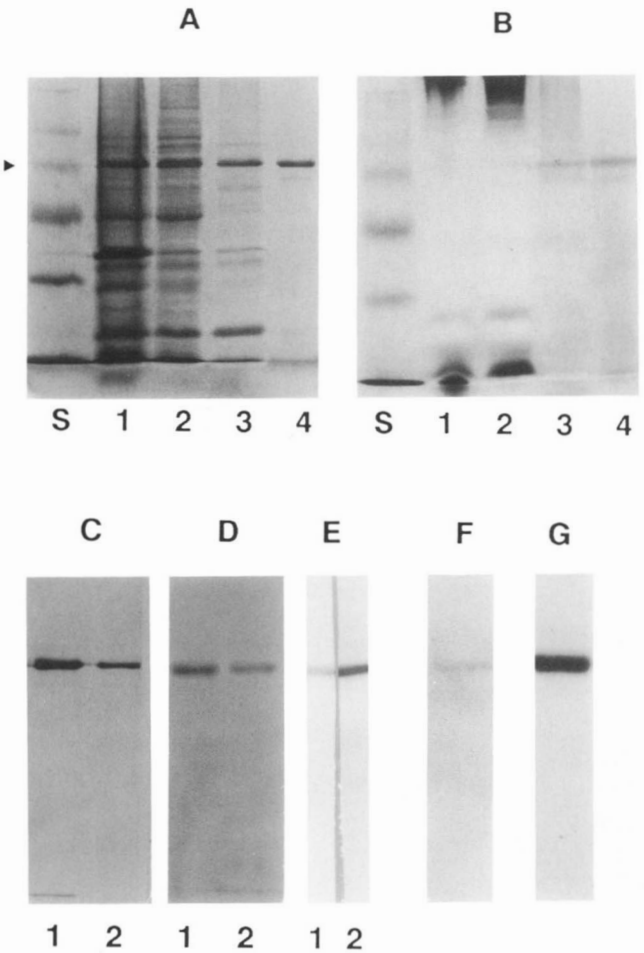

Fig. 1. Analysis of purification of recombinant CGA. Aliquots from different steps of the purification procedure were separated by SDS-PAGE and were revealed using Coomassie blue, Stains-All dye, binding to $^{45}Ca^{2+}$, and phosphorylation by casein kinase II. Then, 0.2% of each step of the preparation was loaded in the individual lanes. A, C: Coomassie blue-stained gels. B, D: Stains-All-stained gels. E: Immunoblots. F: Calcium binding. G: Phosphorylation with casein kinase II. Arrow denotes the position of the bovine serum albumin standard. Lanes in **A, B:** S, molecular weight markers (arrow denotes the migration position of bovine serum albumin); 1, total *Escherichia coli* homogenate; 2, soluble proteins; 3, heat-stable soluble proteins; 4, Superose 12 pool. Lanes in **C, D:** 1, DEAE-pool; 2, pool from C8 column. **E:** Immunoblots of heat-stable proteins in *E. coli* homogenate. The sample used was similar to that in A and B, lane 3. Lane 1 was exposed to a polyclonal sequence-specific antiserum to CGA_{1-76}. Lane 2 was exposed to a polyclonal sequence-specific antiserum to $CGA_{403-429}$. **F:** Autoradiogram of nitrocellulose transfer of pure recombinant CGA after incubation with $^{45}Ca^{2+}$. The sample used was similar to that in panels C and D, lane 2. **G:** Autoradiogram of CGA separated by electrophoresis after incubation with casein kinase and ^{32}P-ATP. The sample used was similar to that in panels C and D, lane 2.

In Figure 1E, the boiled bacterial cytosolic proteins were analyzed with sequence-specific antisera to peptides corresponding to many regions of the bovine CGA sequence. Lane 1 shows the immunoreactivity to an amino-terminal-specific antibody (CGA_{1-76}), and lane 2, the

immunoreactivity to a carboxy-terminal-specific antibody ($CGA_{403-428}$). Both reveal only one immunoreactive band, indicating a lack of appreciable proteolytic degradation, possibly aided by the boiling step. Similar results were obtained using antisera to other internal sequences, e.g., CGA_{44-76}, $CGA_{251-295}$, and $CGA_{316-329}$. No additional immunoreactive bands are formed during purification.

To demonstrate the Ca^{2+}-binding properties of CGA, the purified protein was subjected to SDS-PAGE and transferred to nitrocellulose for the $^{45}Ca^{2+}$ overlay procedure. The purified recombinant CGA shows a distinct band of bound calcium (Fig. 1F). In addition, natural CGA is known to be phosphorylated on serine residues. The purified recombinant CGA was incubated with casein kinase II and ^{32}P-labeled ATP, followed by electrophoretic separation. CGA is a substrate for this enzyme (Fig. 1G).

Verification of the sequence of recombinant CGA

It was important to determine that the primary structure of the expressed CGA was correct, because of the anomalous behavior of CGA on denaturing gel electrophoresis. Automated Edman degradation showed the expected amino-terminal sequence, MLPVNSPMK, with no evidence of contaminating polypeptides.

Tryptic peptides of recombinant CGA protein were analyzed by fast atom bombardment mass spectrometry using an HPLC interface. Additional analysis was performed on the tryptic peptide mixture further digested by the addition of an asparaginyl endoprotease. At least two analyses were performed on each experimental peptide mixture, as well as on control digests, containing only enzyme. Peptides containing a total of 399 residues out of 432 were positively identified. All peptides were identified in at least two experiments. Of the 33 residues that could not be located in the chromatographic profiles, three were single arginine residues, located at positions 115, 331, and 366, two were dipeptides, $CGA_{352-353}$ and $CGA_{430-431}$, and one was a tripeptide, $CGA_{224-226}$. These peptides are not easily detected in the type of experiment performed because of the presence of high background ions at this low mass range. The region $CGA_{207-221}$ was not detected, most likely because of the large negative charge of the peptides in this region. Because the region $CGA_{142-172}$ was not detected in the tryptic digests, an asparaginyl endoprotease was added to cleave the two internal asparagine residues. Two of the three predicted peptides, $CGA_{150-161}$ and $CGA_{162-172}$, were positively identified. The third peptide, $CGA_{142-149}$, was identified, but an ion at a similar m/z was also identified in the control digest.

Figure 2E shows the TIC chromatogram of an HPLC analysis of a tryptic digest of recombinant CGA. The mass chromatograms for four selected ions from this separation are shown in Figure 2A–D. The four ions were located at the extremes of the the HPLC separation and demonstrate that the peptide separation achieved is better than can be observed from the total ion current chromatogram. Panels C and D show the elution of the penultimate carboxy-terminal peptide and the amino-terminal peptide, respectively. The data from this individual experiment permitted the identification of 75.7% of the recombinant CGA sequence.

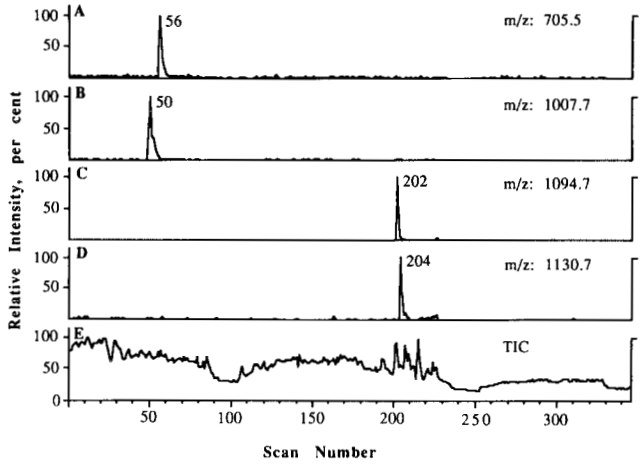

Fig. 2. Ion chromatograms obtained from continuous flow fast atom bombardment mass spectrometry of HPLC-separated tryptic peptides of recombinant CGA. Panel E is the TIC chromatogram of the HPLC separation. Representative mass chromatograms showing the elution positions of four individual protonated molecular ions are shown in panels A–D. The scan numbers are shown adjacent to the peaks. **A:** m/z = 705.5, $CGA_{319-324}$. **B:** m/z = 1,007.7, $CGA_{101-109}$. **C:** m/z = 1,094.7, $CGA_{421-429}$. **D:** m/z = 1,130.7, CGA_{-1-9}.

Figure 3 shows the accumulated mass spectra of the peptides identified in the mass chromatograms in Figure 2. Note that the observed molecular ions are within 0.1–0.2 amu of the predicted monoisotopic ions for these peptides. The background signal caused by the thioglycerol matrix can obscure the positive identification of smaller peptides. Figure 4B shows the accumulated scans that permitted the identification of low molecular weight peptides of m/z 476.2 and 533.4. Data from adjacent scans, shown in Figure 4A, demonstrate that these peaks are significantly above the background ions contributed by the matrix.

Comparison of recombinant CGA to natural CGA preparations

Figure 5 shows the relative HPLC elution profiles of the recombinant CGA and of a typical natural CGA preparation purified from bovine adrenal chromaffin granules by heat treatment and ion-exchange chromatography. The four peaks in the natural CGA preparation were analyzed by automated Edman degradation. Peak 1 is a fragment

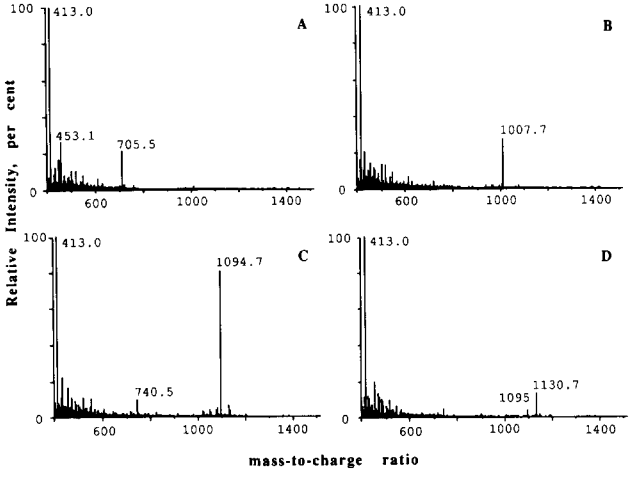

Fig. 3. Mass spectra of tryptic peptides. Accumulated scans of the protonated molecular ions shown in Figure 2. **A:** $CGA_{319-324}$, scans 55–58, predicted protonated monoisotopic m/z = 705.36. **B:** $CGA_{101-109}$, scans 49–54, predicted protonated monoisotopic m/z = 1,007.49. **C:** $CGA_{421-429}$, scans 202–204, predicted protonated monoisotopic m/z = 1,094.6. **D:** CGA_{-1-9}, scans 204–205, predicted protonated monoisotopic m/z = 1,130.6.

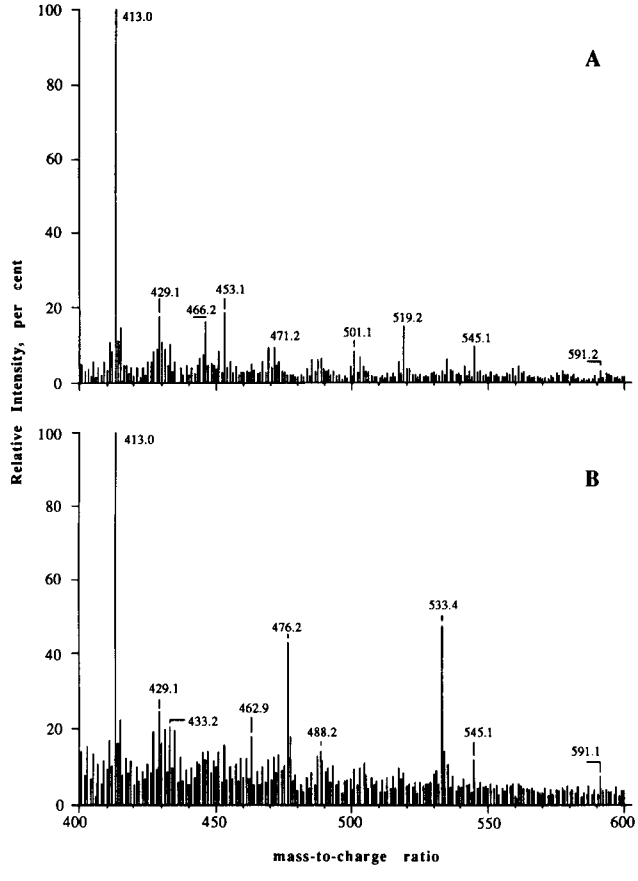

Fig. 4. Mass spectra of low m/z tryptic peptides to show the discrimination from background. **A:** Scans 20–25 from the TIC chromatogram shown in Figure 2E. **B:** Scans 26–27 from the total ion current chromatogram shown in Figure 2E. The ion at m/z 476.2 corresponds to CGA_{44-47}, predicted protonated monoisotopic m/z = 476.21. The ion at m/z 533.4 could correspond to $CGA_{110-114}$, $CGA_{203-206}$, or $CGA_{244-247}$, predicted protonated monoisotopic m/z = 533.27.

of preproenkephalin beginning at residue 234, as demonstrated by automated Edman degradation of the first 10 residues, AEPLPSEEEG. Peaks 2 and 3 exhibit a single amino acid sequence, corresponding to the amino-terminus of intact CGA. Analysis by SDS-PAGE (data not shown) suggests that both of these peaks are amino-terminal fragments of CGA. These fragments are endogenously processed in chromaffin granules at the first two pairs of basic residues and are the molecular species active in vasodilation assays (Aardal et al., 1992) and in assays for inhibition of the secretion of parathyroid hormone (Drees et al., 1991). Peak 4 also exhibits a single amino-terminus characteristic of CGA and is eluted 2.1 min earlier than the recombinant CGA under the same elution conditions. When analyzed by SDS-PAGE, peak 4 showed a predominance of a band of M_r 72,000, with several faster migrating species also immunoreactive with anti-CGA antibodies. The natural CGA protein of M_r 72,000 is known to be phosphorylated on serine residues (Settleman et al., 1985a), and is also glycosylated (Smith & Winkler, 1967; Geissler et al., 1977; Fischer-Colbrie et al., 1982). Therefore, the slightly later elution time of the recombinant CGA is expected.

CD of recombinant CGA

CGA is a very acidic protein, with an isoelectric point of 4.8. In vivo, it experiences two physiological pH conditions: the low pH of the secretory vesicles, between pH 5 and 6; and neutral pH during passage through the Golgi apparatus, and after secretion into the extracellular en-

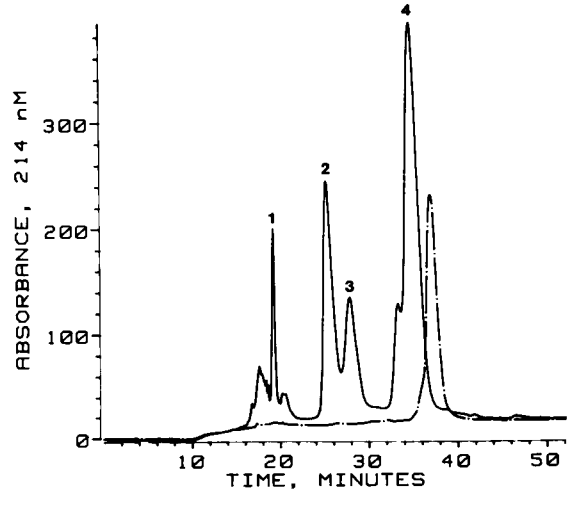

Fig. 5. Comparison of recombinant CGA with natural bovine CGA on C8 reversed-phase HPLC column. Solid line, profile for separation of natural CGA; dashed-dotted line, profile for recombinant CGA.

vironment and into the bloodstream. One report indicates that CGA differs in secondary structure at neutral and acidic pHs, as evidenced by changes in CD spectra (Yoo & Albanesi, 1991). We also note a difference in secondary structure of CGA at neutral and acidic pH, with a much higher component of α-helix at neutral pH (Fig. 6; Table 1). There is also a concentration-dependent effect of the CD spectra. This is not reflected in the calculated content of periodic secondary structure but in the extent of the measured ellipticity. Concentrations of protein greater than 2 μM had limited solubility at acidic pH. Differences in the spectra can also be noted at neutral pH. The curves shown in Figure 6 were measured in the presence of 50 mM NaCl, to eliminate any charge effects in the subsequent calcium titrations. However, the spectral characteristics were the same when performed in the absence of added salt, in the presence of 150 mM NaCl, or in several different buffers.

At acidic pH, addition of calcium over a wide concentration range caused marked changes in the spectrum of recombinant CGA (Fig. 6B,C). At the protein concentrations used, there was no turbidity or aggregation of CGA. The calcium content of the chromaffin granules is 20 mM (Winkler & Westhead, 1980). Yet, differences in the spectra could be noted with addition of as little as 5 μM calcium. However, these spectral changes were not accompanied by changes in the calculated percentages of periodic structural components (Table 1), using the fitting procedures of Yang (1986). At neutral pH (Fig. 6D), addition of calcium to recombinant CGA causes a decrease in α-helical content, and a corresponding increase in the amount of random coil (Table 1), to give values more similar to those obtained for CGA at low pH.

Table 1. *Secondary structure estimations (percentages) of recombinant CGA*

	α-Helix	β-Sheet	β-Turns	Random coil
0.4 μM rCGA, pH 5.8	5.0	17.2	29.9	48.0
+0.1 mM Ca^{2+}	7.1	15.6	30.4	46.8
+2.5 mM Ca^{2+}	6.1	15.0	30.8	48.0
+5.0 mM Ca^{2+}	6.3	14.0	31.0	48.7
+10.0 mM Ca^{2+}	6.2	17.5	28.8	47.6
+25.0 mM Ca^{2+}	4.5	20.7	27.8	46.9
1 μM rCGA, pH 5.8	6.9	17.1	27.4	48.6
+0.5 mM Ca^{2+}	6.9	16.2	28.1	48.8
+5.0 mM Ca^{2+}	6.2	15.7	28.3	49.8
+20.0 mM Ca^{2+}	6.7	14.6	28.4	50.3
0.35 μM rCGA, pH 7.2	37.7	–	28.5	33.8
2.4 μM rCGA, pH 7.2	34.0	3.1	32.4	30.5
+0.5 mM Ca^{2+}	12.1	14.0	27.9	46.0
+2.5 mM Ca^{2+}	11.1	14.9	27.7	46.2
+10.0 mM Ca^{2+}	12.6	14.4	27.7	45.3

Discussion

The reported biological activities of CGA fragments appear to reside wholly within the primary structure, as ev-

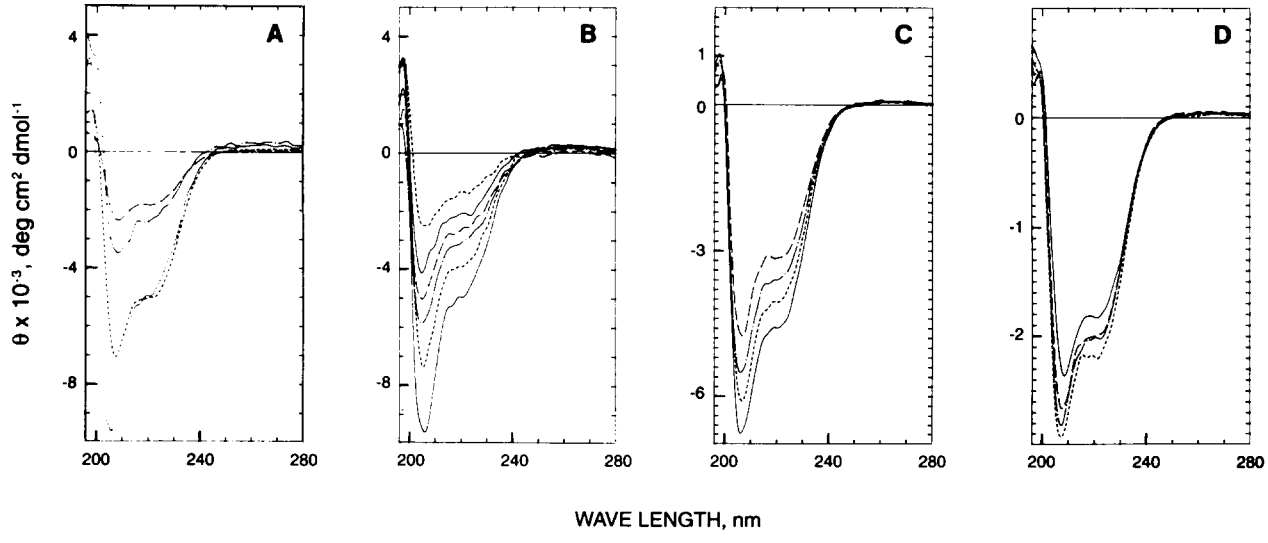

Fig. 6. CD spectra of recombinant CGA at acidic and neutral pH, in the absence and presence of calcium. **A:** Solid line, 0.4 μM CGA, pH 5.8; dotted line, 1 μM CGA, pH 5.8; dashed-dotted line, 0.35 μM CGA, pH 7.2; dashed line, 2.2 μM CGA, pH 7.2. **B:** All measurements were made of 0.4 μM CGA at pH 5.8. Lower solid line, no calcium; lower dotted line, 0.1 mM calcium; dashed-dotted line, 2.5 mM calcium; dashed line, 5 mM calcium; upper solid line, 10 mM calcium; upper dotted line, 25 mM calcium. **C:** All measurements were made of 1 μM CGA at pH 5.8. Solid line, no calcium; dotted line, 0.5 mM calcium; dashed-dotted line, 5 mM calcium; dashed line, 20 mM calcium. **D:** All measurements were made of 2.4 μM CGA at pH 7.2. Solid line, no calcium; dotted line, 0.5 mM calcium; dashed-dotted line, 2.5 mM calcium; dashed line, 10 mM calcium.

idenced by studies using synthetic peptides (Tatemoto et al., 1986; Galindo et al., 1991; Angeletti et al., unpubl.). Yet, the natural CGA is posttranslationally modified in diverse ways (Giessler et al., 1977; Fischer-Colbrie et al., 1982; Falkensammer et al., 1985; Rosa et al., 1985; Settleman et al., 1985a; Wohlfarter et al., 1988; Gowda et al., 1990). These modifications could affect proteolytic processing, binding of small molecules, or biological turnover. Availability of pure CGA of defined structure will permit the study of these processing events and biological function. Further unusual properties of CGA have been reported. For example, it binds tightly to secretory vesicle membranes even though it is a soluble protein (Settleman et al., 1985b). The molecular basis of this property is unknown. The availability of recombinant CGA will permit a careful analysis of this phenomenon, which has been postulated to be involved in secretory granule formation and condensation (Pimplikar & Huttner, 1992).

With the CGA expressed in *E. coli* using the pET vector system, a simple purification procedure could be devised, analogous to that used for purification of natural CGA (Fischer-Colbrie & Schober, 1987). This protocol resulted in a polypeptide with the correct primary structure, as determined by amino-terminal sequencing and mass spectrometry of the tryptic peptides. In the purification of undegraded natural CGA, a critical step is heat treatment for 5 min at neutral pH in a boiling water bath (Rosa et al., 1985; Fischer-Colbrie & Schober, 1987). Most of the chromaffin granule proteins precipitate, leaving a preparation rich in CGA and the related protein, chromogranin B. Heat treatment is a common method used to purify heat-stable proteins, e.g., calmodulin (Wallace et al., 1980). This procedure was also effective in the purification of recombinant CGA. As seen in Figure 1, CGA remains in the supernatant after a heat treatment step, which removes most of the *E. coli* proteins. Yoo & Albanesi (1991) have suggested that CGA prepared in this manner has an altered structure. In contrast, Videen & coworkers (1992) noted no difference in the interactions of natural CGA with either calcium or catecholamines, regardless of whether the protein had been heat treated or not. The CD spectra shown in Figure 6A are similar to those of natural bovine CGA purified without a heat treatment (Yoo & Albanesi, 1991; Angeletti, data not shown).

The CD spectra of recombinant CGA titrated with calcium showed marked changes at calcium concentrations as low as 5 μM. This is in contrast to the report that the changes in CD spectra only occurred in the range of 5–10 mM (Yoo & Albanesi, 1991). It is likely that starting preparations used by these investigators retained bound calcium not removed by the Chelex chromatography step (Holmquist, 1988; Riordan & Vallee, 1988). Several previous investigations have noted the presence of two classes of calcium-binding sites in CGA (Reiffen & Gratzl, 1986a,b; Yoo & Albanesi, 1991), with K_D values for CGA calcium binding of ca. 50 μM and 2 mM as measured by calcium electrode. The Ca^{2+}-dependent spectral changes that we observed are consistent with these values. The previously observed calcium-dependent aggregation of CGA occurs at protein concentrations higher than those used in the present study.

An unusual feature of the CD spectra (Fig. 6) is that the calcium interactions at low pH were not reflected by significant changes in calculations of secondary structural elements. At low pH, the recombinant CGA has approximately 50% random coil structure and 30% β-turns, with lesser amounts of calculated α-helix and β-sheet. The amino acid composition of CGA is unusual, with over 30% acidic residues, and a high proline content (9%). In Figure 7, the amino acid sequence of CGA is represented simply as proline and glycine residues (in bold letters), which disrupt α-helical structure, plus glutamic acid and aspartic acid. The term random coil structure is often loosely interpreted as disordered structure or as a lack of a fixed tertiary structure (Videen et al., 1992). However, a random coil is perhaps more simply defined as a lack of known ordered periodic, or asymmetric, secondary structural elements. The presence of proline and glycine residues, interspersed by stretches of acidic residues, may impose a structure on CGA that would produce CD spectra not easily recognized by present curve-fitting approaches. CGA does not show significant primary structure similarity to other calcium-binding proteins (Iacangelo et al., 1986).

Most other studies have focussed on the effects of calcium on CGA at high protein concentrations, monitoring protein aggregation (Leiser & Sherwood, 1989; Yoo & Albanesi, 1990; Pimplikar & Huttner, 1992; Videen et al., 1992). This property is currently thought to have relevance to the formation of acidic secretory vesicles from the Golgi apparatus (Huttner et al., 1991; Pimplikar

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-P---P---GD-E------E---D----P-P-P---E--E---G-E----
---------E--D----G--E-----------EDE--E--E-P-D--EP-
E--EE----D--E--DD--E-E--DED-DGD-P--P-G-G-GP--EED--
-PGEEEE------P----P-P--PGP---ED-EGP--GP---E-G---E-
G---E-EEEEE--EE-E--E---PEEE-PP-----PPP--G--E-----P
G-PEDG-G--G-EE--PPEG-GE------EEEE----P-----GG--GEP
E-EE----E-ED-------D--------E---EGEEEEEEDPD-------
---G-G--GPG-----G--P---ED--E-G-P----G-PEE--EEEG---
--PED-E-E-----E-E-E------EE---G
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Fig. 7. Representation of CGA amino acid sequence with only proline, glycine, glutamic acid, and aspartic acid shown. Proline and glycine residues are printed in bold.

& Huttner, 1992). Our preparations of recombinant CGA could potentially be used to test elements of this hypothesis in a cell-free system. However, we have initially focussed on the effects of calcium on CGA at lower protein concentrations. Yoo and Lewis (1992) have recently reported that natural CGA is a tetramer at low pH in the presence of calcium, but is a dimer at neutral pH in the absence of calcium, using concentrations of CGA similar to those used in the experiments described in the present communication. It is at these concentrations that we will be able to further explore the physical properties of CGA and to relate these properties to the generation of biologically active peptides.

CGA and the structurally related chromogranin B (Winkler & Fischer-Colbrie, 1992) are members of a small, but novel family of calcium-binding neuroendocrine peptide precursor proteins. The recombinant protein obtained in this study will be used to study the posttranslational processing, receptor binding, and molecular properties of CGA. It will be of interest in the future to see if structural homologies to other families of proteins exist on a three-dimensional level, if not on the level of primary structure.

Materials and methods

Materials

Chemicals and biochemicals were obtained from Sigma or Baker Chemical Co. High purity sodium chloride was purchased from Aldrich Chemical Co. Solvents were Burdick and Jackson HPLC grade. Trifluoracetic acid was obtained from Pierce Chemical Co. Water was distilled and passed through a Milli-Q$^+$ apparatus (Millipore) and used fresh, without storage, only at 18 MΩ/cm (25°) (Riordan & Vallee, 1988). New England Biolabs restriction enzymes were used in all experiments. *Taq* polymerase and polymerase chain reaction reagents from Cetus-Perkin Elmer were used in construction of the expression plasmids. Asparaginyl endoprotease was purchased from Takara Biochemicals (Matsushita et al., 1991). Trypsin, treated with L-1-tosylamide-2-phenylethylchloromethyl ketone, was obtained from Worthington Corp. The cDNA for bovine CGA in a pUC13 vector was a generous gift of Lee Eiden. The pET vectors and bacterial stocks were a gift of Wm. Studier (Studier & Moffat, 1986; Studier et al., 1990). Antibodies to synthetic peptides corresponding to amino acid sequences along the entire length of the CGA polypeptide were prepared in the authors' laboratory and have been used in numerous studies. Recombinant casein kinase II was the generous gift of Charles Rubin (Hu & Rubin, 1990). Casein kinase II assays were performed using ^{32}P-γATP and casein as a positive control substrate, as described by Hu and Rubin (1990).

Construction of pET-CGA

Polymerase chain reaction was used to add Nde I restriction sites to the cDNA for CGA. Oligonucleotide primers were made to add an Nde I site to the 5′ end of the region coding for the CGA protein without the leader sequence (GGATCCCATATGCGCTCCGCCGCGGTCC TGGCGCTTCT), and another was made to convert a site 64 bp beyond the 3′ end of the reading frame (GGATCC CATATGGACCTGCAGAGGGGGCAACAGA). After completion of 24 cycles at a melting temperature of 94 °C (1 min), an annealing temperature of 50 °C (2 min), and an extension temperature of 72 °C (3 min, 10 s extension added/cycle), the correct-sized products were purified from low melting agarose gels and cut with Nde I before ligating the insert into the pET3a vector. The DNA was transformed into the *E. coli* DH5α strain. The DNA from colonies isolated from this strain was restriction mapped and sequenced before the final transformation into the BL21(DE3) strain of *E. coli*. Carbenycilin antibiotic was used throughout this procedure. Bacteria were grown to an absorbance of 0.6 at 590 nm before induction with isopropyl β-D-thiogalactopyranoside.

Protein purification

Cells were collected by centrifugation, frozen at −80 °C and thawed, treated with lysozyme (0.5 mg/g cells, 10 min at 20 °C), and then further lysed by sonication at 4 °C. After centrifugation at 15,000 rpm in a Sorvall SS35 rotor, the supernatant was heated in a boiling water bath for 5 min, chilled on ice for 10 min, and recentrifuged. This final supernatant was dialyzed for 6 h against 10 mM potassium phosphate, pH 7.2, lyophilized, and redissolved in prechilled 10 mM potassium phosphate, pH 7.2, containing 0.1 M NaCl. The proteins were then separated on a Superose 12 column, 2.5 × 150 cm, followed by both DEAE (4.6 mm × 5 cm) and C8 (4.6 mm × 20 cm) reversed-phase HPLC columns (Fischer-Colbrie & Schober, 1987). Protein was monitored by absorbance at 220 nm and 280 nm, and by SDS-PAGE, followed by immunoblotting with a sequence-specific antiserum to bovine CGA. Calcium binding was performed on nitrocellulose according to published methods (Reiffen & Gratzl, 1986a). An Applied Biosystems 477A sequencer was used for protein sequence analysis. Amino acid content was quantitated after acid hydrolysis on a Hewlett-Packard AminoQuant system. A precise quantitation of the sort used in purifying enzymes is more difficult with either recombinant or natural CGA purifications, because there is no direct assay for the intact polypeptide, only immunological assays. The yield of CGA protein at the boiling step was approximately 90%, 75% at the DEAE chromatography step, and 70% from the reversed-phase column. An overall yield of ca. 0.5 mg protein/g culture was obtained.

Mass spectrometry

Mass spectrometry of tryptic peptides from recombinant CGA was performed using a Finnigan MAT-90 mass spectrometer at a resolution of ca. 1,200, and equipped with a microbore HPLC interface. Fast atom bombardment was used for sample ionization, and tryptic peptides were separated on a 0.32-mm-diameter C18 column and analyzed directly by the mass spectrometer. The column was eluted with a gradient of acetonitrile in the presence of 0.1% trifluoracetic acid and 2.5% thioglycerol. A minimum of two runs was made on each digestion mixture. Correct identifications were called on only those ions with a signal-to-noise ratio of 3:1 or greater, which were within 1.5 amu of the predicted protonated monoisotopic m/z, were present in at least two scans, and were not found in the control digests. For purposes of comparison to the natural CGA sequence, the same residue numbers are used for recombinant CGA. The amino-terminal methionine is thus designated as residue −1.

CD

CD spectroscopy was carried out on a JASCO J-720 instrument with a 1-cm cell at designated protein concentrations. Measurements were carried out at a 1-nm band width, 0.125-s response time, and 0.5-nm step resolution. Ten accumulated scans were taken for each sample at a scan speed of 50 nm/min. The curve-fitting techniques of Yang (1986) were used for secondary structure estimation. Protein concentration was measured by absorbance at 280 nm. Measurements were made in both 2 mM sodium acetate and potassium phosphate buffers, at either pH 5.2 or 5.8, or in MOPS (3-[*N*-morpholino]propanesulfonic acid) buffer at pH 7.2.

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References

Aardal, S., Helle, K.B., Elsayed, S., Reed, R.K., & Serck-Hanssen, G. (1992). Vasostatin, a 20–23 kd triplet of the fragmented bovine chromogranin A, suppresses tension development in isolated segments of human blood vessels. *Reg. Peptides 41*, 9–18.

Banks, P. & Helle, K.B. (1965). The release of protein from the stimulated adrenal medulla. *Biochem. J. 97*, 40C–41C.

Benedum, U.M., Baeuerle, P.A., Konecki, D.S., Frank, R., Powell, J., Mallet, J., & Huttner, W.B. (1986). The primary structure of bovine chromogranin A: A representative of a class of acidic secretory proteins common to a variety of peptidergic cells. *EMBO J. 5*, 1495–1502.

Benedum, U.M., Lamouroux, A., Konecki, D.S., Rosa, P., Hille, A., Baeuerle, P.A., Frank, R., Lottspeich, F., Mallet, J., & Huttner, W.B. (1987). The primary structure of human secretogranin I (chromogranin B): Comparison with chromogranin A reveals homologous terminal domains and a large intervening variable region. *EMBO J. 6*, 1203–1211.

Blaschko, H., Comline, R.S., Schneider, F.H., Silver, M., & Smith, A.D. (1967). Secretion of a chromaffin granule protein, chromogranin from the adrenal gland after splanchnic stimulation. *Nature 215*, 58–59.

Campbell, K.P., Maclennan, D.H., & Jorgensen, A.O. (1983). Staining of the calcium-binding proteins, calsequestrin, calmodulin, troponin C, and S-100, with the cationic carbocyanine dye "Stains-All." *J. Biol. Chem. 258*, 11267–11273.

Cohn, D.V., Morrissey, J.J., Hamilton, J.W., Shofstall, R.E., Smardo, F., & Chu, L.L.H. (1981). Isolation and partial characterization of secretory protein I from bovine parathyroid glands. *Biochemistry 20*, 4135–4140.

Drees, B.M., Rouse, J., Johnson, J., & Hamilton, J.W. (1991). Bovine parathyroid glands secrete a 26-kDa N-terminal fragment of chromogranin-A which inhibits parathyroid cell secretion. *Endocrinology 129*, 3381–3387.

Eiden, L.E. (1987). Is chromogranin A a prohormone? *Nature 325*, 301.

Falkensammer, G., Fischre-Colbrie, R., & Winkler, H. (1985). Biogenesis of chromaffin granules: Incorporation of sulfate into chromogranin B and into a proteoglycan. *J. Neurochem. 45*, 1475–1480.

Fischer-Colbrie, R., Schachinger, M., Zangerle, R., & Winkler, H. (1982). Dopamine β-hydroxylase and other glycoproteins from the soluble content and the membranes of adrenal chromaffin granules: Isolation and carbohydrate analysis. *J. Neurochem. 38*, 725–732.

Fischer-Colbrie, R. & Schober, M. (1987). Isolation and characterization of chromogranins A, B and C from bovine chromaffin granules and from a rat pheochromocytoma. *J. Neurochem. 48*, 262–270.

Galindo, E., Mendez, M., Calvo, S., Gonzalez-Garcia, C., Cena, V., Hubert, P., Bader, M.-F., & Aunis, D. (1992). Chromostatin receptors control calcium channel activity in adrenal chromaffin cells. *J. Biol. Chem. 267*, 407–412.

Galindo, E., Rill, A., Bader, M.-F., & Aunis, D. (1991). Chromostatin, a 20-amino acid peptide derived from chromogranin A, inhibits chromaffin cell secretion. *Proc. Natl. Acad. Sci. USA 88*, 1426–1430.

Geissler, D., Martinek, A., Margolis, R.U., Margolis, R.K., Skrivanek, J.A., Ledeen, R., König, P., & Winkler, H. (1977). Composition and biogenesis of complex carbohydrates of ox adrenal chromaffin granules. *Neuroscience 2*, 685–693.

Gowda, D.C., Hogue-Angeletti, R., Margolis, R.K., & Margolis, R.U. (1990). Chromaffin granule and PC12 cell chondroitin sulfate proteoglycans and their relation to chromogranin A. *Arch. Biochem. Biophys. 281*, 219–224.

Helle, K.B. (1966). Some chemical and physical properties of the soluble protein fraction of bovine adrenal chromaffin granules. *Mol. Pharmacol. 2*, 298–310.

Holmquist, B. (1988). Elimination of adventitious metals. *Methods Enzymol. 158*, 6–12.

Hu, E. & Rubin, C.S. (1990). Expression of wild-type and mutated forms of the catalytic (α) subunit of *Caenorhabditis elegans* casein kinase II in *Escherichia coli*. *J. Biol. Chem. 265*, 20609–20615.

Huttner, W.B. & Benedum, U.M. (1987). Chromogranin A and pancreastatin. *Nature 325*, 305.

Huttner, W.B., Gerdes, H.H., & Rosa, P. (1991). The granin (chromogranin/secretogranin) family. *Trends Biochem. Sci. 16*, 27–30.

Iacangelo, A., Affolter, H.U., Eiden, L.E., Herbert, E., & Grimes, M. (1986). Bovine chromogranin A: Its sequence and the distribution of its messenger RNA in endocrine tissues. *Nature 323*, 82–86.

Leiser, M. & Sherwood, L.M. (1989). Calcium-binding proteins in the parathyroid gland. *J. Biol. Chem. 264*, 2792–2800.

Matsushita, H., Kato, I., Abe, Y., & Ishii, S. (1991). Asparaginylendopeptidase. *Protein Nucleic Acid Enzyme 36*, 730–736.

O'Connor, D.T., Burton, D., & Deftos, L.J. (1983). Chromogranin A: Immunohistology reveals its universal occurrence in normal polypeptide hormone producing endocrine glands. *Life Sci. 33*, 1657–1663.

Pimplikar, S.W. & Huttner, W.B. (1992). Chromogranin B (secreto-

granin I), a secretory protein of the regulated pathway, is also present in a tightly membrane-associated form in PC12 cells. *J. Biol. Chem. 267*, 4110–4118.

Reiffen, F.U. & Gratzl, M. (1986a). Chromogranins, widespread in endocrine and nervous tissue bind calcium. *FEBS Lett. 195*, 327–330.

Reiffen, F.U. & Gratzl, M. (1986b). Calcium binding to chromaffin vesicle matrix proteins: Effect of pH, magnesium, and ionic strength. *Biochemistry 25*, 4402–4406.

Riordan, J.F. & Vallee, B.L. (1988). Preparation of metal-free water. *Methods Enzymol. 158*, 3–6.

Rosa, P., Hille, A., Lee, R.W.H., Zanini, A., DeCamilli, P., & Huttner, W.B. (1985). Secretogranins I and II: Two tyrosine-sulfated secretory proteins common to a variety of cells secreting peptides by the regulated pathway. *J. Cell Biol. 101*, 1999–2011.

Settleman, J., Nolan, J., & Angeletti, R.H. (1985a). Chromogranin. An integral membrane protein. *J. Biol. Chem. 260*, 1641–1642.

Settleman, J., Nolan, J., Fonseca, R., & Angeletti, R.H. (1985b). Relationship of multiple forms of chromogranin. *J. Biol. Chem. 260*, 1645–1650.

Smith, A.D. & Winkler, H. (1967). Purification and properties of an acidic protein from chromaffin granules of bovine adrenal medulla. *Biochem. J. 103*, 483–492.

Smith, W.J. & Kirshner, N. (1967). A specific soluble protein from the catecholamine storage vesicles of bovine adrenal medulla. Purification and chemical characterization. *Mol. Pharmacol. 3*, 52–62.

Studier, F.W. & Moffat, B.A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high level expression of clones genes. *J. Mol. Biol. 189*, 113–130.

Studier, F.W., Rosenberg, A.H., & Dubendorff, J.W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol. 185*, 60–89.

Tatemoto, K., Efendic, S., Mutt, V., Makk, G., Feistner, G.J., & Barchas, J.D. (1986). Pancreastatin, a novel pancreatic peptide that inhibits insulin secretion. *Nature 324*, 476–478.

Videen, J.S., Mezger, M.S., Chang, Y.M., & O'Connor, D.T. (1992). Calcium and catecholamine interactions with adrenal chromogranins. *J. Biol. Chem. 267*, 3066–3073.

Wallace, R.W., Tallant, E.A., & Cheung, W.Y. (1980). Assay, preparation and properties of calmodulin. In *Calcium and Cell Function*, Vol. 1. *Calmodulin* (Cheung, W.Y., Ed.), pp. 13–40. Academic Press, New York.

Winkler, H. & Fischer-Colbrie, R. (1992). Commentary. The chromogranins A and B: The first 25 years and future perspectives. *Neuroscience 49*, 497–528.

Winkler, H. & Westhead, E. (1980). The molecular organization of chromaffin granules. *Neuroscience 5*, 1803–1823.

Wohlfarter, T., Fischer-Colbrie, R., Hogue-Angeletti, R., Eiden, L.E., & Winkler, H. (1988). Processing of chromogranin A within chromaffin granules starts at C- and N-terminal sites. *FEBS Lett. 231*, 67–70.

Yang, C. (1986). Prediction of protein structure. *Methods Enzymol. 130*, 185–273.

Yoo, S.H. & Albanesi, J.P. (1990). Ca^{++}-induced conformational change and aggregation of chromogranin A. *J. Biol. Chem. 265*, 14414–14421.

Yoo, S.H. & Albanesi, J.P. (1991). High capacity, low affinity Ca^{++} binding of chromogranin A. Relationship between the pH-induced conformational change and Ca^{2+} binding property. *J. Biol. Chem. 266*, 7740–7745.

Yoo, S.H. & Lewis, M.S. (1992). Effects of pH and Ca^{2+} on monomer–dimer and monomer–tetramer equilibria of chromogranin A. *J. Biol. Chem. 267*, 11236–11241.