Purification and pH-Dependent Secretory Vesicle Membrane Binding of Chromogranin B

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ABSTRACT: Chromogranins A and B have been suggested to play crucial roles in the sorting of vesicular matrix proteins into secretory vesicles during vesicle biogenesis. Chromogranin A (CGA), a high-capacity, low-affinity Ca^{2+}-binding protein, is the major protein in the secretory vesicles, while chromogranin B (CGB) is present in the vesicle at a significantly lower concentration. Chromogranin B has not been purified in its native form so far, thus severely limiting detailed studies of this protein. In the present study, chromogranin B was purified to complete homogeneity in its native state from the secretory vesicle lysates of bovine adrenal chromaffin cells using several chromatographic and electrophoresis steps. Recently, several intravesicular matrix proteins including chromogranins A and B have been shown to interact with the vesicle membrane at the intravesicular pH of 5.5 and to be released at a near-physiological pH of 7.5. However, since the experiment was done with the total vesicle lysate proteins, it was not clear whether CGB bound to the vesicle membrane directly or not. Hence, the pH-dependent binding of CGB to the vesicle membrane was tested using purified CGB, and it was found that pure CGB directly bound to the vesicle membrane at the intravesicular pH of 5.5. However, unlike the vesicle membrane-bound CGA, which can be easily eluted by a change of pH in the elution buffer from 5.5 to 7.5, the change of pH from 5.5 to 7.5 was not enough to elute the vesicle membrane-bound CGB. Its elution required a combination of both the pH change and a high salt concentration (1 M KCl), indicating that CGB had a higher affinity for the vesicle membrane than CGA. The tighter interaction of CGB with the vesicle membrane was further confirmed by vesicle membrane-coupled column chromatography using the mixture of CGA and CGB. In this regard, it appears that the high affinity of CGB for the membrane compensates its low concentration in the vesicle to ensure its interaction with the membrane, and the pH-dependent membrane binding of CGB may reflect the critical roles chromogranins A and B are suggested to play during vesicle biogenesis.

Chromogranins had orginally been discovered as the soluble matrix proteins of secretory vesicles of adrenal medullary chromaffin cells (Helle, 1966; Smith & Winkler, 1967; Smith & Kirschner, 1967; Blaschko et al., 1967). The secretory vesicles contain 500−600 mM catecholamine, 130 mM ATP, and 40 mM Ca^{2+} in addition to chromogranins and many other proteins and peptides (Winkler et al., 1986; Winkler & Westhead, 1980). Chromogranin A (CGA)[1] is the major matrix protein, constituting 40% of all the soluble matrix proteins of the secretory vesicles, and its high-capacity, low-affinity Ca^{2+}-binding property (Yoo & Albanesi, 1991) has been attributed to the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store role of the secretory vesicles (Yoo & Albanesi, 1990a). Subsequent studies indicated that chromogranin A undergoes pH- and Ca^{2+}-dependent conformational changes (Yoo & Albanesi, 1990b; Yoo & Ferretti, 1993), and exists in a predominantly dimeric state at a near-physiological pH of 7.5 and in a predominantly tetrameric state at the intravesicular pH of 5.5 (Yoo & Lewis, 1992).

Moreover, chromogranin A has been shown to bind to the vesicle membrane at the intravesicular pH of 5.5, but to be released at a near-physiological pH of 7.5 (Yoo, 1993a). In particular, chromogranin A interacted with membrane proteins, including the inositiol 1,4,5-trisphosphate receptor/Ca^{2+} channel (Yoo, 1994). This pH-dependent vesicle membrane-binding property of CGA is considered critical for an appropriate sorting of intravesicular matrix proteins and the vesicle membrane proteins to secretory vesicles during secretory vesicle biogenesis (Yoo, 1993a,b). Our recent studies indicated that several intravesicular matrix proteins besides CGA also interact with the vesicle membrane at pH 5.5 (Yoo, 1993b). One of the membrane-interacting matrix proteins was identified as chromogranin B (Yoo, 1993b). Since several matrix proteins, including CGB, are known to interact with CGA at the intravesicular pH 5.5 (Yoo, 1995a), it was not clear whether CGB bound directly to the vesicle membrane or interacted with the vesicle membrane through its binding to CGA which in turn bound to the vesicle membrane. In previous studies, chromogranins A and B have been shown to aggregate in a Ca^{2+}- and pH-dependent manner, aggregating at acidic pH levels in the presence of Ca^{2+} (Gorr et al., 1989; Yoo & Albanesi, 1990b; Chanat & Huttner, 1991; Yoo, 1995b). This Ca^{2+}- and pH-dependent aggregation property of chromogranins A and B is considered to be an essential feature for the proper sorting of the chromogranin aggregation complex (Gerdes et al., 1989; Gorr et al., 1989; Yoo & Albanesi, 1990b; Chanat &

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[1] Abbreviations: CGA, chromogranin A; CGB, chromogranin B; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

Huttner, 1991). Furthermore, the binding of the vesicular matrix protein complex to the vesicle membrane is suggested to be critical for a successful sorting of these matrix proteins into secretory vesicles during vesicle biogenesis (Huttner et al., 1988, 1991; Yoo, 1993a,b). Hence, in view of the potential physiological importance the pH-dependent membrane binding by chromogranins might have during vesicle biogenesis (Yoo, 1993a,b), it was of considerable importance to discern the nature of CGB binding to the vesicle membrane.

To address this question, it was necessary to test the possibility of direct pH-dependent membrane binding by purified CGB. Accordingly, chromogranin B was purifed from the secretory vesicles of adrenal medullary chromaffin cells, and its membrane-binding property was studied. Although purified CGB has previously been obtained either by elution from SDS−polyacrylamide gels (Benedum et al., 1987; Fischer-Colbrie & Schober, 1987; Gorr et al., 1989; Gill et al., 1991) or by employing a boiling step (Fischer-Colbrie & Schober, 1987) which is known to change the native conformation of chromogranin A (Yoo & Albanesi, 1990b), chromogranin B has never been purified in its native state; thus, the present result represents the first purification of chromogranin B in its native state. It was further demonstrated in the present study that purified CGB directly interacted with the vesicle membrane at pH 5.5, as had been the case with chromogranin A (Yoo, 1993a). In addition, the pH-dependent membrane-binding property of CGB was compared with that of CGA, and its physiological implications are discussed.

EXPERIMENTAL PROCEDURES

Materials. DE-52, phenyl-Sepharose, and cyanogen bromide (CNBr)-activated Sepharose 4B were from Pharmacia LKB Biotechnology Inc. Molecular size standards, polyacrylamide, and Tris/glycine and Tris/glycine/SDS electrophoresis buffers were from Bio-Rad. EGTA and EDTA were from Sigma.

Secretory Vesicle Lysate and Vesicle Membrane Preparation. To obtain the secretory vesicle lysates and the vesicle membrane, the secretory vesicles from bovine adrenal chromaffin cells were prepared as described previously (Yoo & Albanesi, 1990a). The vesicles were lysed by resuspending in 40 volumes of 15 mM Tris-HCl, pH 7.5, and frozen and thawed twice. The vesicle lysates and vesicle membranes were prepard from this lysate as described previously (Yoo, 1993a).

DE-52 Column Chromatography. A 595 mg sample of vesicle lysate proteins was loaded onto a DE-52 column (5 × 15 cm) equilibrated with 10 mM imidazole, pH 6.1. Elution was carried out with a 650 mL KCl gradient (0.2−0.4 M) in 10 mM imidazole, pH 6.1. The flow rate was 80 mL/h, and 10.2 mL fractions were collected. Fractions eluted at 0.27−0.34 M KCl were pooled and subjected to phenyl-Sepharose column chromatography.

First Phenyl-Sepharose Column Chromatography. The pooled DE-52 fractions were adjusted to 0.3 M $(NH_4)_2SO_4$, 15 mM Tris-HCl, pH 7.5, and 0.2 mM $CaCl_2$, and half (150 mg) of the proteins was loaded on a phenyl-Sepharose column (2.5 cm × 8.5 cm). The proteins were eluted with a 300 mL reverse gradient of $(NH_4)_2SO_4$ (0.3 M−0 M) in 15 mM Tris-HCl, pH 7.5, 0.2 mM $CaCl_2$. The flow rate was 150 mL/h, and 12 mL fractions were collected. An additional 200 mL of buffer with no ammonium sulfate was added to complete the elution. Chromogranin B-containing fractions obtained from the trailing end of the peak were pooled for a second fractionation on the same column.

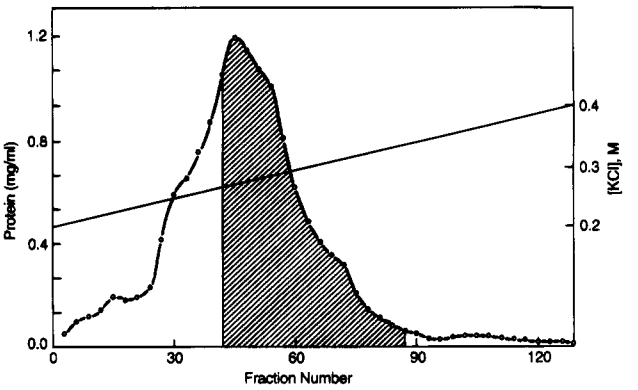

FIGURE 1: DE-52 column chromatography of the vesicle lysate proteins. The vesicle lysate proteins were directly loaded onto a DE-52 column (5 × 15 cm) and eluted with 0.2−0.4 M KCl in 10 mM imidazole, pH 6.1. Proteins eluted at 0.27−0.34 M KCl were pooled (shaded area) for further purification on a phenyl-Sepharose column.

Second Phenyl-Sepharose Column Chromatography. Half (64 mg) of the pooled proteins from the first phenyl-Sepharose chromatography step was adjusted to 50 mM $(NH_4)_2SO_4$ in 15 mM Tris-HCl, pH 7.5, 0.2 mM $CaCl_2$, and loaded onto the same phenyl-Sepharose column. The proteins were eluted with a 200 mL reverse gradient of $(NH_4)_2SO_4$ (50 mM−0 mM) in the same buffer. The flow rate was 120 mL/h, and 10 mL fractions were collected. An additional 200 mL of buffer with no ammonium sulfate was added to complete the elution. Fractions eluted at 5 mM−0 mM $(NH_4)_2SO_4$ were pooled for further fractionation by continuous elution electrophoresis.

Preparative Continuous Elution Electrophoresis. The CGB-containing fractions from the second phenyl-Sepharose chromatography step were further separated by continuous elution electrophoresis on a 5% native polyacrylamide gel using a Model 491 Prep Cell of Bio-Rad. Sixteen milligrams of proteins in 18 mL of 80 mM Tris-HCl, pH 7.5, 4 mM EDTA, 8% glycerol, and 0.025% bromphenol blue was loaded onto a 5% native gel tube (6 cm long, 37 mm i.d.) and subjected to continuous elution electrophoresis at 4 °C. The eluted fractions were collected, and the protein content in each fraction was individually determined according to Bradford (1976).

Native PAGE and Electroelution. One-half milligram of proteins in 4.2 mL of 45 mM Tris-HCl, pH 7.5, 8% glycerol, 2 mM EGTA, and 0.025% bromphenol blue was loaded onto a 7% native preparative polyacrylamide slab gel (3 mm × 12 cm) and electrophoresed. The location of the intact CGB band was identified by staining the lanes at both ends of the gel in Coomassie blue after the completion of electrophoresis. The intact CGB band was cut out, and the protein was electroeluted in Tris/glycine electrophoresis buffer using a Bio-Rad Model 422 electroeluter at 4 °C.

Coupling the Secretory Vesicle Membrane to CNBr−Sepharose 4B. Coupling of the vesicle membrane to the CNBr-activated Sepharose 4B was done as described previously (Yoo, 1993a).

Chromogranin A Preparation. Chromogranin A was purified as described previously (Yoo & Albanesi, 1990b) using the vesicle lysates (see above) as the starting material.

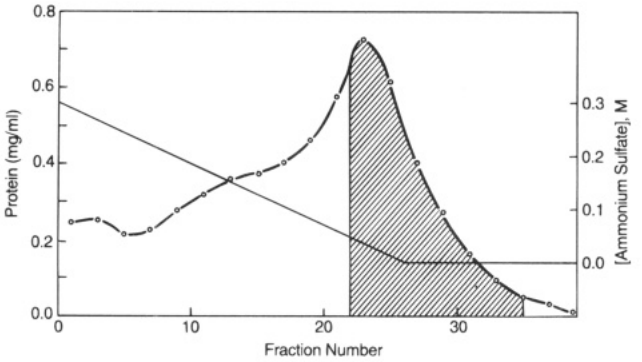

FIGURE 2: First phenyl-Sepharose column chromatography of the vesicle lysate proteins. The pooled protein fractions from the DE-52 column were loaded on a phenyl-Sepharose column (2.5 cm × 8.5 cm) and eluted with 0.3−0 M $(NH_4)_2SO_4$ buffer in 15 mM Tris-HCl, pH 7.5, 0.2 mM $CaCl_2$. Proteins eluted at 40−0 mM $(NH_4)_2SO_4$ were pooled (shaded area) and subjected to a second phenyl-Sepharose column chromatography.

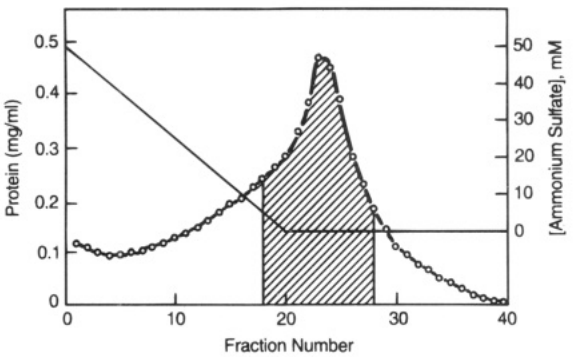

FIGURE 3: Second phenyl-Sepharose column chromatography of the vesicle lysate proteins. The chromogranin B-containing fractions from the first phenyl-Sepharose column chromatography were adjusted to 50 mM $(NH_4)_2SO_4$ in 15 mM Tris-HCl, pH 7.5, 0.2 mM $CaCl_2$, and loaded on the same phenyl-Sepharose column. The proteins were eluted with a reverse gradient of $(NH_4)_2SO_4$ (50−0 mM) in the same buffer, and fractions eluted at 5 mM $(NH_4)_2SO_4$ or lower were pooled (shaded area) for further purification.

RESULTS

Purification. Soluble proteins from the vesicle lysates were fractionated on a DE-52 column as shown in Figure 1. Fractions containing chromogranin B at the trailing edge of the peak (shaded area) were pooled. This pool was fractionated by hydrophobic chromatography on phenyl-Sepharose (Figure 2). Chromogranin B was eluted at the end of a reverse $(NH_4)_2SO_4$ gradient, indicating a strong hydrophobic interaction with the resin. Appropriate fractions containing CGB were pooled for a second chromatography on the same phenyl-Sepharose column (Figure 3) in a narrower range of reverse $(NH_4)_2SO_4$ gradient. Chromogranin B started to be eluted at approximately 5 mM $(NH_4)_2SO_4$ and the majority of CGB was eluted at zero $(NH_4)_2SO_4$ concentration, reflecting its strong hydrophobic interaction with the resin. The chromogranin B-containing fractions were pooled (shaded area in Figure 3) and electrophoresed on a native 5% polyacrylamide gel using a Model 491 Prep Cell (Bio-Rad) for a continuous elution electrophoresis. As indicated in Figure 4 by the shaded area, chromogranin B was eluted ahead of CGA which was represented by the trailing large peak. Considering that CGA exists in a dimeric state at pH 7.5 (Yoo & Lewis, 1992), elution of CGB ahead of dimeric CGA on the native polyacrylamide gel indicates a monomeric existence of CGB.

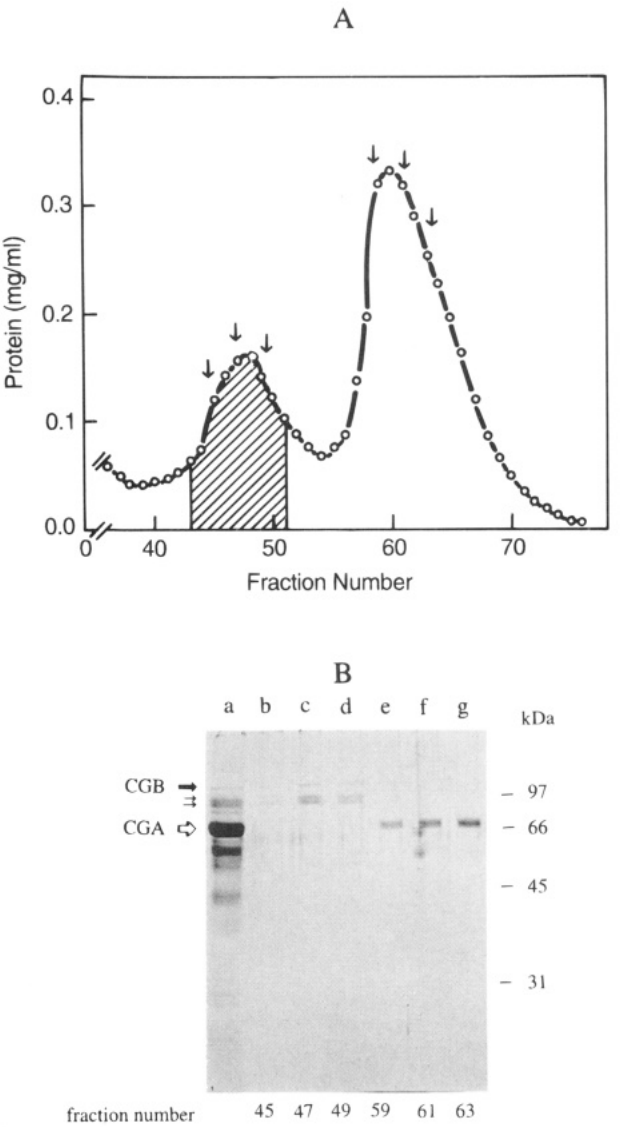

FIGURE 4: Preparative continuous elution electrophoresis. (A) Sixteen milligrams of pooled protein fractions from the second phenyl-Sepharose chromatography in 18 mL of 80 mM Tris-HCl, pH 7.5, 4 mM EGTA, 8% glycerol, and 0.025% bromphenol blue was loaded on a 5% native tube gel of a Model 491 prep cell (6 cm long, 37 mm i.d.) (Bio-Rad), and fractionated by continuous elution electrophoresis. The arrows indicate the fractions from which 5−10 μL aliquots were taken for SDS−PAGE in (B). (B) The eluted protein fractions were analyzed on a 10% SDS−polyacrylamide gel by loading 5−10 μL aliquots from appropriate fractions. Lane a, vesicle lysate (6 μg); lanes b−d, fractions 45, 47, and 49 (10 μL each); lanes e−g, fractions 59, 61, and 63 (5 μL each). Pure CGB, a mixture of intact CGB (indicated by a thick arrow) and CGB fragments without C-terminal ends (two thin arrows), was eluted as a small peak (shaded area) ahead of CGA (open arrow), which is supposed to be in a dimeric state and trailed CGB as a large peak.

The pooled CGB fractions in Figure 4A represented pure CGB as shown in Figure 4B. However, these fractions contained not only intact CGB but also CGB fragments without the C-terminal regions as the isolation and sequencing of these two CGB fragments showed the N-terminal sequences of MPVDIR, identical to that of bovine CGB (Bauer & Fischer-Colbrie, 1991). The mixture of intact CGB and CGB fragments without the C-terminal regions was further fractionated by electrophoresis on a 7% native polyacrylamide gel. The CGB fragments without the C-terminal ends moved faster than the intact CGB on the native polyacrylamide gel (not shown). The slow-moving band

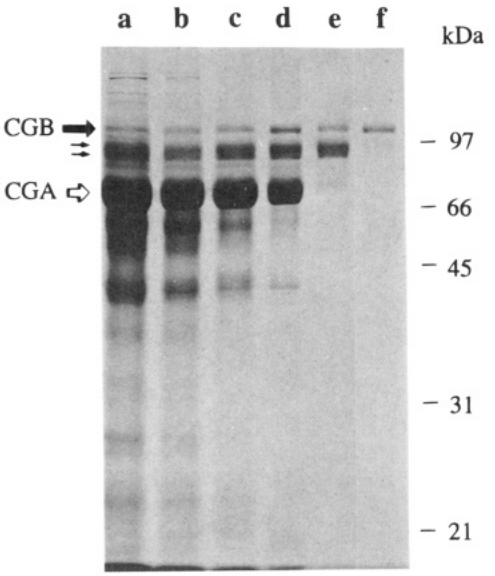

FIGURE 5: SDS−PAGE of chromogranin B fractions. The lysate proteins from each purification step were visualized on a 10% SDS−polyacrylamide gel: vesicle lysate protein (14 μg, lane a); protein after the DE-52 step (9.5 μg, lane b); protein after the first phenyl-Sepharose step (5 μg, lane c), protein after the second phenyl-Sepharose step (3.5 μg, lane d); CGB after continuous elution electrophoresis (1.5 μg, lane e); purified intact CGB after the native PAGE step (0.6 μg, lane f). Intact CGB and two CGB fragments are indicated by a thick arrow and two thin arrows, respectively. Chromogranin A is indicated by an open arrow.

Table 1: Summary of Chromogranin B Purification from the Secretory Vesicle Lysate of Bovine Adrenal Medulla

	total protein (mg)	% recovery	% purity	chromogranin (mg)
vesicle lysate	595	100	1.8	10.71
DE-52	299	84.4	3.0	9.04
first phenyl- Sepharose	128	57.1	4.8	6.11
second phenyl-Sepharose	32.8	42.9	14	4.59
Model 491 prep cell	11.8	34.3	31	3.67
native PAGE	2.60	24.3	100	2.60

containing intact CGB was cut out, and the intact CGB was electroeluted. This gave pure intact CGB as shown in lane f of Figure 5. The purification procedures are summarized in Table 1. Starting with approximately 595 mg of total intravesicular lysate proteins, 2.6 mg of completely homogeneous intact CGB was obtained.

pH-Dependent Vesicle Membrane Binding. The pooled CGB fractions containing purified CGB (the mixture of intact CGB and two CGB fragments with cleaved C-terminal ends) from Figure 4 were dialyzed extensively against 20 mM sodium acetate, pH 5.5, 0.1 M KCl, and this mixture of intact CGB and CGB fragments was used in the study of pH-dependent vesicle membrane binding by chromogranin B. To determine whether purified CGB can bind to the vesicle membrane at the intravesicular pH of 5.5, 65 μg of the above CGB preparation was applied to the vesicle membrane-coupled Sepharose 4B column (0.6 mL volume) equilibrated with 20 mM sodium acetate, pH 5.5, 0.1 M KCl. As shown in Figure 6, elution of the column with a 1 M KCl buffer released a small amount of protein from the column, suggesting the possibility that CGB bound to the vesicle membrane at pH 5.5. Further elution of the same column with a buffer of pH 7.5 instead of 5.5 released a significantly larger amount of protein from the column, apparently indicating that the change of pH from 5.5 to 7.5 caused the

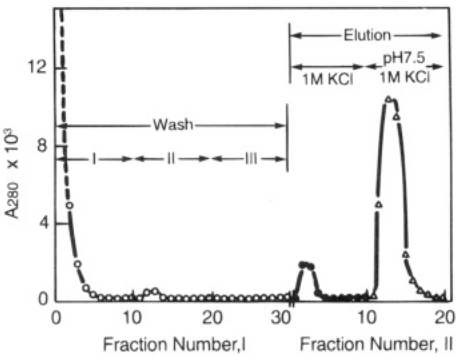

FIGURE 6: Vesicle membrane-coupled Sepharose 4B chromatography of chromogranin B. 65 μg of chromogranin B (the mixture of intact CGB and CGB fragments) in 0.3 mL of 20 mM sodium acetate, pH 5.5, 0.1 M KCl was loaded onto a vesicle membrane-coupled Sepharose 4B column (0.6 mL volume) equilibrated with 20 mM sodium acetate, pH 5.5, 0.1 M KCl. The column was washed with 0.1 M KCl (Wash I), followed by 0.15 M KCl (Wash II) and 0.1 M KCl (Wash III), all in 20 mM sodium acetate, pH 5.5. The protein then was first eluted with 1 M KCl in the same acetate buffer. After the high-salt elution, the pH of the elution buffer was changed to 7.5 (20 mM Tris-HCl, pH 7.5, 1 M KCl), and the elution was continued. The fraction size was 1 mL/fraction for fraction I and 0.5 mL/fraction for fraction II, and the chromatography was carried out at room temperature.

dissociation of the bound proteins. The amount of protein eluted was ~19 μg, accounting for 29% of the loaded CGB.

To analyze the content of the proteins released from the column, the eluted protein fractions were analyzed by SDS−PAGE. As shown in Figure 7, there was virtually no CGB in the fractions eluted with the 1 M KCl buffer with no change in pH, but a significant amount of CGB was present in the fractions eluted with the pH 7.5−1 M KCl buffer. This result indicated that CGB bound to the vesicle membrane at pH 5.5 and was released from it at pH 7.5. The pH-dependent binding of CGB appeared to be a specific interaction between the vesicle membrane and CGB for the CNBr-activated Sepharose 4B resin that had gone through all the coupling routine but without the vesicle membrane was not able to bind any CGB.

Since it has been previously shown that a large portion of the bound CGA can be released from the vesicle membrane-coupled columns by changing the pH of the elution buffer to 7.5 without changing the salt concentration (Yoo, 1993a), it was of interest to determine whether CGB can also be released from the vesicle membrane-coupled column by changing the pH from 5.5 to 7.5. Hence, 130 μg of CGB was loaded onto the vesicle membrane-coupled Sepharose 4B column, washed, and eluted with a pH 7.5 buffer as shown in Figure 8. Elution with a pH 7.5 buffer alone released only a small amount of protein. However, elution of the same column with the pH 7.5−1 M KCl buffer released a large amount of protein, indicating that the pH change alone is not sufficient to release the bound protein. The analysis of the eluted proteins by SDS−PAGE as shown in Figure 9 shows that little or no bound CGB was eluted by the pH 7.5 buffer alone, but a large amount of CGB was released by the pH 7.5−1 M KCl buffer, implying a stronger interaction between CGB and the vesicle membrane than that between CGA and the vesicle membrane. The amount of protein eluted was 42−43 μg, accounting for approximately 33% of the loaded CGB. Given that the present column used had 0.6 mL (wet volume) of resin with 0.39 mg of coupled membrane protein, this result indicates that 0.39 mg of

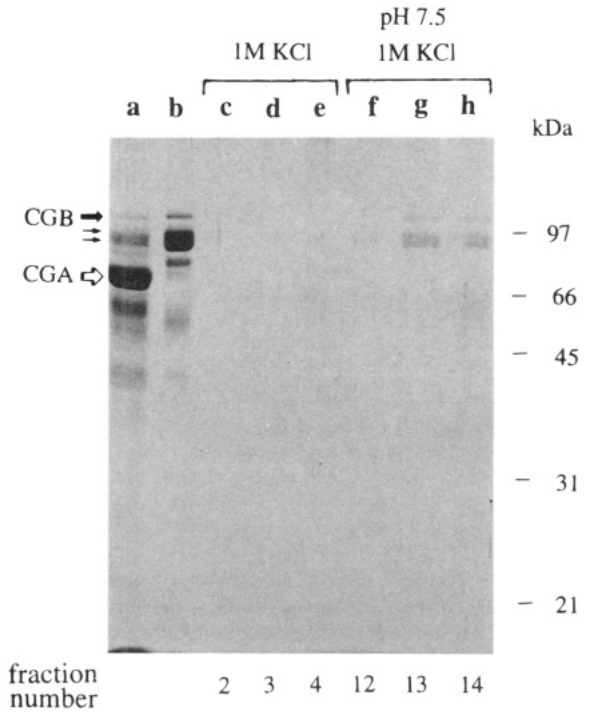

FIGURE 7: SDS−PAGE of the eluted proteins from the vesicle membrane-coupled Sepharose 4B chromatography. The eluted fractions were diluted 5-fold with water to reduce the salt concentration and concentrated 25-fold using a Centricon-10 (Amicon, Beverly, MA). 25 μL aliquots from fraction II of Figure 6 are analyzed on a 10% SDS−polyacrylamide gel (Laemmli, 1970). Lane a, preloading vesicle lysate (6 μg); lane b, mixture of intact CGB and CGB fragments (2.5 μg); lanes c, d, and e, fractions 2, 3, and 4; lanes f, g, and h, fractions 12, 13, and 14 of fraction II, Figure 6. Intact CGB and two CGB fragments are indicated by a thick arrow and two thin arrows, respectively. Chromogranin A is indicated by an open arrow.

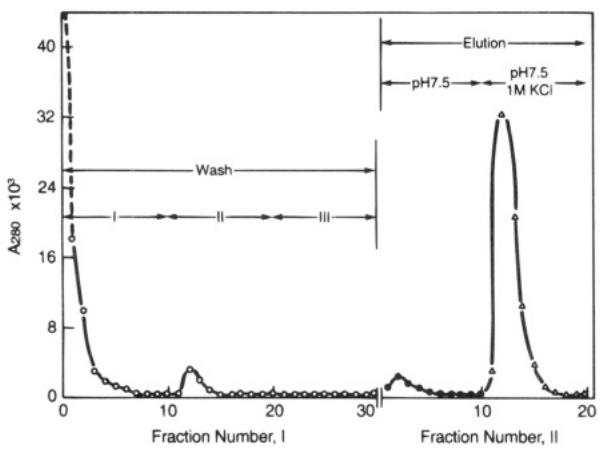

FIGURE 8: Vesicle membrane-coupled Sepharose 4B chromatography of chromogranin B. 130 μg of chromogranin B (the mixture of intact CGB and CGB fragments) in 0.3 mL of 20 mM sodium acetate, pH 5.5, 0.1 M KCl was loaded onto a vesicle membrane-coupled Sepharose 4B column (0.6 mL volume) equilibrated with 20 mM sodium acetate, pH 5.5, 0.1 M KCl. The chromatography was carried out as described in Figure 6 except that the first elution after the washing step was carried out with a pH 7.5 buffer (20 mM Tris-HCl, pH 7.5) with no added salt. The second elution was carried out with 1 M KCl in 20 mM Tris-HCl, pH 7.5. Other conditions are the same as for Figure 6.

coupled membrane protein bound and released 42−43 μg of CGB.

In order to determine the potential difference between CGA and CGB in their interaction with the vesicle membrane, 20 μg of purified CGA was mixed with 100 μg of

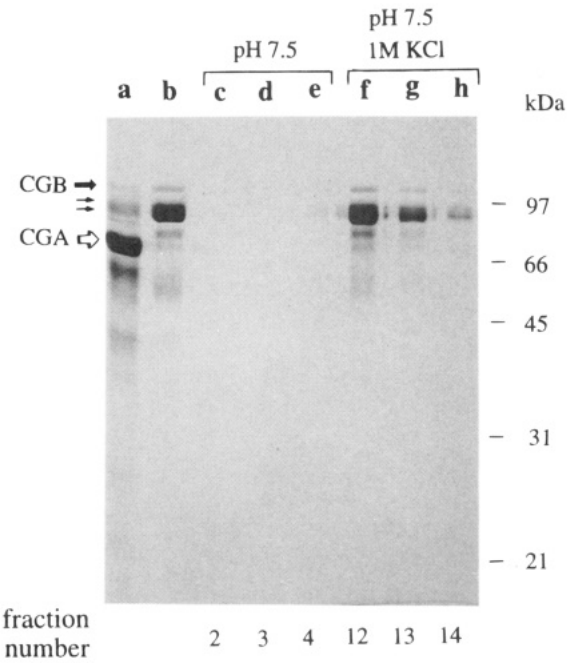

FIGURE 9: SDS−PAGE of eluted chromogranin B from the vesicle membrane-coupled Sepharose 4B chromatography. The eluted fractions were diluted 5-fold with water to reduce the salt concentration and concentrated 25-fold. 25 μL aliquots from fraction II of Figure 8 are analyzed on a 10% SDS−polyacrylamide gel (Laemmli, 1970). Lane a, preloading vesicle lysate (6 μg); lane b, mixture of intact CGB and CGB fragments (2.5 μg); lanes c, d, and e, fractions 2, 3, and 4; lanes f, g, and h, fractions 12, 13, and 14 of fraction II, Figure 8. Other conditions are the same as for Figure 7.

the purified CGB and loaded onto the vesicle membrane-coupled column. After the usual washing, the column was eluted with the 1 M KCl buffer without changing the pH. As shown in Figure 10, the 1 M KCl buffer released a small amount of protein from the column, which was shown to consist primarily of CGA and some CGB fragments (see lanes c−f, Figure 11). It is noteworthy that little or no intact CGB was eluted, consistent with the result in Figure 7. However, further elution of the same column with the pH 7.5−1 M KCl buffer eluted a larger additional amount of protein, which included intact CGB along with the CGB fragments and CGA (lanes g−j, Figure 11), thereby establishing the fact that CGB interacts with the vesicle membrane significantly more strongly than CGA. The amount of protein bound and released was ~40 μg.

DISCUSSION

In view of the observation that several intravesicular matrix proteins including chromogranin B interacted with the vesicle membrane at pH 5.5 (Yoo, 1993b), it was not clear whether CGB interacted with the vesicle membrane directly or not. In this regard, the present results clearly demonstrate the fact that CGB directly interacts with the vesicle membrane at the intravesicular pH of 5.5, but is freed from it at a near-physiological pH of 7.5. However, unlike the results obtained with purified CGA in which most of the vesicle membrane-bound CGA was released by the pH change of the elution buffer from 5.5 to 7.5 (Yoo, 1993a), the results in Figures 8 and 9 show that the pH change alone is not sufficient to elute the bound CGB from the vesicle membrane-coupled column. This implied a potentially stronger affinity of CGB for the vesicle membrane than CGA and also contrasted with the previous observation which showed the

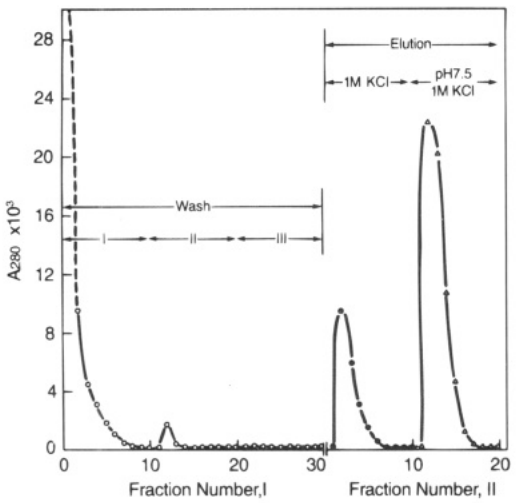

FIGURE 10: Vesicle membrane-coupled Sepharose 4B chromatography of the mixture of chromogranin A and chromogranin B. 120 μg of chromogranins A and B (20 μg of CGA was mixed with 100 μg of CGB) in 0.4 mL of 20 mM sodium acetate, pH 5.5, was loaded onto a vesicle membrane-coupled Sepharose 4B column (0.6 mL volume) equilibrated with 20 mM sodium acetate, pH 5.5, 0.1 M KCl. The column was washed with 0.1 M KCl (Wash I), followed by 0.15 M KCl (Wash II) and 0.1 M KCl (Wash III), all in 20 mM sodium acetate, pH 5.5. The protein then was first eluted with 1 M KCl in the same acetate buffer. After the high-salt elution, the pH of the elution buffer was changed to 7.5 (20 mM Tris-HCl, pH 7.5, 1 M KCl) and the elution was continued. The fraction size was 1 mL/fraction for fraction I and 0.5 mL/fraction for fraction II, and the chromatography was carried out at room temperature.

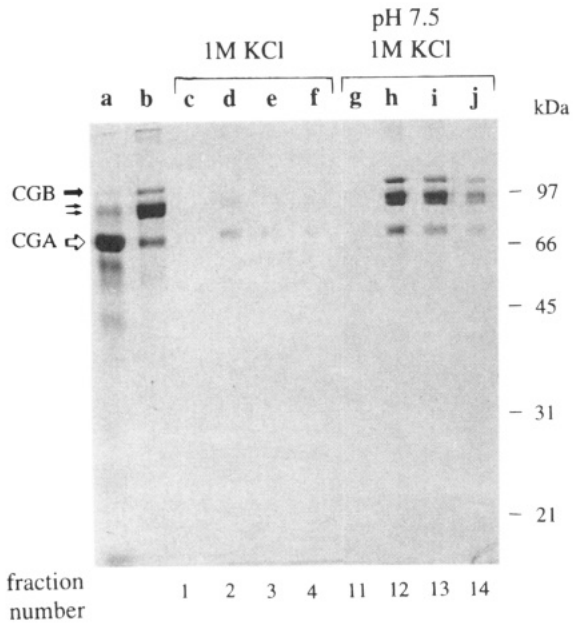

FIGURE 11: SDS−PAGE of the eluted chromogranins A and B from vesicle membrane-coupled Sepharose 4B chromatography. The eluted fractions were diluted 5-fold with water to reduce the salt concentration and concentrated 25-fold. 25 μL aliquots from fraction II of Figure 10 are analyzed on a 10% SDS−polyacrylamide gel. Lane a, preloading vesicle lysate (5 μg); lane b, mixture of CGA and CGB (2.5 μg); lanes c, d, e, and f, fractions 1, 2, 3, and 4; lanes g, h, i, and j, fractions 11, 12, 13, and 14 of fraction II, Figure 10. Other conditions are the same as for Figure 7.

elution of most of the bound intravesicular matrix proteins, including chromogranins A and B, by the same pH 7.5 buffer (Yoo, 1993b). In view of the fact that the previous experiments had been done with total vesicle lysate proteins compared to purified CGB of the present experiments, it appears that other intravesicular matrix proteins, especially CGA, contributed to the dissociation of CGB from the vesicle membrane at pH 7.5. In particular, given that CGB interacts with CGA at pH 5.5 and dissociates from it at pH 7.5 (Yoo, 1995a) and a large portion of the vesicle membrane-bound CGA can be eluted by the pH change alone (Yoo, 1993a,b), the elution of CGB, along with other matrix proteins, by the pH 7.5 buffer appears to be due to the interaction of CGB with CGA in the vesicle lysate. This could also explain why some of the CGB fragments without the C-terminal ends were eluted by the pH 7.5 buffer from the membrane-coupled column which had been loaded with the mixture of CGA and CGB (Figure 11) while virtually no intact CGB or CGB fragments were eluted from the column loaded with CGB only (Figure 9).

Tighter interaction of CGB with the vesicle membrane than CGA had in fact been implied before (Yoo, 1993b); when the secretory vesicles were lysed in 10 volumes of buffer at pH 5.5, more than 80% of CGB remained bound to the vesicle membrane while a little more than 50% of CGA remained bound to the vesicle membrane [cf. Figure 5 of Yoo (1993b)], suggesting a relatively stronger CGB interaction with the vesicle membrane. Combined with the fact that neither the 1 M KCl buffer (Figure 7) nor the pH 7.5 buffer alone (Figure 9) was able to eulte either intact CGB or the CGB fragments without the C-terminal ends from the column, the results in Figure 11 clearly indicate that intact CGB interacts with the vesicle membrane significantly more tightly than CGA at pH 5.5. In addition, considering that chromogranin A exists in a dimeric state at pH 7.5 and in a tetrameric state at pH 5.5 (Yoo & Lewis, 1992), and chromogranin B appears to exist only in a monomeric state as it migrates ahead of dimeric CGA on native PAGE (Figure 4), it is probable that CGB interacts with the vesicle membrane in a monomeric state, unlike the interaction of tetrameric CGA. Moreover, in view of the fact the CGB concentration in the vesicle is only a fraction of the CGA concentration, it appears likely that the stronger interaction by CGB will enable the relatively lower concentration of CGB to effectively compete with dominant CGA in the vesicle to come in contact with the vesicle membrane, thus ensuring CGB binding to the vesicle membrane. Nevertheless, given that most of the vesicle membrane-bound matrix proteins, including chromogranins A and B, were released by the change of pH from 5.5 to 7.5 (Yoo, 1993b), it appears certain that the stronger affinity of CGB for the vesicle membrane does not prevent CGB from being released into the bloodstream during exocytosis.

Moreover, in light of the suggestion that the conserved near N-terminal region [residues 17(18)−36(37)] of chromogranins A and B might serve as the anchor region for interaction with the vesicle membrane (Yoo, 1993a,b), the binding of the two CGB fragments without the C-terminal regions to the vesicle membrane is consistent with the idea of interaction of chromogranins A and B through the anchor role of the conserved near-N-terminal region. In the case of the chromogranin A structure, the N-terminal side of the molecule forms a compact structure complemented by the disulfide bond formed in the conserved near-N-terminal region while the C-terminal side is considered to be structurally loose and more exposed than the N-terminal side (Yoo & Ferretti, 1993). As had been proposed with CGA (Yoo, 1993a), it may be that the conserved near-N-terminal region of CGB also is extended to the vesicle membrane at the

intravesicular pH of 5.5 (Yoo, 1993b), thus interacting with the vesicle membrane components until the pH level changes to that of the extracellular fluid during exocytosis, which is likely to cause withdrawal of the anchor region.

In light of the proposed roles of chromogranins A and B during vesicle biogenesis, the physical contact between the aggregated chromogranins and the potential vesicle membrane in the trans-Golgi network where secretory vesicle biogenesis occurs is a crucial step (Huttner et al., 1988, 1991; Yoo, 1993a,b). Accordingly, the pH-dependent interaction of CGB with the vesicle membrane underlines the proposed model (Yoo, 1993b) which suggested the pH-dependent interaction between chromogranins and the vesicle membrane as a crucial step in vesicle biogenesis. In our recent study, the purifed native CGB was shown to aggregate at a significantly lower Ca^{2+} concentration than was required for CGA aggregation at the intravesicular pH of 5.5 (Yoo, 1995b), indicating a significantly higher Ca^{2+} sensitivity of CGB aggregation than that of CGA. The higher Ca^{2+} sensitivity for aggregation and the stronger interaction of CGB with the vesicle membrane appear to suggest that CGB, despite its low concentration in the vesicle compared to that of CGA, might play active roles in joining the early phase of the intravesicular matrix complex to the potential vesicle membrane in the trans-Golgi network. Furthermore, the differences in the Ca^{2+} sensitivity for aggregation and in the vesicle membrane binding affinity of CGA and CGB might contribute to the nonuniform distribution of CGA and CGB in different secretory vesicles of different cell types (Schmid et al., 1989; Lahr et al., 1992). For example, in human tissues, CGB is costored with CGA in the adrenal medulla and the anterior pituitary, whereas only CGA is found in the parathyroid gland. On the other hand, no immunostaining for CGA and only weak immunostaining for CGB were observed in some cells of the spinal cord of the nervous system while the Purkinje cells of the cerebellum were strongly positive for CGB (Schmid et al., 1989). In this regard, the purification of native CGB would be the first step toward a better understanding of the potential roles CGB might play in the cell, i.e., in vesicle biogenesis, in the storage of hormones and ions, and as precursors of other bioactive peptides.

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