Chromogranins A and B, two widespread neuroendocrine secretory proteins, contain a homologous N-terminal disulfide-bonded loop that is required for sorting to secretory granules. Here we have investigated the role of this loop in the oligomerization of chromogranin A. Reduction of the disulfide bond or the addition of an excess of an N-terminal chromogranin A fragment containing the loop (CgA1–60) resulted in the dissociation into monomers of the chromogranin A dimer found at pH 7.4 and 6.4 and of the chromogranin tetramer found at pH 5.4. The addition of an excess of a synthetic peptide corresponding to the conserved C-terminal domain of chromogranin A (CgA306–431) had no effect on the chromogranin dimers at pH 7.4 and 6.4 and resulted in the dissociation of the chromogranin A tetramers at pH 5.4 into dimers. Fluorescence energy transfer experiments using fluorescently labeled CgA1–60 showed that the N-terminal disulfide-bonded loop has a high affinity for homodimerization (KD = 20 nM at pH 6.4), which was sufficient to mediate dimerization of full-length chromogranin A. Association and dissociation of loop-mediated chromogranin A dimerization approached completion within a few seconds. Our results imply that chromogranin A homodimerizes shortly after synthesis in the endoplasmic reticulum and that the loop-mediated homodimeric state is an essential prerequisite for its sorting, in the trans-Golgi-network, to secretory granules.

Protein secretion is a fundamental cellular function. Virtually all eukaryotic cells secrete proteins in parallel with their synthesis via the constitutive pathway. Certain cells such as exocrine cells, endocrine cells, and neurons in addition secrete proteins via the regulated pathway; they store a subset of their secretory proteins in a specialized vesicular organelle, the secretory granules. Here we have investigated the role of a homologous disulfide-bonded homodimeric loop that is required for sorting to secretory granules. In the course of their conversion to mature secretory granules, immature secretory granules give rise to vesicles thought to mediate constitutive-like secretion. Protein sorting takes place at both the level of the TGN and the level of immature secretory granules (1–3).

Concerning the sorting of secretory proteins at the level of the TGN, two important features have been elucidated. One is the selective aggregation of regulated secretory proteins in the lumen of the TGN, which is a means of segregating them from constitutive secretory proteins (4–7). The model proteins in many of these studies have been the granins (chromogranins, secretogranins), a family of regulated secretory proteins that are widespread constituents of neuroendocrine secretory granules (8–9). Their aggregation is promoted by multivalent cations and low pH, two parameters of the TGN lumenal milieu (7, 10).

The second feature concerns discrete structures in regulated secretory proteins essential for sorting. N-terminal disulfide-bonded loops have been identified as such structures in chromogranin B (CgB) (11) and pro-opiomelanocortin (12, 13). In the case of pro-opiomelanocortin, the N-terminal disulfide-bonded loop has been shown to bind to the membrane-associated form of carboxypeptidase E (14). This interaction has been implicated in sorting (14, 15), although the claim of an essential role of CPE in sorting is controversial (16).

In the case of CgB, reduction of the disulfide bond is sufficient to cause complete missorting to constitutive secretory vesicles (11). Understanding the molecular mechanism underlying this missorting requires determination whether or not the disulfide-bonded loop participates in an intermolecular interaction and, if so, the identification of the interacting molecule. Chromogranin A (CgA), another member of the granin family, contains an N-terminal disulfide-bonded loop highly homologous to that of CgB (17), which as in the case of CgB is encoded by a separate exon (18–20). Taking advantage of the bovine adrenal medulla as a suitable source for the purification of CgA, we demonstrate here that the disulfide-bonded loop plays the crucial role for oligomerization of CgA, strongly suggesting a link between chromogranin oligomerization and sorting.

MATERIALS AND METHODS

Purification of CgA

All steps were performed at 4 °C. Chromaffin granule pellets, obtained from bovine adrenals as described by Bartlett and Smith (21), were resuspended in a buffer containing 20 mM MES, pH 6.4, 20 mM CaCl2, 0.5 mM phenylmethylsulfonyl fluoride, and 100 μg/ml leupeptin and lyzed by freeze-thawing and sonication. The acidic pH and the high calcium concentration were chosen to maintain CgB, secretogranin II, and other soluble proteins in an aggregated state, while most of the CgA was found to be soluble under these conditions. The lysate was centrifuged at 100,000 × g for 30 min, and the supernatant (80 ml, 10 mg/ml formed, constitutive secretory vesicles and immature secretory granules. In the course of their conversion to mature secretory granules, immature secretory granules give rise to vesicles thought to mediate constitutive-like secretion. Protein sorting takes place at both the level of the TGN and the level of immature secretory granules (1–3).

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The abbreviations used are: TGN, trans-Golgi network; CgA, chromogranin A; CgB, chromogranin B; DTT, dithiothreitol; FRET, fluorescence resonance energy transfer; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; MES, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; BSA, bovine serum albumin;
protein) was supplemented with 40 mM EDTA and brought to pH 7.4 with NaOH. After extensive dialysis against 50 mM sodium phosphate, pH 7.4, proteins were bound to an anion exchange column (Pharmacia Q-Sepharose, 26 × 100 mm) and eluted using a gradient of 0–1 M NaCl in 50 mM sodium phosphate, pH 7.4. The fractions containing CgA (as determined by SDS-PAGE) were pooled, and CgA was further purified by gel filtration (Superdex 200 HR, 10 × 300 mm, 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1 mM EDTA). CgA was concentrated by chromatography on a small anion exchange column (MONO Q, 5 × 50 mm, elution by a gradient of 0–1 M NaCl in 50 mM sodium phosphate, pH 7.4), which typically yielded ~2 ml containing ~10 mg of virtually pure CgA (see top panel of Fig. 2).

Preparation of CgA

Unless otherwise indicated, all steps were performed at 4 °C. Chromaffin granule pellets were resuspended in a buffer containing 20 mM MES, pH 6.4, and 20 mM CaCl₂ and lysed by repeated freeze-thawing. The lysate was centrifuged at 200,000 × g for 30 min. The supernatant was collected and supplemented with 40 mM EDTA. Following extensive dialysis against 50 mM NH₄HCO₃, pH 7.9, and 1 mM EDTA, the dialyzed solution (80 ml, 5 mg/ml protein) received 200 µg of S. aureus V-8 protease. After 2 h of incubation at 37 °C, 3,4-dichloroisocoumarin (100 µl of 10 mM in dimethylformamide) was added to inhibit the protease solution was filtered through a 0.2-

Fluorescent Derivatives of CgA

Purified CgA (1 mg) was dissolved in 900 µl of 50 mM sodium borate, pH 9.0. FITC was added to a final concentration of 1 mM. After incubation for 2 h at room temperature, ethanolamine (1 µl) was added (18.5 mM final concentration). After 30 min of incubation at room temperature, the FITC-CgA was purified by gel filtration (Superdex 200 HR, 10 × 300 mm, 50 mM sodium phosphate, pH 7.4). The stoichiometry of labeling was calculated from the extinction at 494 nm and was found to be 3.5 mol of FITC/mol of CgA. The same protocol was used for labeling of CgA with TRITC (554 nm, stoichiometry of 2.8).

Fluorescent Derivatives of CgA

CgA₁₋₆₀ (1 mg/ml in 50 mM sodium borate, pH 9.0) was reacted with 1 mM TRITC for 2 h at room temperature. Ethanolamine was added to a final concentration of 10 mM. After 1 h, the peptide was isolated by gel filtration as above. TRITC-CgA₁₋₆₀ was separated from the unlabeled peptide by reverse phase chromatography as above. Several peaks of fluorescent CgA₁₋₆₀ were obtained. The peak containing TRITC-CgA₁₋₆₀ modified in a 1:1 stoichiometry, as determined by electrospray mass spectrometry, was used for the experiments. FITC-CgA₁₋₆₀ was prepared accordingly.

Analytical Gel Filtration

Gel filtration of purified CgA was performed at 4 °C using an FPLC system (Pharmacia) equipped with a Superdex 200 HR column (26 × 300 mm) run with a flow rate of 0.7 ml/min. The buffers used for the preincubation and incubation of the samples prior to FPLC and for the FPLC itself were 100 mM NaCl, 1 mM EDTA, and either 50 mM sodium phosphate (pH 7.4 and 6.4) or 50 mM sodium acetate (pH 5.4) as indicated in the legend to Fig. 1. DTT and CgA peptides were added to the buffer as indicated in the legend to Fig. 1. The elution of proteins was monitored by UV absorption at 280 nm.

Sucrose Gradient Centrifugation

A solution (150 µl) containing either purified TRITC-CgA or a mixture of TRITC-CgA plus CgA₁₋₆₀ was layered on top of a 4-ml linear 5–20% (w/v) sucrose gradient and centrifuged at 4 °C in a Beckman SW60 rotor for 15 h at 60,000 rpm. Both sample and sucrose gradient contained 100 mM NaCl, 1 mM EDTA, and either 50 mM sodium phosphate (pH 6.4 for CgA, pH 7.4 for marker proteins) or 50 mM sodium acetate (pH 5.4 for CgA). In the case of centrifugation of the TRITC-CgA/CgA₁₋₆₀ mixture, the sucrose gradient in addition contained 30 mM CgA₁₋₆₀. Fractions (170 µl) were collected from the top of the gradient and analyzed in a Shimadzu FR 1502 spectrophotometer (for TRITC-CgA) or by SDS-PAGE followed by Coomassie Blue staining (for marker proteins). The S values and molecular masses of the marker proteins (23) were plotted as a function of their migration from the top of the sucrose gradient in a linear and semilogarithmic plot, respectively. For each condition, the apparent S value and apparent molecular mass of TRITC-CgA was calculated using linear regression.

Fluorescence Resonance Energy Transfer (FRET) Studies

Determination of Dissociation Constants

The FITC-CgA or FITC-CgA₁₋₆₀ donor and the TRITC-CgA or TRITC-CgA₁₋₆₀ acceptor were dissolved in FITR buffer (150 mM sodium phosphate, pH 7.4, 1 mM EDTA, and either 0.5 mM sodium borate (pH 7.4 for CgA, pH 6.4 for marker proteins) or 50 mM sodium acetate (pH 5.4 for CgA). In the case of centrifugation of the TRITC-CgA/CgA₁₋₆₀ mixture, the sucrose gradient in addition contained 30 mM CgA₁₋₆₀. Fractions (170 µl) were collected from the top of the gradient and analyzed in a Shimadzu FR 1502 spectrophotometer (for TRITC-CgA) or by SDS-PAGE followed by Coomassie Blue staining (for marker proteins). The S values and molecular masses of the marker proteins (23) were plotted as a function of their migration from the top of the sucrose gradient in a linear and semilogarithmic plot, respectively. For each condition, the apparent S value and apparent molecular mass of TRITC-CgA was calculated using linear regression.

Determination of Kinetic Constants

Dissociation of the Dimer—A relatively concentrated mixture of FITC-CgA and TRITC-CgA (ratio 1:4, total concentration 9 µM in FITR buffer), present in a cuvette placed in the spectrophotometer, was
rapidly mixed with 50–200 volumes of FRET buffer. The dissociation of the dimer was immediately monitored at 25 °C by measuring the time-dependent increase of the donor fluorescence at 516 nm.

The total increase in donor fluorescence intensity caused by dimer dissociation is given as the difference between the donor fluorescence intensity \(I_d\) in the equilibrium and the donor fluorescence intensity \(I_d\) measured immediately after mixing. The kinetics of the increase in donor fluorescence and hence the dissociation of the dimer can be described by a first order rate equation,

\[
I_{eq} - I_d \propto (I_{eq} - I_d) \times e^{-kt}
\]

expressed alternatively as the equation,

\[
\ln(I_{eq} - I_d) = \ln(I_{eq} - I_d) - k_d t
\]

with \(I_d\) being the donor fluorescence intensity at the time \(t\).

The experimental data were plotted according to Equation 7, and the first order rate constant of dissociation, \(k_d\), was determined by linear regression of the data points obtained during the first six seconds after mixing.

**Formation of Dimers:—A mixture (1.39 ml) of FITC-CgA and TRITC-CgA (ratio 1:1, total concentration 70–210 nm in 10 mM sodium phosphate, pH 7.9, 0.5 mg/ml BSA) was pipetted into a cuvette placed in the spectrophotometer. This solution was rapidly mixed with 110 \(\mu\)l containing 0.5 \(\mu\)M NaH_2PO_4 and 0.5 mg/ml BSA, yielding a final buffer composition of 50 mM sodium phosphate, pH 6.4. The formation of dimers was immediately monitored at 25 °C by measuring the time-dependent decrease in the donor fluorescence intensity at 516 nm.

The slope \(dI/dt\) of the curve obtained is proportional to the reaction rate \(v\). The initial \(dI/dt\) was used to calculate the rate of consumption of monomeric donor, which equals the rate of formation of donor-acceptor dimer, according to the equation,

\[
v = \frac{dI/dt \times [\text{donor}]}{I\text{\text{eq}} \times [\text{final donor}]} \tag{8}
\]

where \([\text{donor}]\) is the concentration of FITC-CgA after mixing. The second order rate constant of dimer formation, \(k_d\), was calculated according to the equation,

\[
k_d = \frac{v}{[\text{donor}][\text{acceptor}]} \tag{9}
\]

**Miscellaneous Procedures**

Protein in solution was determined by the Bradford assay (22), using BSA as a standard. Proteins fixed and Coomassie Blue-stained in gels were quantitated by densitometric scanning.

**RESULTS**

The N-terminal Domain of CgA Containing the Disulfide-bonded Loop Has an Essential Role in Homodimerization—To investigate a possible role of the N-terminal domain of CgA in homo-oligomerization, we used full-length CgA and CgA_{1–60}, which extends 22 amino acid residues beyond the disulfide-bonded loop (24, 25). CgA obtained from bovine chromaffin granules by lysis at pH 6.4 in the presence of 20 mM calcium is a polypeptide without post-translational modifications is 48 kDa (24). At pH 7.4 (Fig. 1B, solid line), and even more so at pH 6.4 (Fig. 1F, solid line), the addition of a 10-fold molar excess of CgA_{1–60} caused a shift in the peak of full-length CgA toward larger elution volumes, corresponding to a 2-fold reduction in apparent molecular mass. Analysis of the eluted material by SDS-PAGE showed that the shifted peak contained both full-length CgA and CgA_{1–60} (Fig. 2). SDS-PAGE under reducing and nonreducing conditions of full-length CgA alone and of a mixture of full-length CgA plus CgA_{1–60} identical to that subjected to gel filtration indicated the absence of any disulfide-linked homo- or hetero-oligomers (data not shown). We conclude that the addition of CgA_{1–60} reduced the amount of homodimeric CgA and resulted in the formation of a heterodimer between full-length CgA and CgA_{1–60}.

We then examined whether the single intramolecular disulfide bond in the N-terminal domain of CgA (17) was required for the dimerization of CgA. After treatment with the thiol-reducing agent DTT (Fig. 1, A and E, solid lines), full-length CgA became monomeric, eluting from the gel filtration column at a position very similar to that of the heterodimer between full-length CgA and CgA_{1–60}. Given that the dimers of full-length CgA (27) are not due to intermolecular disulfide bridges (17) (data not shown), we conclude that the disulfide-bonded loop plays a crucial role in the noncovalent dimerization of CgA.

**Loop-mediated Homodimerization Is a Prerequisite for Homotetramerization of CgA—Upon gel filtration at pH 5.4 (the luminal pH of mature secretory granules of neuroendocrine cells (28)) (Fig. 1, I–L, dashed lines), full-length CgA eluted earlier than at pH 6.4 and 7.4, consistent with CgA being a homotetramer at pH 5.5 (27). Treatment of full-length CgA with DTT at pH 7.4 followed by gel filtration at pH 5.4 resulted in the appearance of a second peak (Fig. 1I, solid line). By comparison with monomeric CgA at pH 6.4 (Fig. 1E, solid line), this second peak was likely to correspond to monomeric full-length CgA, indicating a tetramer-monomer equilibrium at pH 5.4. No peak was detected in a position expected of a CgA dimer. Similar observations were made after the addition of CgA_{1–60} at pH 5.4. Here, the second peak was likely to correspond to the heterodimer between full-length CgA and CgA_{1–60} (Fig. 1J, solid line), indicating a homotetramer-heterotetramer equilibrium at pH 5.4. No peak was detected in a position expected of a (CgA-CgA_{1–60})_2 heterotetramer. We conclude that the disulfide-bonded loop is of critical importance in maintaining the homotetrameric state of CgA that exists at pH 5.4.

**Homotetramerization of CgA Involves an Interaction of Its C-terminal Domain—When gel filtration of full-length CgA at pH 5.4 was performed in the presence of the synthetic peptide CgA_{406–431}, which corresponds to the C-terminal domain of CgA, a protein complex of smaller size was eluted (Fig. 1K, solid line). This complex, however, was larger than the CgA-CgA_{1–60} heterodimer and presumably corresponded to the (CgA-CgA_{406–431})_2 heterotetramer. Upon the addition of both CgA_{1–60} and CgA_{406–431}, full-length CgA was recovered as a peak with a shoulder, which reflected an equilibrium between the (CgA-CgA_{1–60})_2 heterotetramer and a CgA_{1–60}-CgA_{406–431} heterotrimer, respectively (Fig. 1L, solid line). None of the effect of the CgA_{406–431} peptide, alone or in combination with CgA_{1–60}, was detected at pH 6.4 (Fig. 1, G and H, solid lines) and pH 7.4 (Fig. 1, C and D, solid lines). We conclude that at pH 5.4, the C-terminal domain of CgA mediates the assembly of CgA homotetramers from CgA homodimers; assembly of the latter depends on the disulfide-bonded loop.

In addition to the above changes in the oligomeric state of CgA, we noticed that the apparent size of full-length CgA in any given oligomeric state decreased upon reduction in pH from pH 7.4 to 5.4. Similar pH-induced shifts in elution volume were observed when full-length CgA was present as monomer...
Loop-mediated Chromogranin A Oligomerization

Fig. 1. Gel filtration of CgA under various conditions. Purified CgA (4 μM) was incubated at 37 °C in the absence (—, dashed lines) and presence (+, solid lines) of 20 mM DTT (A, E, and I), 40 μM CgA1–60 (B, F, and J), 400 μM CgA406–431 (C, G, and K), or 40 μM CgA1–60 plus 400 μM CgA406–431 (D, H, and L). Incubations were carried out for 30 min at pH 7.4 (A–D), pH 6.4 (E–H), or pH 5.4 (I–L). Samples containing DTT had been preincubated in the presence of DTT for 30 min at pH 7.4 prior to the 30-min incubation at either pH 7.4, 6.4, or 5.4. After incubation, samples were subjected to gel filtration by FPLC at 4 °C using a Superdex 200 HR column, with on-line recording of the absorbance at 280 nm. FPLC was carried out at the same pH and with the same additions as in the incubations, except that DTT was reduced to 5 mM, CgA1–60 was omitted, and CgA406–431 was reduced to 10 μM. Numbers above peaks and shoulders indicate the presumptive oligomeric state with respect to full-length CgA and ignore the contribution of CgA1–60 and CgA406–431 to the oligomers. The small peak at 16–17 ml of elution is due to free CgA1–60 (compare Fig. 2), which does not contain tryptophan and tyrosine and therefore contributes little to the absorbance at 280 nm; the relatively high peak at this position in panel L is due to presence of trace amounts of CgA1–60 modified by an aromatic group. Note that in panel C the solid line and the dashed line are exactly superimposed. Solid squares at the top of panels A–D indicate the position of elution of marker proteins (from left to right: thyroglobulin, 660 kDa; ferritin, 440 kDa; IgG, 150 kDa; apotransferrin, 81 kDa; ovalbumin, 43 kDa; myoglobin, 18 kDa).

Gel filtration of CgA under various conditions. Purified CgA (4 μM) was incubated at 37 °C in the absence (—, dashed lines) and presence (+, solid lines) of 20 mM DTT (A, E, and I), 40 μM CgA1–60 (B, F, and J), 400 μM CgA406–431 (C, G, and K), or 40 μM CgA1–60 plus 400 μM CgA406–431 (D, H, and L). Incubations were carried out for 30 min at pH 7.4 (A–D), pH 6.4 (E–H), or pH 5.4 (I–L). Samples containing DTT had been preincubated in the presence of DTT for 30 min at pH 7.4 prior to the 30-min incubation at either pH 7.4, 6.4, or 5.4. After incubation, samples were subjected to gel filtration by FPLC at 4 °C using a Superdex 200 HR column, with on-line recording of the absorbance at 280 nm. FPLC was carried out at the same pH and with the same additions as in the incubations, except that DTT was reduced to 5 mM, CgA1–60 was omitted, and CgA406–431 was reduced to 10 μM. Numbers above peaks and shoulders indicate the presumptive oligomeric state with respect to full-length CgA and ignore the contribution of CgA1–60 and CgA406–431 to the oligomers. The small peak at 16–17 ml of elution is due to free CgA1–60 (compare Fig. 2), which does not contain tryptophan and tyrosine and therefore contributes little to the absorbance at 280 nm; the relatively high peak at this position in panel L is due to presence of trace amounts of CgA1–60 modified by an aromatic group. Note that in panel C the solid line and the dashed line are exactly superimposed. Solid squares at the top of panels A–D indicate the position of elution of marker proteins (from left to right: thyroglobulin, 660 kDa; ferritin, 440 kDa; IgG, 150 kDa; apotransferrin, 81 kDa; ovalbumin, 43 kDa; myoglobin, 18 kDa).

(Stem text from the image)
Fig. 2. CgA_{1–60} binds to monomeric full-length CgA. Purified CgA_{4.8 μM} and CgA_{1–60} (40 μM) were incubated at pH 7.4 and subjected to gel filtration as described in Fig. 1B (solid line). After 7.5 ml of elution, 700-μl fractions were collected (7.5–8.2 ml of elution = fraction 1). Fractions were analyzed by Tris-Tricine SDS-PAGE followed by Coomassie Blue staining (top). Full-length CgA (arrow) and CgA_{1–60} (arrowhead) are indicated; positions of molecular mass markers are given on the right. Full-length CgA (open circles) and CgA_{1–60} (filled circles) were quantitated (bottom). Note the correspondence between Coomassie Blue-stained CgA (peak in fractions 4–7, 9.5–12 ml of elution) and the absorbance shown in Fig. 1B (solid line, peak at 9.5–12 ml of elution). To accommodate the peak of free CgA_{1–60} (fractions 14 and 15, 16.6 ml of elution) in the bottom panel, the ordinate scale has been adjusted.

60, FRET will only occur if the two labeled peptides are brought together by a binding interaction, i.e. dimerize.

Fig. 4 shows the fluorescence spectrum upon excitation at 485 nm of FITC-CgA_{1–60} in the presence of either unlabeled CgA_{1–60} plus free TRITC-ethanolamine (unquenched, line with arrow) or TRITC-CgA_{1–60} (quenched, line with open arrowhead). In the presence of 0.64 μM TRITC-CgA_{1–60}, fluorescence at 516 nm emitted from 0.16 μM FITC-CgA_{1–60} was quenched by 67% (FRET value of 0.67).

The FRET values showed a concentration dependence consistent with a reversible dimerization of CgA_{1–60} (Fig. 5A, open circles). If this dimerization reflected the mechanism underlying the dimerization of full-length CgA, the reduction of the disulfide bridge should suppress the dimerization of CgA_{1–60} as it did for full-length CgA in the gel filtration experiments. Indeed, no FRET between FITC-CgA_{1–60} and TRITC-CgA_{1–60} was detectable after treatment with DTT at pH 7.4 (Fig. 5A, solid circles) or pH 6.4 (data not shown). The lack of FRET was not due to the loss of the chromophore from either labeled CgA_{1–60} caused by the DTT treatment, as indicated by SDS-PAGE of the peptides followed by analysis of the fluorescence (data not shown).

The FRET values obtained at various concentrations of labeled CgA_{1–60} at pH 7.4 and pH 6.4 (Fig. 5B) were used to calculate the equilibrium binding constants of dimerization (K_{d}), which were found to be 85 and 20 nm, respectively (Table I). Extrapolation of the individual FRET values yielded an E_{transfer} value of ≈0.9, indicating that the distance between the two chromophores in the CgA_{1–60} dimer was significantly less than 5.4 nm.

FRET Analysis of the Dimerization of Full-length CgA—We extended the FRET analysis to full-length CgA to obtain equilibrium binding constants and to corroborate the effects, observed in gel filtration, of DTT, CgA_{1–60}, and CgA_{406–431} on the dimerization of full-length CgA. Purified full-length CgA was conjugated with either FITC or TRITC, and labeled dimeric CgA was isolated at pH 7.4. The molar ratio of chromophore to protein in either labeled CgA was ~3:1. As with CgA_{1–60}, the binding constant of full-length CgA dimerization (calculated from the individual FRET values; Fig. 6A) was lower at pH 6.4 (214 nm) than at pH 7.4 (1.6 μM) (Table I). However, half-maximal dimerization of full-length CgA occurred at a 10–20-fold higher concentration than that of CgA_{1–60}. Consistent with the results of gel filtration, DTT treatment (Fig. 6C) or the addition of excess unlabeled CgA_{1–60} (Fig. 6B), but not unlabeled CgA_{406–431} (Fig. 6B), abolished most of the FRET between full-length CgA at pH 6.4. We conclude that the N-terminal domain of CgA is the major determinant for its dimerization and that the dimerization mediated by this domain is promoted at mildly acidic pH, a characteristic feature of the lumen of the TGN (10), where sorting to the regulated pathway of protein secretion begins (1, 3).

Kinetics of CgA Dimer Dissociation and Monomer Association—FRET occurs in the nanosecond range and therefore allows measurement of protein interaction kinetics that are too
Fig. 4. FRET between FITC-CgA1–60 and TRITC-CgA1–60. Stock mixtures containing FITC-CgA1–60 (0.16 μM) was incubated at pH 7.4 with a mixture of CgA1–60 (0.64 μM) and TRITC-ethanolamine (0.64 μM) (line with arrow) or with TRITC-CgA1–60 (0.64 μM) (line with open arrowhead). The fluorescence emission spectrum was obtained using an excitation wavelength of 485 nm; fluorescence intensity is given as arbitrary units. The arrow and open arrowhead indicate the intensity of FITC fluorescence at 516 nm, respectively; the energy transfer (E) values shown in Figs. 4 and 5 were obtained according to the equation

\[ E = (U - Q)/U. \]

Fig. 5. FRET analysis of CgA1–60 dimerization. Stock mixtures containing FITC-CgA1–60 (0.6 μM) and either TRITC-CgA1–60 (2.4 μM) (for the determination of the quenched fluorescence) or CgA1–60 (2.4 μM) plus TRITC-ethanolamine (2.4 μM) (for the determination of the unquenched fluorescence) were diluted to the indicated concentration (values on the abscissas refer to the sum of FITC-CgA1–60 and TRITC-CgA1–60 concentration) and incubated for 30 min at 37 °C, and the binding of FITC-CgA1–60 to TRITC-CgA1–60 was determined by FRET analysis as described in Fig. 4, under the following conditions. A, binding of FITC-CgA1–60 to TRITC-CgA1–60 at pH 7.4 in the absence (open circles) and presence (filled circles) of DTT. For DTT treatment, an aliquot of each stock mixture was preincubated for 15 min at 37 °C in the presence of 25 mM DTT, and the buffer for dilution contained 5 mM DTT. B, binding of FITC-CgA1–60 to TRITC-CgA1–60 at pH 7.4 (open circles) and pH 6.4 (open squares). The curves shown were the result of least square fitting to the individual FRET values using Equation 5, which describes a monomer-dimer equilibrium.

Table I

<table>
<thead>
<tr>
<th>pH 7.4</th>
<th>K_D (nM)</th>
<th>k_1 (s^-1)</th>
<th>t_1/2 (s)</th>
<th>k_2 (s^-1 μM^-1)</th>
<th>K_P (nM)</th>
<th>k_1 (s^-1)</th>
<th>t_1/2 (s)</th>
<th>k_2 (s^-1 μM^-1)</th>
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</thead>
<tbody>
<tr>
<td>CgA</td>
<td>1600 ± 900 (2)</td>
<td>6.6 ± 0.6 (2)</td>
<td>10.5</td>
<td>0.4*</td>
<td>214 ± 7 (2)</td>
<td>2.2 ± 0.1 (2)</td>
<td>32</td>
<td>1.3 ± 0.3 (3)</td>
</tr>
<tr>
<td>CgA1–60</td>
<td>85 ± 4 (2)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>19.5 ± 0.5 (2)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Equilibrium binding constants and kinetic constants of dimerization of full-length CgA and CgA1–60 as determined by FRET analysis

The equilibrium binding constants, K_D, were determined according to Equation 5, and the rate constants of association, k_1, were determined according to Equation 7. Values for t_1/2 were calculated from the mean k_1 values according to t_1/2 = ln 2/k_1. The rate constants of association of CgA monomers, k_2, were either determined experimentally according to Equation 9 or calculated from the mean k_1 and K_D values according to k_2 = k_1/K_D (asterisks). The number of experiments is given in parentheses. Either the variation of the individual values from the mean (n = 2) or the S.D. (n = 3) is given. The kinetic constants for CgA1–60 were not determined (ND); they were too fast to be determined reliably with the monitoring system available.

DISCUSSION

The Oligomeric Structure of CgA—Our results, obtained by gel filtration, sedimentation, and FRET analyses, show that the two highly conserved domains of CgA, the N-terminal disulfide-bonded loop and the C-terminal amphipathic α-helix, are of critical importance for the homo-oligomerization of the protein. The N-terminal disulfide-bonded loop is both necessary and sufficient for the homodimerization that occurs at pH 7.4.
and 6.4 (Fig. 9B), the pH values in the lumen of the secretory pathway from the endoplasmic reticulum (pH 7.4) to the immature secretory granule (pH 6.4) (32). In contrast, the C-terminal amphipathic α-helix showed no detectable (using gel filtration) or only a very minor (using FRET, Fig. 6, B and C, triangles) contribution to the homodimerization of CgA at pH 7.4 and 6.4. Previously, Yoo and Lewis (33) proposed that the C-terminal amphipathic α-helix is the primary domain of CgA mediating the homodimerization at pH 7.5 and homotetramerization at pH 5.5. This conclusion was based on the oligomerization behavior of synthetic peptides corresponding to specific segments of CgA. The apparent disagreement between the previous (33) and our present findings can be explained by the fact that the interaction mediated by the N-terminal disulfide-bonded loop is critically dependent on the oxidized state of the two cysteine residues. The N-terminal synthetic peptide used by Yoo and Lewis (33) did not include these critical two cysteines and, hence, could not adopt a conformation allowing dimer formation. Furthermore, the binding energy of dimerization measured by Yoo and Lewis for the C-terminal peptide (33) was much smaller than that for full-length CgA (27) and therefore insufficient to account for the dimerization of the latter. In contrast, in our hands, the affinity for homodimerization of the N-terminal disulfide-bonded loop was much larger than that of full-length CgA and hence sufficient to account for the dimerization of full-length CgA.

At pH 5.4, the pH value of mature secretory granules of neuroendocrine cells (28), the intermolecular interactions leading to CgA oligomerization are more complex, involving both the N- and C-terminal domains. Our data support a homotetrameric structure at this pH (Fig. 9C), consistent with previous findings (27). In the formation of the tetramer, the interactions mediated by the N- and C-terminal domains behave in a cooperative manner, with the N-terminal one being a prerequisite for efficient interaction via the C-terminal domain. The follow-
ing lines of evidence lead to this conclusion. Treatment with DTT or the addition of N-terminal peptide, both of which interfere with the N-terminally mediated interaction between full-length CgA molecules, is sufficient to cause the dissociation of CgA tetramers into monomers. No dimers are seen under these conditions. Dimers, but no monomers, however, are observed when the C-terminally mediated interactions between CgA molecules are blocked by the addition of C-terminal peptide. These observations suggest that C-terminal interactions lead to stable complexes only if they link two preexisting N-terminally bound CgA dimers to one another (Fig. 9C). In this case, the parallel interactions of two pairs of C-terminal domains would contribute twice the binding energy, similar to an antibody binding an oligomeric antigen via its two Fab domains. Another indication for the greater stability of the C-terminal than the C-terminal interaction is that upon gel filtration of CgA, the dissociation of tetrameric CgA into dimers was observed only if the C-terminal peptide was added to both the sample and the running buffer, demonstrating the relatively weak and transient nature of a single C-terminal interaction. In contrast, the dissociation of oligomeric CgA into monomers was observed already if the N-terminal peptide was added to the sample only, showing that a single N-terminal interaction provides sufficient binding energy to give stable complexes.

**Dimerization of CgA and Sorting**—The N-terminal disulfide-bonded loop is not a feature exclusive to CgA. A highly homologous domain is also found in CgB (17). In PC12 cells, which express CgB but not CgA, reduction of the disulfide bond in *vivo* prevents the sorting of CgB from the TGN into immature secretory granules and results in its exit from the TGN in constitutive secretory vesicles (11). The sorting of secretogranin II, a member of the granin family devoid of cysteine residues (4), was unaffected by the reductive treatment (11). These observations indicated that the disulfide-bonded loop of CgB, and by analogy that of CgA, is necessary for sorting of the chromogranins (11). Together with the present finding that the disulfide-bonded loop mediates the formation of homodimers, we are left with two possible explanations. First, two distinct structural motifs are necessary for dimer formation and sorting, and both are localized to the disulfide-bonded loop. Opening the loop by reduction would independently disturb both dimerization and sorting. This possibility seems unlikely, since the 22-amino acid residue-containing loop is presumably too small to encompass two structural motifs capable of binding to their interaction partners simultaneously. Second, the disulfide-bonded loop is required for sorting of chromogranins because the homodimerization mediated by this structural motif is essential for sorting to occur, a possibility that we favor.

How would the loop-mediated homodimerization of the chromogranins have an essential role in sorting? We would like to discuss this question with respect to the two principal levels of the sorting process in the TGN: (i) the segregation of constitutive and regulated secretory cargo from one another in the lumen of the TGN and (ii) the interaction of regulated secretory cargo with membrane components in the TGN. As to the first level, segregation of regulated from constitutive secretory cargo is thought to be achieved by milieu-induced selective aggregation of the former (7), followed by the exit from the TGN of aggregated secretory proteins in immature secretory granules and of soluble secretory proteins in constitutive secretory vesicles (3). In cells with a very high sorting efficiency such as PC12 cells, this implies that for any given regulated secretory protein, the vast majority of molecules undergoes aggregation during passage through the TGN. Any delay in aggregation during this passage, i.e. at t₁⁄₂ of aggregate formation approaching that of exit in constitutive secretory vesicles, would lead to substantial missorting of the regulated secretory protein. It is conceivable that the loop-mediated dimerization of CgB, although not a prerequisite for its calcium- and low pH-induced aggregation as such (34), provides the nucleating core units necessary for aggregation to approach completion within the time span available for sorting in the TGN.

As to the second level of sorting, the disulfide-bonded loop may be critical for the interaction of chromogranins with membrane components destined to secretory granules. Both direct and indirect roles of the loop in the membrane binding of the chromogranins are conceivable. One such direct role would be a loop-mediated homodimerization, i.e. the binding of the loop of a soluble chromogranin molecule to the loop of a tightly membrane-associated chromogranin molecule (35, 36). Another di-

![Diagram of oligomeric states of full-length CgA](Image)
rect role would be the loop-mediated heterodimerization, i.e. the binding of the loop of a soluble chromogranin molecule to a membrane receptor. A precedent for a loop-mediated heterodimerization is provided by the finding that pro-opiomelanocortin binds, via its N-terminal disulfide-bonded loop, to the membrane-associated form of carboxypeptidase E (14). An indirect role of the loop in membrane binding of the chromogranins would be the case if dimers, but not monomers, of soluble chromogranin molecules were able to bind with sufficient affinity to either membrane-associated chromogranin (homophilic interaction) or a membrane receptor (heterophilic interaction).

Dynamics and Compartments of CgA Oligomerization—Newly synthesized CgB is transported to the sorting compartment, the TGN, with a $t_{1/2}$ of $\sim 7$ min (11). If, as discussed above, loop-mediated dimerization of CgB is essential for sorting, the observation that reduction of the disulfide-bonded loop prevents sorting implies that newly synthesized CgB has dimerized by the time it reaches the TGN. Consistent with this, our results on the kinetics of CgA monomer association as determined by FRET reveal that already at a protein concentration much below that likely to exist in the endoplasmic reticulum (the presumptive site of disulfide bond formation), association is complete within $\sim 2$ min. At a low estimate for the chromogranin concentration in the endoplasmic reticulum of a neuroendocrine cell, $1 \mu M$, association would be complete within a few seconds. It is therefore likely that chromogranins already dimerize in the endoplasmic reticulum and are transported to the TGN as dimers.

The CgA dimers are not only formed rapidly, they also exhibit a fast rate of dissociation, resulting in a $t_{1/2}$ for the decay of $\sim 30$ s (at 25 °C). This implies that although only a small proportion of the CgA in the secretory pathway exists in monomeric form, there is rapid interconversion between the monomeric and dimeric state. This could be relevant for the interaction of soluble CgA with the membrane discussed above.

Finally, our observation that upon lowering the pH from 6.4 to 5.4, CgA homodimers formed homotetramers via C-terminal interactions is intriguing. Such a change in pH corresponds to the acidification known to occur concomitant with secretory granule maturation in neuroendocrine cells (32). It is tempting to speculate that this form of interaction of CgA molecules is involved in the condensation of the secretory cargo during secretory granule maturation.

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REFERENCES