Structural Studies of EDTA-Induced Fibrillation of Salmon Calcitonin

Stefan Seyferth[1] and Geoffrey Lee[1,2]

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Purpose. The purpose of this work was to determine the structure of an insoluble precipitate formed when mixing approximately equimolar amounts of ethylenediamine tetraacetic acid (EDTA) and salmon calcitonin (sCT).

Methods. The interaction between EDTA and sCT was examined by measuring solution turbidity kinetics as a function of pH, ionic strength, and addition of ferric ions. Fourier-transformation infrared spectroscopy (FT-IR) identified changes in peptide secondary structure in presence of EDTA. Scanning and transmission electron spectroscopy revealed the macromolecular structure of the sCT/EDTA precipitate.

Results. Aggregation of sCT in a time frame up to 1200 min cannot be induced by either pH (range 3.0–7.0) or ionic strength (up to 200 mM) alone, but is a noncovalent interaction between sCT and EDTA. In the pH range 5.0–7.0, a molar binding stoichiometry of sCT/EDTA in the precipitate of 1–3 was determined. We suggest coulombic binding of the free acidic groups of the EDTA to the side chains of the basic amino acids present in the sCT primary sequence. This results in bridging aggregation of the sCT molecules and their precipitation in aqueous solution. The aggregation reaction was blocked by the addition of ferric ions, which bind preferentially to the acidic groups of the EDTA. The sCT/EDTA precipitate redissolves in water in a pH-dependent manner. FT-IR measurements showed a progressive loss of the random coil structure of sCT in water in the presence of EDTA and a simultaneous strong increase in β-structure. Scanning electron microscopy revealed a fine, sponge-like morphology of the isolated, solid sCT/EDTA precipitate. Transmission electron microscopy delivered final proof of the existence of extensive fibrillation in the sCT/EDTA mixture.

Conclusions. EDTA induces rapid fibrillation of sCT in water and the partially reversible formation of a noncovalent, insoluble sCT/EDTA precipitate.

KEY WORDS: calcitonin; EDTA; precipitate; fibrillation.

INTRODUCTION

In the patent literature, there is a single, obscure report of the formation of a poorly water-soluble precipitate when mixing aqueous solutions of peptides and ethylenediamine tetraacetic acid (EDTA) in an approximately equimolar ratio (1). This phenomenon was reported to occur with salmon calcitonin (sCT) to yield a precipitate that on redispersion in water gave only 20% redissolved sCT after 22 h. The claimants suggested that a "chelate" was reversibly formed between peptides such as sCT and the EDTA. There is, however, no further information available about this interaction between EDTA and peptides.

sCT has, in contrast to human calcitonin (hCT), only a weak propensity to aggregate and form fibrils in aqueous solution at pHs ≤ neutral (2). This is a result of its lack of secondary structure to promote association (3) and also its high iso-electric-point (iep) of 10.4 (2,4). The reported precipitation of sCT indicates that these hindrances to sCT molecular aggregation are in some manner overcome in the presence of EDTA. In this work we present the first part of our systematic study of the interaction between sCT and EDTA. Using measurements of turbidity we follow the kinetics of the interaction between sCT and EDTA. Analysis of the supernatant yields the stoichiometry of the binding between sCT and EDTA in dependence of pH. The use of Fourier-transformation infrared spectroscopy (FT-IR), scanning, and transmission electron microscopy illustrates the changes occurring in the secondary and macromolecular structures of the sCT during its precipitation with EDTA and shows that these are associated with fibrillation of the peptide. The use of EDTA to precipitate peptides in a reversible fashion could have potential application in the field of controlled release.

MATERIALS AND METHODS

Materials

sCT (molecular weight 3.4 kDa) and hCT (molecular weight 3.4 kDa) were kindly provided by Novartis (CH-Bern, Germany). EDTA-disodium salt (molecular weight 372 Da) was obtained from Sigma Chemicals (D-Munich, Germany) and used as received. Acetonitrile required for the high-performance liquid chromatography (HPLC) analysis of EDTA and sCT and trifluoracetic acid required for the HPLC analysis of sCT were both obtained from Aldrich Chemicals (D-Munich, Germany), and tetrabutyl ammonium bromide from Sigma. Remaining buffer salts, reagents, and KBr for preparing IR windows were all obtained from Sigma. Water was double distilled from an all-glass apparatus.

Turbidity Kinetics of the Interaction between sCT and EDTA

Separate solutions of sCT and EDTA were prepared in water and the pH adjusted by addition of NaOH/HCl. One hundred microliters of each solution was then pipetted into the wells of a quartz microtiter 96-well plate standing in a Shimadzu CS-9301PC Densitometer at 25 ± 0.5°C. The absorbance of the resulting mixed solution was then measured at $\lambda = 320$ nm for up to 1200 min, and the result expressed as a plot of absorbance vs. time (2). All experiments were performed at least in duplicate.

Binding Stoichiometry of sCT with EDTA

Separate solutions of sCT and EDTA were mixed in a 1.5-mL Eppendorf cup. The resulting solution was then adjusted to the pH and ionic strength values required and made up to 500 μL. On completion of the reaction, the contents of the cup were centrifuged at 5,000 rpm for 5 min to obtain a clear supernatant. The unbound amounts of EDTA and sCT present in the supernatant were then determined using HPLC. The identical experiment but without either EDTA or sCT was performed as a control. EDTA was quantified using a reversed-phase ion-pairing method with Fe-(III)-EDTA

[1] Department of Pharmaceutics, Friedrich-Alexander-University, Cauerstr. 4, 91058 Erlangen, Germany.

[2] To whom correspondence should be addressed. (e-mail: lee@pharmtech.uni-erlangen.de)

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and tetra butyl ammonium bromide (5). The sCT was determined using the gradient HPLC analysis described by Lee *et al.* (6).

The sCT/EDTA precipitate was isolated by mixing separate solutions prepared as described above, followed by centrifugation through a Centricon filter device (Millipore) having molecular weight cut-off (MWCO) of 10,000. The precipitate so obtained was washed with 100 μl water and then dried for 4 days under vacuum.

FT-IR

Liquid samples were examined in a CaF_2 cuvette of pathlength 5.6 μm (7). Solid samples were mixed with KBr (1 + 98 mg) and pressed to windows using an International Crystal Laboratories KL press. FT-IR spectra were obtained at 25 ± 0.2°C on a Nicolet Magna IR 550 FT-IR system continually purged with dry air. From each sample a total of 256 interferograms were collected in single beam mode with 4 cm^{-1} resolution. The solvent or vapor components were subtracted from the sample spectrum, after which the difference spectrum was imported into Galactic Grams and truncated to the region of the amide I bands between 1720 and 1580 cm^{-1}. After baseline correction, the second derivative spectrum was calculated and smoothed using a nine-point binomial function, a standard procedure for elucidating the secondary structural features of proteins (8,9).

Scanning and Transmission Electron Microscopy

Scanning electron micrographs (SEMs) of solid samples were obtained by Au sputtering, followed by examination on an Amray 1810T SEM. Transmission electron micrographs (TEMs) of EDTA/sCT reaction mixtures were obtained by absorption of a 10-μL drop on to a pioloform-coated Cu grid for 5 min. Excess liquid was then removed and the residue negatively stained with a 2% w/w uranyl acetate solution for 5 min. After vacuum drying, the sample was examined with a Zeiss EM10 TEM.

RESULTS AND DISCUSSION

Turbidity Kinetics of Interaction between sCT and EDTA

The extent of interaction between sCT and EDTA is strongly pH dependent. A pH 3.3 aqueous solution containing 2 mM sCT shows no change in absorbance of the original, clear solution (t = 0) over 1200 min at any of the EDTA concentrations of 5–20 mM examined (Fig. 1A). At pH 4.5 (Fig. 1B), however, an increase in absorbance is seen after 500 min at the highest EDTA concentrations examined, i.e., 15 and 20 mM, as a result of visually observable precipitation of the sCT. At pH values of 5.5 (Fig. 1C) and 7.0 (Fig. 1D), an increase in absorbance caused by precipitation of the sCT occurs after approximately 200 min at all EDTA concentrations ≥5 mM. At all pH values and also in pure water, a 2 mM solution of sCT without EDTA shows no absorbance or visible precipitation up to 1200 min (cf. Fig. 1A–D). This is indeed the expected behavior for sCT in water because it has only a weak propensity to aggregate and form fibrils. Arvinte *et al.* (2) found, for example, that a 3 mM sCT aqueous solution at pH 7.4 required >50 days of storage before incipient aggregation and fibrillation occurred. Gilchrist and Bradshaw (10) used TEM to detect the presence of primary fibrils in a 3 mM sCT aqueous solution formed after 9 days at pH 5–8 and high temperature. Much higher sCT concentrations than those used here (2 mM) are required for ready fibrillation, for example, a 15 mM aqueous solution of sCT at pH 3.3 gelled and formed birefringent fibrils on storage (3). The weak propensity of sCT to aggregate and form fibrils at pHs less than or equal to neutral is a result of its lack of secondary structure to promote association (3) and also its high iep of 10.4 (2,4). It is evident from Fig. 1A–D, however, that EDTA rapidly induces precipitation of sCT at pH values ≥4.5. At pH values of 5.5 and 7.0, 5 mM EDTA is sufficient to precipitate the 2 mM sCT, whereas at pH 4.5, at least 15 mM EDTA is required. This suggests that electrostatic interactions play a role in the observed precipitation. We note that the sigmoidal shape of the absorbance vs. time curves in Fig. 1A–D is the same as that determined for fibrillation of hCT in water (2). An initial lag time ("fibrillation" time) is followed by increasing absorbance, which then levels off to a maximum value. The fibrillation time of the sCT/EDTA interaction is approximately 500 min at pH 4.5, reducing to 200 min at pH ≥5.5. Although 2 mM sCT alone shows no turbidity at any pH level examined here, Arvinte et al. (2) reported fibrillation time of >50 days for sCT at pH 7.4 greatly exceeds the measurement time of 1200 min used in our experiments. Only at pH 7 could an incipient precipitation be observed at times of >1500 min. The visual appearances of the final solutions (t = 1200 min) were also similar to those reported for fibrillation of hCT. At pH values of 4.5 and 5.5, the sCT/EDTA precipitate appeared as punctuate aggregates, whereas at pH 7.0, a turbid gel was formed. These two, distinctive appearances were reported for fibrillation of increasing concentrations of hCT between 0.3 mM and 4.5 mM (2).

Studies of fibrillating hCT in water have shown that initial molecular aggregation is initiated by intermolecular hydrophobic association of the Cys1–Cys7 N-terminal loop and central Met8–Pro23 α-helix regions (11). This is followed by association of the C-terminal Gln24–Pro32 β-sheets to form a template for fibril elongation (2,12). sCT exists, however, in water as a random coil at the concentration (2 mM) used in our study (13). The presence of the EDTA in the concentrations used here did not shift pH, ruling out effects on electrostatic repulsion. We suggest that the EDTA causes "bridging aggregation" of the sCT molecules in water, akin to the well-known phenomenon of bridging flocculation of colloidal particles by some polymers (14). With its iep of 10.4 (4), sCT will carry an overall positive charge at those pH values ≤7.0 where the sCT/EDTA interaction occurs. In the absence of EDTA, this will inhibit aggregation of the individual sCT molecules by reason of electrostatic repulsion of the random coils in solution (2). The sCT primary sequence contains basic amino acids at Lys11, Lys18, His17, and Arg24 (15). The pKa of the ε-NH_3^+ side group of the Lys is 10.5, that of the guanido group of the Arg is 12.5, and the imidazole group of the His is 6.0 (16). Thus, at pH values ≤7, both the Lys and Arg have fully protonated side groups and also the His will be partially charged. These charged, basic amino acid residues could form coulombic interactions with the dissociated acidic groups of one EDTA molecule. This idea is supported by an early proton nuclear magnetic resonance study demonstrating that the addition of EDTA to an aqueous solution of pancreatic ribonuclease caused strong perturbation of the protein's

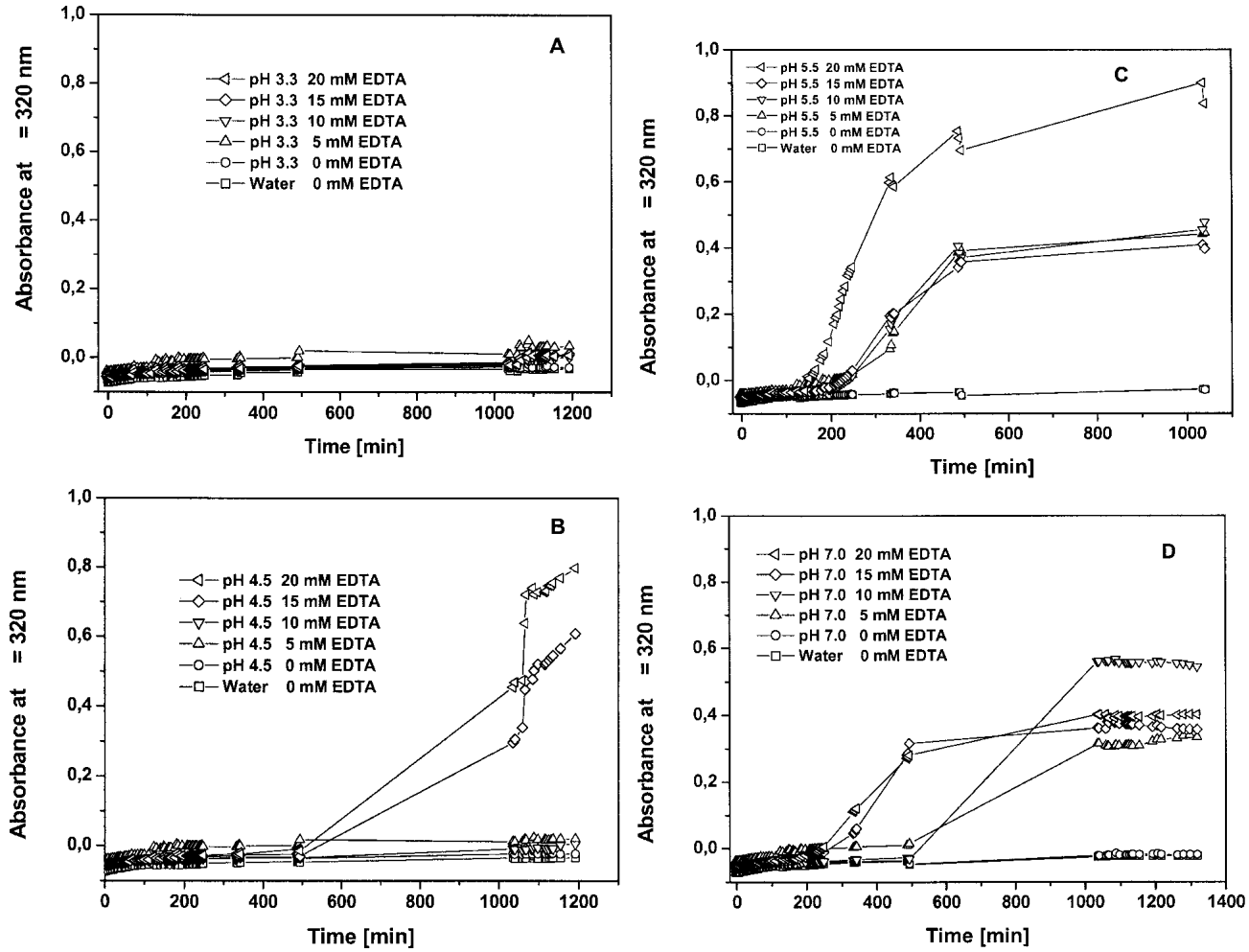

Fig. 1. Turbidity kinetics of the interaction between sCT (2 mM) and EDTA-Na_2 in aqueous solution. (A) pH 3.3, (B) pH 4.5, (C) pH 5.5, and (D) pH 7.0. Absorbance was measured at $\lambda = 320$ nm in 100-μL volumes present in the wells of a microtiter well plate standing in a densiometer.

His residues (17), although no precipitation was reported in this case. The authors envisaged that EDTA could bind to the His and possibly also Lys residues of a single protein molecule. We suggest that this coulombic binding occurs between EDTA's dissociable acidic groups (we used EDTA-Na_2) and more than one sCT molecule to induce bridging aggregation of the sCT and hence also the observed precipitation. The importance of coulombic interactions between the sCT and EDTA molecules can be demonstrated by adding ferric ions to the reaction medium. These posses large binding constants to the acidic groups of EDTA (18) and should therefore prevent, or at least impede formation of coulombic interactions with sCT. Indeed, in the presence of 6 mM Fe^{2+}, there is now no measurable turbidity at pH values of 4.5, 5.5, and 7.0 with EDTA concentrations up to 20 mM over a time of 1200 min (absorbance vs. time plots not shown). There is no aggregation of the sCT by the EDTA in this case, since the dissociable acidic groups of the EDTA are not available to interact coulombically with the basic amino acids of the sCT primary sequence.

It was not possible to induce precipitation and turbidity of the sCT by increasing the ionic strength of the solution in the absence of EDTA. The addition of up to 200 mM NaCl failed to cause measurable turbidity of the 2 mM sCT solution at pH values 3.3, 4.5, 5.5, or 7.0 up to 1200 min (absorbance vs. time plots not shown). Bauer and Merkle (19) found that the fibrillation time of 3 mM hCT was continually shortened from 360 min to 26 min by addition of up to 1 M NaCl, owing to increased screening of coulombic repulsion of the equally changed hCT molecules. The lack of effect with sCT is a result of its higher iep (10.4 compared with 8.7 for hCT; Ref. 4), and also of its much weaker propensity to fibrillation. We conclude that the observed precipitation of sCT is a result of bridging aggregation induced by EDTA and requires the formation of coulombic interactions between the basic amino acid residues of the sCT and the acidic groups of the EDTA molecules. It is not simply a pH or ionic-strength induced phenomenon.

The sCT/EDTA precipitate can redissolve when added to water, depending on the pH. Figure 2A shows the absorbance vs. time curves obtained on addition of either pure sCT or the sCT/EDTA precipitate to buffer. At pH values of 3.4, 5.4, and 7.2, the pure sCT dissolves rapidly, and either no or little (at pH 7.2) turbidity is measured. On addition of the sCT/EDTA precipitate to buffer, however, an absorbance of 0.2–0.3 is initially measured because a turbid dispersion of the

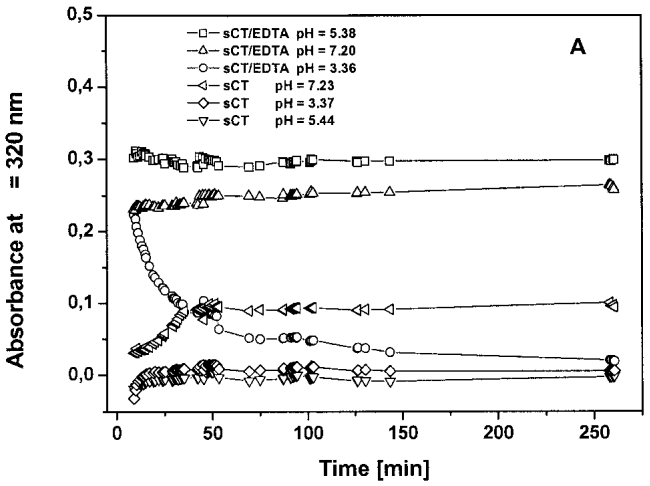

Fig. 2. Dissolution of pure sCT and isolated sCT/EDTA precipitate in water at various pH. Turbidity kinetics of dissolution process determined as given in Fig. 1.

precipitate in the buffer is formed. With time the absorbance at pH 3.4 decreases, and after approximately 250 min the precipitate has completely dissolved. Assuming a dissolution kinetics of first order for this nonstirred system (the small volume of liquid, 100 μL, in the microtiter well plate could not be stirred without impeding the turbidity measurement), the $t_{1/2}$ of dissolution from the pH 3.4 curve is approximately 35 min. At pH 5.4 the absorbance decreases only slightly over the 250 min of the experiment, and at pH 7.2 it shows no decrease over this time. At pH 3.4, the coulombic binding of sCT to the EDTA in the precipitate is weakened by preferential protonation of the EDTA's acidic groups. The sCT/EDTA precipitate then dissolves into its two components. At pH 5.4 and 7.2, the coulombic binding between the less protonated acidic groups of the EDTA and the basic amino acids of the sCT is stronger, and dissolution of the sCT/EDTA precipitate is retarded.

Binding Stoichiometry of sCT with EDTA in Precipitate

The molar amounts of sCT and EDTA bound in the insoluble precipitate were determined from the difference between the original amounts in solution and those measured in the filtrate that passed through a MWCO 10,000 centrifuge filter. At pH 4.5, there is no loss of either sCT or EDTA in the solution containing 2 mM sCT + 2 mM EDTA (Fig. 3A). This result agrees with the absorbance measurements at this pH (cf. Fig. 1B), where no precipitate formed on mixing 2 mM sCT with ≤10 mM EDTA. With 4 and 6 mM/L EDTA, however, there is a loss in both sCT and EDTA (Fig. 3A), also in agreement with the turbidity measurements in Fig. 1B, where precipitation occurred on mixing 2 mM/L sCT with 15 or 20 mM/L EDTA. The resulting EDTA/sCT molar stoichiometry bound in the precipitate is approximately unity (see Fig. 3B). The nonbound sCT detected in the filtrate is either molecularly dispersed or may form a soluble complex with the remaining, nonbound EDTA. This result was confirmed in a separate experiment where 1.17 mg of the sCT/EDTA precipitate prepared at pH 4.5 was completely dissolved in pH 3.3 buffer. The amount of sCT recovered was 0.35 ± 0.02μM (n = 3) and of EDTA recovered was 0.28 ± 0.004 μM (n =

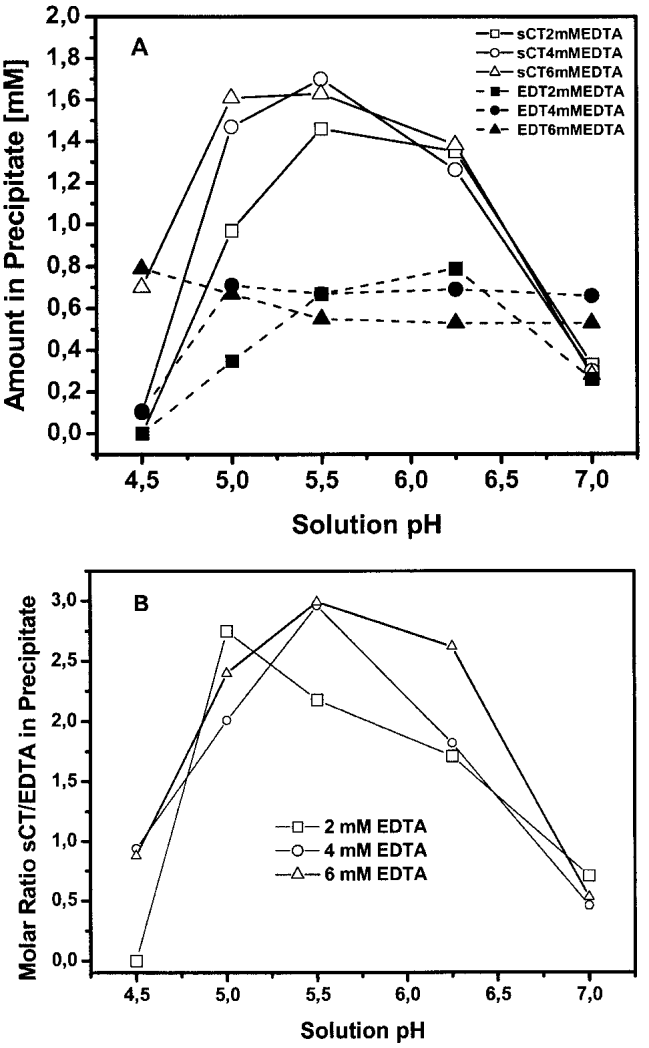

Fig. 3. Results of determination of binding stoichiometry of sCT with EDTA. (A) Amounts of sCT and EDTA bound in precipitate; 2 mM sCT mixed with either 2, 4, or 6 mM EDTA and 2 mM sCT mixed with either 2, 4, or 6 mM EDTA. (B) Resulting molar ratio of sCT/EDTA bound in the precipitate formed when mixing 2 mM sCT and either 2, 4, or 6 mM EDTA.

3). This result gives a binding stoichiometry in the precipitate of 1:1.25 (EDTA/sCT) by molar mass, in good agreement with the difference measurement in Fig. 3B. This molar ratio is equivalent to approximately 1:12 (EDTA/sCT) by weight.

At pH values ≥5.0 precipitation occurs at all the EDTA concentrations examined. The amount of EDTA bound in the precipitate stays, however, broadly constant with initial EDTA concentrations of 4 and 6 mM (see Fig. 3A), whereas the amount of sCT bound in the precipitate increases to a maximum at pH 5.5 and then decreases to pH 7 at all initial EDTA concentrations. As a consequence of these differing binding patterns, the molar stoichiometry of EDTA/sCT bound in the precipitate increases from approximately 1.0 at pH 4.5 up to a maximum of 2.5–3 around pH 5.5, and then decreases to <1.0 at pH 7 (Fig. 3B). In the pH range 5–6.25, one molecule of EDTA binds therefore a maximum of two to three molecules of sCT. A simple, intuitive model of bridging aggregation pictures 1 molecule of EDTA (the Na_2 salt) being able to bind two to four molecules of sCT. Again, coulombic

interactions between EDTAs up to four free acidic groups (pKa_4 is 10.26, however, and unlikely therefore to be involved) and the sCT's basic amino acid residues hold the precipitate together. The weaker interaction between sCT and EDTA at pH 4.5 (molar EDTA/sCT stoichiometry of 1:1) is a result of increasingly preferred protonation of the nonsalt acidic groups of EDTA at this pH. The reduced binding at pH 7.0 (molar EDTA/sCT stoichiometry also of 1:1) occurs despite strong precipitation of the sCT at this pH according to the turbidity result in Fig. 1D.

FT-IR Measurements

sCT (2 mM) in water or at pH 3 shows only a single broad band centered at 1641 cm^{-1} in the second derivative FT-IR spectrum (Fig. 4A). This is attributable to the random coil conformation of the polypeptide below its N-terminal Cys1–Cys7 loop at low solution concentrations (7). At pH values of 6.0 and 7.0, there is a greatly reduced random coil band and now a dominating band at 1632 cm^{-1}, which is assigned to an intramolecular β-sheet structure (3). New bands appear at 1667 cm^{-1} and 1618 cm^{-1}, classifiable as β-turn and intermolecular β-sheet, respectively (3). Increased pH from 3.0 to 7.0 induces therefore some β-sheet conformation of the sCT, although this does not result in any measurable precipitation up to 1200 min (cf. Fig. 1).

Marked changes occur in the FT-IR spectrum of sCT on addition of EDTA. Recall that 2 mM sCT is precipitated by 4 mM EDTA at pHs ≥5.0 (cf. Fig. 3B). Figure 4B shows the second derivative spectra obtained from the reaction mixture 2 mM sCT + 4 mM EDTA at pH 6.0 and various times after their combination. The 30-min spectrum was taken during the lag time observed in the turbidity measurements (cf. Fig. 1C) and shows therefore the conformation of the sCT before visible precipitation. The remaining spectra were taken after precipitation (there was no evident sedimentation in the cuvette) and reflect therefore also the conformation in the sCT/EDTA precipitate. The band at 1620 cm^{-1} assigned to intermolecular β-sheet (3) increases in intensity up to 96 h. The intensity of the β-turn band at 1668 cm^{-1} (3) also increases. The 1632 cm^{-1} intramolecular β-sheet, which dominates at t = 0 (cf. Fig. 4A, pH 6 without EDTA), becomes smaller up to 96 h in solution. Simultaneously, the intensity of the random coil band at 1649 cm^{-1}, which is still substantial at t = 30 min, decreases. A slight α-helix band at 1656 cm^{-1} is also distinguishable at t = 24 h. The spectrum obtained from the redispersed sCT/EDTA precipitate shows the same structural features as the t = 96 h sample. The changes in these bands from t = 0 to t = 96 h indicate a progressive increase in secondary structural order of the sCT in the presence of EDTA, most notably induction of β-conformation. The changes in the intermolecular β-sheet 1620 cm^{-1} and random-coil 1649 cm^{-1} bands are the same as those occurring during fibrillation of hCT in aqueous solution (2), and also during the weak fibrillation of sCT at neutral pH and high concentration (15 mM; Ref. 3). The importance of coulombic interactions involving the acidic groups of the EDTA is again evident from second derivative spectra obtained from the reaction mixture 2 mM sCT + 4 mM EDTA + 4 mM ferric ions over up to > 200 h. Only a dominating, broad random coil band centered at 1641–1644 cm^{-1} is evident during the whole course of the experiment (result not shown). Preferential

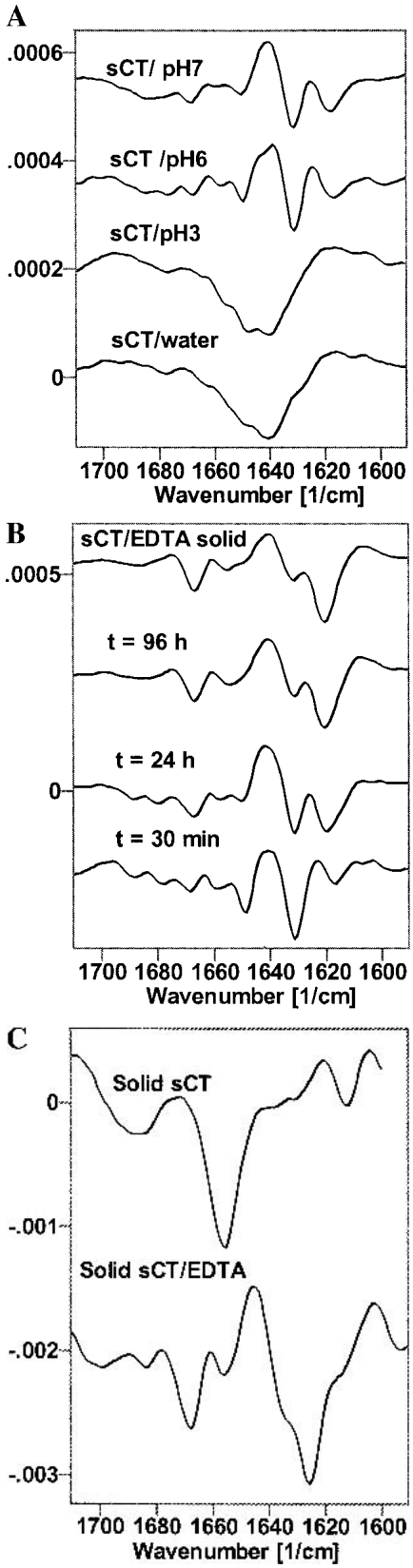

Fig. 4. Second-derivative FT-IR spectra obtained using 5.6-μm path-length CaF_2 cuvette. (A) Amide I region of FT-IR spectra of aqueous solutions of sCT, 2 mM. (B) Amide I region of FT-IR spectra of solution containing 2 mM sCT and 4 mM EDTA in water at various times. (C) Amide I region of solid state FT-IR spectra of pure sCT and the isolated sCT/EDTA precipitate.

binding of the ferric ions with the acidic groups of the EDTA therefore not only prevents aggregation of the sCT (see above); it also prevents EDTA-induced formation of the β-sheet structure observed in the absence of ferric ions.

The second derivative spectrum of the isolated sCT/EDTA-precipitate shows substantial differences to that obtained from solid sCT (Fig. 4C). Pure, solid sCT has a dominating band at 1656 cm^{-1}. This band is much narrower than the random coil 1643 cm^{-1} band seen with the 2 mM sCT solution in Fig. 4A. It can be attributed to α-helix structure (3), indicating that the solid state sCT has a central Met8–Pro23 α-helix region before the Cys1–Cys7 N-terminal loop, as seen with hCT (11). The second band at 1612 cm^{-1} can be assigned to inter-molecular β-sheet. In contrast, the spectrum of the isolated sCT/EDTA precipitate is dominated by two strong bands at 1668 cm^{-1} and 1626 cm^{-1}. The former is assignable to β-turn. The 1626 cm^{-1} band has a shoulder at 1634 cm^{-1}, leading us to assign these two bands to inter- and intramolecular β-sheet, respectively. A third band at 1656 cm^{-1} assigned to α-helix is much reduced compared with pure sCT. The shoulder on the right side of the 1626 cm^{-1} intermolecular β-sheet band corresponds to a 1615 cm^{-1} intramolecular β-sheet. The isolated sCT/EDTA precipitate has therefore predominantly β-sheet character compared with either pure solid sCT (predominantly α-helix) or sCT in aqueous solution (predominantly random coil, Fig. 4A). Circular dichroism and FT-IR studies of the fibrillation of hCT in aqueous solution show similar behavior. There is inter alia the induction of β-sheet conformation, thought to take place in the C-terminal Gln24–Pro32 region (2). Indeed, the isolated residue 20–31 of hCT fibrillated and gelled in aqueous solution and showed β-sheet conformation (12). In contrast, the isolated 20–31 residue of sCT maintained random coil structure in aqueous solution and showed only extremely weak gel formation (12). There is therefore a strong similarity between the secondary structural features of the sCT/EDTA precipitate and those of fibrillated hCT in water. Taken together with the turbidity results the FT-IR data show that EDTA binds with sCT and induces its aggregation, associated with a major change in secondary structure. This results in precipitation of the sCT/EDTA complex. The relevance of this finding lies in the known, very weak propensity of sCT to fibrillate in aqueous solution (2,13). This can be induced by the addition of EDTA in the pH range $\geq$ 4.5–7.0.

SEM and TEM

The isolated sCT/EDTA precipitate prepared from 2 mM sCT + 4 mM EDTA had the identical appearance under

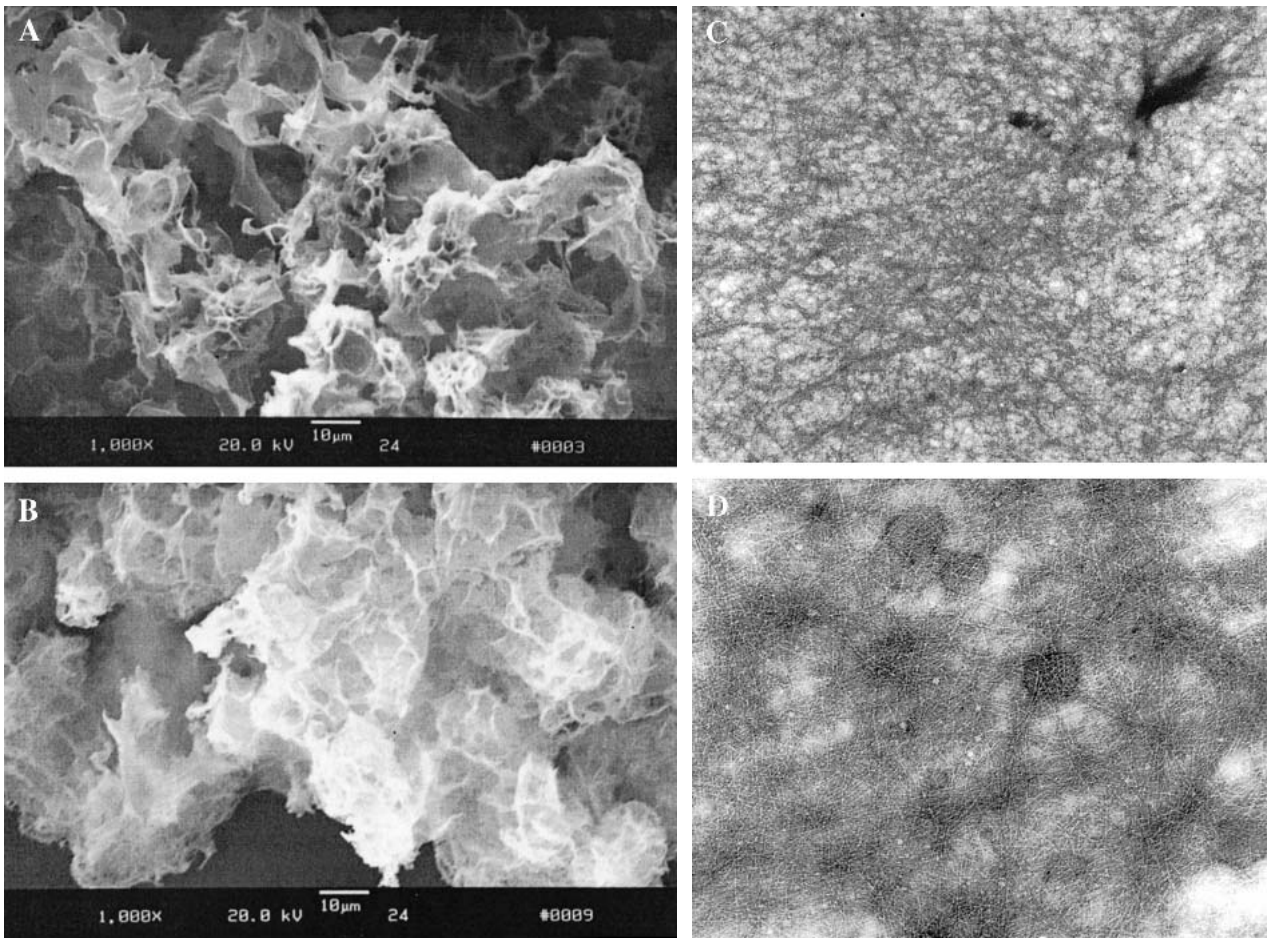

Fig. 5. Electron micrographs of various peptide preparations. (A) SEM of sCT/EDTA precipitate obtained from pH 5.5 solution, magnification 700×. (B) SEM of fibrillation structure of hCT obtained from pH 6.0 solution, magnification 600×. (C) Negatively stained TEM of reaction medium of 2 mM sCT + 6 mM EDTA at pH 5.5 taken 50 h after preparation, magnification 5000×. (D) Negatively stained TEM of reaction medium of 2 mM sCT + 6 mM EDTA at pH 5.5 taken 50 h after preparation, magnification 25,000×.

SEM from reaction solutions at pH 5, 5.5, 6.25, and 7.0. Figure 5A illustrates thin diaphanous sheets with smooth, nontextured surfaces, resembling the structure of amorphous freeze-dried sucrose (20). As a comparison, Fig. 5B shows the SEM of the solid obtained from a fibrillated 2 mM hCT (pH 6.0) solution by centrifugation through the MWCO 10,000 filter. The same diaphanous structure of smooth, nontextured sheets is visible as seen with the sCT/EDTA precipitate at the same magnification in Fig. 5A. The existence of fibrillated sCT in the sCT/EDTA precipitate is proven by the negative staining TEM technique. Figure 5C shows a typical region of a low magnification (5000×) TEM of the reaction mixture 2 mM sCT + mM EDTA at pH 5.5 taken 28 h after its preparation. A dense structure is seen composed of numerous anatomizing filaments, which is identical to that found during the early stages of fibrillation of a concentrated (30 mM) aqueous solution of hCT (2). Under the higher magnification (25,000×) of Fig. 5D, the numerous fibrils are seen to have a uniform thickness of approximately 20–40 nm. They are thicker than the protofibrils of approximately 8-nm thickness observed by Bauer *et al.* (21) under TEM in dilute (<1 mM) aqueous solutions of hCT. Their physical appearance is, however, strikingly similar. Negative staining of a 2 mM aqueous solution of sCT showed no evidence of a fibrillar structure at pH 5.5 (result not shown). We conclude that the observed EDTA-induced precipitation of sCT is a result of strong fibrillation of the peptide.

CONCLUSIONS

We have elucidated the structure of a previously reported (1) EDTA-induced precipitate of sCT in water. The results obtained for turbidity kinetics and binding stoichiometry support the suggestion that the acidic groups of the EDTA bind coulombically with the basic amino acids in sCT's primary sequence. The FT-IR results show that the random coil structure of dissolved sCT is lost an addition of EDTA and that there is a strong increase in β-sheet conformation. These changes in secondary structure of the sCT are essentially the same as those reported previously for the fibrillation of hCT in water. sCT is known, however, to have only a weak propensity to fibrillation in water. The electron micrographs of the EDTA/sCT system clearly show that a dense fibrillated structure of the peptide is present. We suggest that EDTA induces bridging aggregation of the sCT monomers, associated with a parallel strong increase in β-conformation of their C-terminal region. This allows formation of sCT fibrils, which precipitate as an sCT/EDTA complex. The sCT/EDTA precipitate can be redissolved in water to an extent strongly dependent of pH. The partial reversibility of this EDTA-induced precipitation makes a potential application in controlled peptide release feasible. This could be of especial interest in the case that EDTA induces precipitation of other peptides containing exposed basic amino acid residues.

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