Phenothiazine-Binding and Attachment Sites of $CAPP_1$–Calmodulin

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ABSTRACT: In the presence of Ca^{2+} norchlorpromazine isothiocyanate forms a monocovalent complex with calmodulin: $CAPP_1$–calmodulin (Newton et al., 1983). Trypsin digestion of [^{3}H]$CAPP_1$–calmodulin yields as the major radioactive peptide N^{ϵ}-CAPP-Lys-Met-Lys, corresponding to residues 75–77 of calmodulin. Stoichiometric amounts of all other expected tryptic peptides are also found, indicating that norchlorpromazine isothiocyanate selectively acylates Lys 75. A second molecule of CAPP-NCS can react, albeit slowly, with calmodulin to form $CAPP_2$–calmodulin. Fragments 38–74 and 127–148 are completely missing from the trypsin digests of $CAPP_2$–calmodulin without deliberate exposure to UV irradiation. Possibly the lengthy preparation of $CAPP_2$–calmodulin favors photolysis, caused by room lights, of the putative CAPP-binding domains located in these two peptides. Lys 148, the sole lysyl residue in fragment 127–148, is a probable site of attachment of the second molecule of CAPP. UV irradiation of $CAPP_1$–calmodulin, followed by digestion with trypsin, results in the selective loss of 50% each of peptides containing residues 38–74 and 127–148, suggesting that these peptides contain the hydrophobic amino acids that form the phenothiazine-binding sites. The loss of peptides encompassing residues 38–74 and 127–148, located in the amino and carboxyl halves of calmodulin, respectively, suggests that the hydrophobic rings of CAPP can bind at either one of the two phenothiazine sites. Computer modeling of $CAPP_1$–calmodulin with the X-ray coordinates of calmodulin (Babu et al., 1986) indicates that CAPP attached to Lys 75 cannot interact with the carboxyl-terminal phenothiazine-binding site. Thus, in solution, the structure of $CAPP_1$–calmodulin may be different from that of the crystal, and flexibility of the central helix may allow CAPP linked to Lys 75 to bind at either one of the two drug-binding sites with equal probability.

The binding of four atoms of Ca^{2+} to calmodulin induces the formation of two hydrophobic surface patches that are likely binding sites for the calmodulin antagonists, phenothiazines (Levin & Weiss, 1978; LaPorte et al., 1980; Tanaka et al., 1980). Much attention has been devoted to the identification of the phenothiazine-binding sites of calmodulin because the same sites that bind calmodulin antagonists are believed to bind calmodulin-regulated enzymes as well (LaPorte et al., 1980). The identification of these binding sites may therefore shed light on the unique ability of calmodulin to interact with so many different proteins. Proton and ^{113}Cd NMR studies of calmodulin and its fragments confirmed the existence of two phenothiazine-binding sites that were suggested by binding studies of the relatively low affinity phenothiazines (Forsen et al., 1980; Klevit et al., 1981; Krebs & Carafoli, 1982; Dalgarno et al., 1984). The identification of amino acid residues whose environment is affected by phenothiazine binding led to the proposal that the N-terminal helices of sites I and III and the C-terminal helices of sites II and IV participate in the formation of the drug-binding sites (Krebs & Carafoli, 1982; Dalgarno et al., 1984). Covalent modification of calmodulin with the tricyclic compound phenoxybenzamine, which also prevents interaction of calmodulin with its target proteins, yields three modified peptides (residues 38–75, 107–126, and 127–148), which contain most of the amino acid residues that had been proposed to form the interaction sites on the basis of NMR studies (Lukas et al., 1985). The inhibition of acetylation of Lys 75 and Lys 148 by trifluoperazine or their specific modification by a fluorenyl-based spin label (Jackson & Puett, 1984; Giedroc et al., 1985) as well as the specific labeling of Lys 148 with the affinity reagent 10-(3-propionyloxysuccinimido)-2-(trifluoromethyl)phenothiazine (Faust et al., 1987) suggested that these residues may also be part of the binding sites.

In this paper we demonstrate that the monocovalent complex of calmodulin with norchlorpromazine isothiocyanate ($CAPP_1$–calmodulin)[1] (Newton et al., 1983) is the result of the selective acylation of Lys 75. The equivalent loss of peptides 31–74 and 107–148 after UV irradiation of $CAPP_1$–calmodulin provides direct evidence that each of these fragments contains hydrophobic residues located in the vicinity of the aromatic rings of the bound norchlorpromazine.

Materials and Methods

Ram testis calmodulin (Newton et al., 1988), norchlorpromazine hydrochloride (CAPP), norchlorpromazine isothiocyanate (CAPP-NCS), [^{3}H]CAPP-NCS, $CAPP_1$–calmodulin (Newton et al., 1983), CAPP–Affigel (Newton et al., 1984), and calmodulin–Sepharose (Klee & Krinks, 1978) were obtained or prepared as described previously. Trypsin treated with L-1-(tosylamino)-2-phenylethyl chloromethyl ketone was purchased from Worthington. HPLC columns and solvents were from Waters Associates. PD-10 columns were purchased

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[1] Abbreviations: CAPP, 2-chloro-10-(3-aminopropyl)phenothiazine; CAPP-NCS, 2-chloro-10-(3-aminopropyl)phenothiazine isothiocyanate (norchlorpromazine isothiocyanate); $CAPP_1$–calmodulin or $CAPP_2$–calmodulin, 1:1 or 2:1 calmodulin–norchlorpromazine isothiocyanate covalent adduct; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; RT, retention time; dansyl-Cl, 1-(dimethylamino)naphthalene-5-sulfonyl chloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Table I: Extinction Coefficients of Calmodulin Tryptic Peptides at 215 nm[a]

peptide	ϵ_{215}[b]	$\epsilon^{1\%}_{215}$	integrator units[c]	integrator units/$\epsilon^{1\%}$
1–13	11 700	75	1300	17.9
14–30	17 200	94	1700	18.1
31–37	5 400	68	1200	17.6
38–71[d]	34 300	93	1900	20.4
38–74	39 200	97	2100	21.7
38–71[e]	32 100	87	1900	21.8
78–86[f]	5 200	64	1200	18.8
87–90	7 300	145	2600	17.9
91–106	16 200	93	2000	21.5
107–126	21 100	88	1700	19.3
127–148	21 100	84	1500	17.7

[a] Tryptic peptides of calmodulin were purified and analyzed as described under Materials and Methods. Peptide and protein concentrations were determined by amino acid analysis. [b] Extinction coefficients at 215 nm were measured in the HPLC solvents. [c] Integrator units from the Waters data module per microgram of peptide applied to the column. Peptides were resolved on a C18 reverse-phase column using a 0.1% PO_4H_3–CH_3CN gradient as described under Materials and Methods. [d] Peptide 38–71 (RT = 44.5 min). [e] Peptide 38–71 (RT = 47.5 min). [f] Peptide 78–86 was isolated from the tryptic digest of $CAPP_1$–calmodulin.

from Pharmacia Fine Chemicals. [*N-methyl*-^{14}C]Dansyl-Cl (63 000 cpm/nmol) was obtained from Amersham. All other reagents were of the best available grade.

Preparation of $CAPP_2$–Calmodulin. Calmodulin was treated with CAPP-NCS as described for $CAPP_1$–calmodulin (Newton et al., 1987), but the incubation at 37 °C was prolonged for 21–24 h. Calmodulin, CAPP-NCS, and $CAPP_1$– and $CAPP_2$–calmodulins were resolved by chromatography on a μBondapak C18 or a CN reverse-phase column (Newton et al., 1987). To achieve complete purification, $CAPP_2$–calmodulin was rechromatographed on a new C18 column using three successive linear gradients of CH_3CN in 0.1% (v/v) PO_3H_4 (10–35% over 5 min, 35–50% over 20 min to elute contaminating calmodulin and $CAPP_1$–calmodulin, and 50–75% over 20 min to elute $CAPP_2$–calmodulin). Elution was monitored by absorbance at 215 and 280 nm and by radioactivity. Fractions containing $CAPP_2$–calmodulin were pooled, lyophilized, dissolved in 0.05 M NH_4HCO_3, and passed through a PD10 column equilibrated and eluted with the same solvent. Contamination with calmodulin was thus reduced to 0.08 mol %.

Tryptic Digestion. Solutions of calmodulin and $CAPP_1$–calmodulin [1.6–2.3 mg/mL in 0.05 M NH_4HCO_3, 1 mM EGTA, and 0.1% (v/v) thiodiglycol] were brought to 37 °C and trypsin (1 mg/mL in 1 mM HCl) was added to a final concentration of 0.2 mg/mL. After 1 h at 37 °C, an identical aliquot of trypsin was added, and the incubation was continued for another hour. The peptides were purified by HPLC on C18 reverse-phase columns (10-μm resin diameter, 3.9 mm × 30 cm) with a 0–50% linear gradient of CH_3CN in 0.1% (v/v) H_3PO_4 over 50 min (Fullmer & Wasserman, 1979). The flow rate was 1.5 mL/min, and fractions were collected every 30 s. The column was monitored by absorbance at 215 and 280 nm and, when indicated, by radioactivity. Analytical runs were done with 25-μL aliquots; up to 1.9-mL samples were used for preparative purposes.

Identification and Quantification of Calmodulin Peptides. The fractions containing calmodulin peptides were flash evaporated and dissolved in water. A new C18 column, run under the same conditions, was used to rechromatograph the peptides (23–116 nmol). Peptides 1–13, 31–37, and 107–126 required a third chromatography to remove residual contamination with trypsin autolytic fragments. Peptides were identified and quantitated by amino acid analysis.[2] The absorbance at 215 nm of the solutions used for amino acid analyses was measured on a Beckman DU-7 spectrophotometer to determine the extinction coefficients (Table I). With the exception of peptide 87–90, the $\epsilon^{1\%}_{215nm}$ are similar. The extinction coefficients at 215 nm of most amino acids are low (20–100 and 140 for glutamine and asparagine). At the low pH used for these determinations, those of tyrosine, phenylalanine, histidine, and methionine are 5700, 4800, 5300, and 1000, respectively (Saidel et al., 1952). As shown in Figure 1 the value of $\epsilon^{1\%}_{215nm}$ increases linearly with the number of tyrosine, phenylalanine, histidine, and methionine residues. If one assumes that the molar absorbance at 215 nm of the peptides (ϵ) is the sum of the absorbances of the individual residues

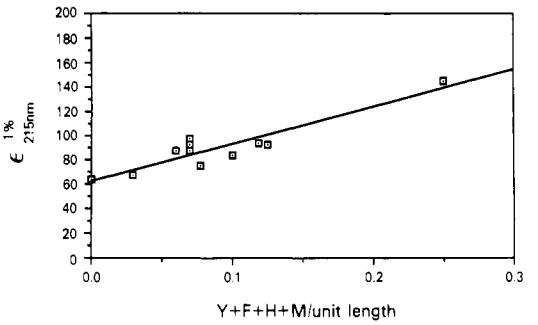

FIGURE 1: Effect of amino acid composition on the $\epsilon^{1\%}_{215nm}$ of calmodulin peptides. The $\epsilon^{1\%}_{215nm}$ of calmodulin peptides is plotted against the sum of tyrosines, phenylalanines, histidines, and methionines per unit length of the corresponding peptides. Since the ϵ_{215nm} of methionine is only 20% of that of the other three amino acids, methionine was assigned a value of 0.2.

$$\epsilon = p(N - 1) + 5700N_Y + 4800N_F + 1000N_M + 5300N_H \quad (1)$$

where N is the total number of residues and N_Y, N_F, N_M, and N_H are the numbers of tyrosines, phenylalanines, methionines, and histidines, respectively, the molar absorbance of the peptide bond (p) calculated with the dataplot program is 637 ± 36. This value is lower than the molar absorbance of the peptide bond (958 ± 85) determined by Goldfarb (1953) for polylysine (N = 6–83). The absorbance at 215 nm of most peptides, with chain length above 10, is not significantly affected by the amino acid composition and reflects mainly the absorbance of the peptide bond. Since absorbance at 215 nm can provide a good estimate of peptide concentration, integrator units obtained from a Waters data module (Model 730) could be used to quantitate the tryptic fragments of calmodulin. Aliquots of peptide solutions were chromatographed on the C18 column to determine the number of integrator units per microgram of peptide (100% peptide recovery was assumed). As shown in Table I, the ratio integrator units/microgram of peptide to $\epsilon^{1\%}_{215nm}$ varies between 17.6 and 21.8. This variation reflects variable recoveries of peptides from the column.[3]

Amino Acid Analyses. Hydrolysis was for 22 h at 110 °C as described previously (Spackman et al., 1958). Analyses of 2–9 nmol of peptide were performed on a Waters amino acid analyzer equipped with a CAT EX resin column (0.4 × 25 cm) at 58 °C using the Pickering sodium eluent buffers, pH 3.15 (adjusted to pH 3.08) and pH 7.4. Two nonlinear gradients [100 to 20% pH 3.08 buffer for 45 min (programmer

[2] Data made available to the reviewers.

[3] Calmodulin eluted at pH 2 exhibits a ratio of 11.5, but when the pH of the eluting buffer is 6.3, the recovery of calmodulin is increased and the value of the ratio is raised to 17.3.

setting 8) and 20 to 0% pH 3.08 buffer for 15 min (programmer setting 9)] were used. Under these conditions, trimethyllysine elutes 4 min before lysine. Postcolumn derivatization with orthophthalaldehyde was performed in the presence of hypochlorite. Analyses of picomole amounts of peptides were performed with the Pico Tag prederivatization system (Heinrikson & Meredith, 1984) according to the manufacturer's instructions. Equal volumes of HPLC column fractions, devoid of 215-nm absorbance, that eluted immediately before or after the peptides were hydrolyzed in the same way and the values from these analyses used as blanks.

Amino-Terminal Group Determinations. The procedure of Weiner et al. (1972) was used. Peptide fractions (200–250 pmol) were lyophilized, dissolved in 10 μL of 0.2 M $NaHCO_3$, pH 9.8, and lyophilized. The dried samples were dissolved in 10 μL of the same buffer made 1% (w/v) sodium dodecyl sulfate and mixed with 12 μL of 1 mM [^{14}-C]dansyl-Cl in acetone. The samples were incubated in the dark for 2 h at 37 °C. After hydrolysis for 20 h at 110 °C in 6 N HCl, the dried amino acids were dissolved in 50% pyridine (sequenal grade from Pierce Chemical Co.) and mixed with 2 μL of a mixture of dansylated amino acids. One-microliter samples were spotted on Cheng Chin polyamide sheets (Pierce Chemical Co.) and chromatographed in solvents I and II of Weiner et al. (1972). Radioactive spots were identified by comigration with fluorescent spots of known standards.

Identification of Monodansylated Lysines. N^{ϵ}-Acetyl-DL-lysine and N^{α}-acetyl-L-lysine (25 nmol) were dansylated at pH 9.8 as described above. Lyophilized dansylated acetyl amino acids were converted to N^{ϵ}-dansyllysine and N^{α}-dansyllysine by acid hydrolysis. The monodansylated derivatives were resolved from each other in the first chromatographic system by addition of 0.1% (w/v) $CuSO_4$ to the first solvent, 1.5% HCOOH (Greenstein & Winitz, 1961). The R_f values of the α and ε derivatives were 1 and 0.94, respectively.

UV Irradiation of Calmodulin and $CAPP_1$–Calmodulin. Aliquots (70 μL) of calmodulin and $CAPP_1$–calmodulin (1.1 mg in 0.05 M NH_4HCO_3, 3 mM Ca^{2+}) were placed in quartz microcuvettes (1-cm light path) and irradiated at 4 °C for 10, 20, and 30 min. The light source was a 450-W Hanovia mercury arc lamp with 5% $CuSO_4$ as a filter. After addition of EGTA (6 mM) and thiodiglycol (0.06%), the samples were digested with 0.34 mg/mL trypsin. The resulting peptides were resolved by HPLC.

Results

We previously demonstrated the Ca^{2+}-dependent, covalent attachment of 1 mol of CAPP to 1 mol of calmodulin (Newton et al., 1983). As shown in Figure 2A, if the incubation time is increased from 6 to 21–24 h (without further addition of CAPP), a second derivative is formed that is partially resolved from $CAPP_1$–calmodulin. The formation of this derivative is Ca^{2+} dependent (Figure 2B). It was identified as a 2:1 adduct ($CAPP_2$–calmodulin) on the basis of its absorbance at 280 nm and its specific radioactivity. The UV absorbance spectra of the proteins are shown in Figure 3. Since the extinction coefficient of CAPP at 259 nm is more than 10 times that of calmodulin, the near-UV spectrum of $CAPP_1$–calmodulin is dominated by the absorbance of CAPP with a broad band between 300 and 330 nm and a sharp peak at 259 nm. The molar extinction coefficients of $CAPP_2$–calmodulin at 310 and 259 nm are 2.1 and 1.9 times those of $CAPP_1$–calmodulin at the same wavelengths. The far-UV spectra of the two calmodulin derivatives are dominated by the absorbance of calmodulin, and therefore the absorbances

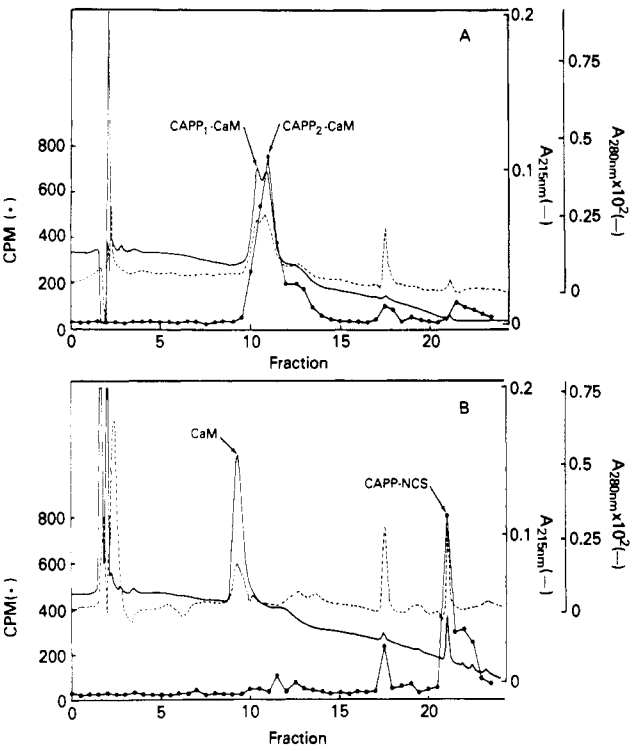

FIGURE 2: Ca^{2+}-dependent formation of $CAPP_2$–calmodulin. Calmodulin was incubated with CAPP-NCS in the presence of Ca^{2+} (A) or EGTA (B) for 20 h as described under Materials and Methods. Aliquots (150 μL) were analyzed by HPLC on a 0.39 × 30 cm CN reverse-phase column (10-μm resin diameter) eluted over 20 min with a 5–65% linear gradient of CH_3CN in 0.01 M potassium phosphate buffer, pH 6.25, containing 2 mM EGTA. Fractions collected every 30 s were analyzed for radioactivity and the column monitored for absorbance at 215 and 280 nm. Recovery of radioactivity was 88% (A) and 97% (B). The UV-absorbing and radioactive peaks are identified in the figure.

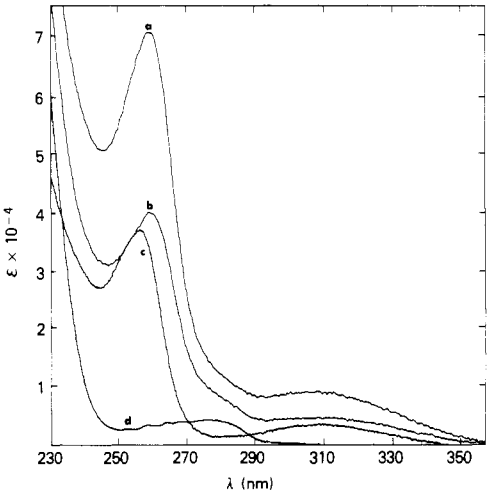

FIGURE 3: UV absorbance spectra of calmodulin, CAPP, and $CAPP_1$– and $CAPP_2$–calmodulins. The solutions used for the UV spectra were (a) 2.8 μM $CAPP_2$–calmodulin in 0.05 M NH_4HCO_3, (b) 3.4 μM $CAPP_1$–calmodulin in 0.05 M NH_4HCO_3, (c) 3.4 μM CAPP in ethanol, and (d) 48 μM calmodulin in 10 mM Hepes buffer, pH 7.7. The protein concentrations were determined by amino acid analysis.

of $CAPP_1$– and $CAPP_2$–calmodulins at 215 nm are similar (214, 256, and 297 × 10^3 for calmodulin and $CAPP_1$– and $CAPP_2$–calmodulin). The stoichiometry of the CAPP–calmodulin adducts determined at 259 nm was 1 mol/mol for $CAPP_1$–calmodulin and 1.9 mol/mol for $CAPP_2$–calmodulin. The stoichiometries based on specific radioactivities were also 1.1 and 1.9.

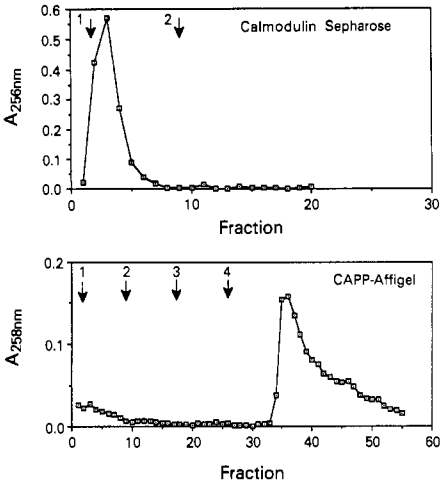

FIGURE 4: Interaction of $CAPP_1$–calmodulin with calmodulin and CAPP. (Top) ^3H-Labeled $CAPP_1$–calmodulin [200 μg in 0.38 mL of 40 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM dithiothreitol, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$ (buffer 1)] was applied to a calmodulin–Sepharose column (0.75 × 1.6 cm). After standing at 4 °C for 40 min, the column was washed with buffer 1 (first arrow) and with the same buffer containing 0.4 M KCl, 1 mM $MgCl_2$, and 2 mM EGTA instead of $CaCl_2$ (second arrow). Recoveries of radioactivity and UV-absorbing material were 100 and 92%, respectively. (Bottom) $CAPP_1$–calmodulin (175 μg in 0.08 mL of 0.05 M NH_4HCO_3, 1 mM $CaCl_2$, was applied to a CAPP–Affigel column (0.7 × 2.6 cm) equilibrated in buffer 1. After the column was washed with buffer 1 (first arrow), buffer 1 made 0.2 M KCl (second arrow), and buffer 1 made 0.4 M KCl (third arrow), $CAPP_1$–calmodulin was eluted by replacing $CaCl_2$ with 2 mM EGTA in the last buffer (fourth arrow). Recovery of $CAPP_1$–calmodulin in the last peak, measured by absorbance at 258 nm, was 94%.

The Ca^{2+}-dependent attachment of 2 mol of CAPP/mol of calmodulin is consistent with the reported presence of two phenothiazine-binding sites on calmodulin (Levin & Weiss, 1978; Marshak et al., 1985). The rate of formation of the ternary adduct, $CAPP_2$–calmodulin, was much slower than that of $CAPP_1$–calmodulin. Attempts to increase this rate by starting the reaction with $CAPP_1$–calmodulin[2] or by increasing the temperature to 40 °C (data not shown) were not successful.

Interaction of $CAPP_1$–Calmodulin and $CAPP_2$–Calmodulin with Phenothiazine and Calmodulin. As shown in Figure 4 (upper panel) CAPP, when covalently bound to calmodulin, no longer binds to a calmodulin–Sepharose column. In contrast, $CAPP_1$–calmodulin, applied to a CAPP–Affigel 10 column in the presence of Ca^{2+}, was quantitatively retained on the column and eluted upon removal of Ca^{2+} by EGTA. Thus, the remaining phenothiazine-binding site of $CAPP_1$–calmodulin has preserved its integrity and Ca^{2+} dependence. $CAPP_2$–calmodulin was partially retained on the CAPP–Affigel column but was not dissociated with EGTA. Quantitative elution of $CAPP_2$–calmodulin required denaturing conditions such as 6 M urea. Similar Ca^{2+}-independent binding was observed upon chromatography of $CAPP_2$–calmodulin on phenyl-Sepharose.

Identification of the CAPP Attachment Sites of Calmodulin. To identify the site of attachment of CAPP to calmodulin, [^3H]$CAPP_1$–calmodulin was digested with trypsin. $CAPP_1$–calmodulin was more resistant to proteolysis than calmodulin, which was completely proteolyzed after 1.5 h at 37 °C in the presence of 0.25 mg/mL trypsin. $CAPP_1$–calmodulin was not fully digested after 6 h under the same conditions. A second addition of trypsin was required to achieve near complete cleavage of $CAPP_1$–calmodulin. Since the peptide bonds at Lys 21 and 94, both flanked by aspartyl residues, are resistant to trypsin (Watterson et al., 1980), only

Table II: Recovery of Peptides from Calmodulin and $CAPP_1$– and $CAPP_2$–Calmodulin[a]

peptide	calmodulin (mol %)	$CAPP_1$–calmodulin[b] (mol %)	$CAPP_2$–calmodulin (mol %)
1–13	113[c]	133 (1.2)[d]	113 (1.0)
14–30	126[c]	139 (1.1)	135 (1.1)
31–37	103	118 (1.2)	130 (1.3)
38–74[e]	83	118 (1.4)	ND[f]
75–86[g]	86	126 (1.5)	155 (1.8)
87–90	88	124 (1.4)	123 (1.4)
91–106	91	106 (1.2)	103 (1.1)
107–126	102	85 (0.8)	93 (0.9)
127–148	97	106 (1.1)	12 (0.1)

[a] Tryptic digests of calmodulin (2.65 nmol), $CAPP_1$–calmodulin (3.31 nmol), and $CAPP_2$–calmodulin (0.6 nmol) were analyzed by HPLC as shown in Figure 5. The amounts of $CAPP_1$– and $CAPP_2$–calmodulin used for the analyses were calculated on the basis of radioactivity by using their respective specific activities. [b] The recovery of individual peptides, expressed in mole percent, was based on the integrator units listed in Table I. The ratio of the recovery of a given peptide in the modified calmodulin to that of the same peptide in calmodulin is indicated in parentheses. [c] The anomalously high yields were attributed to contaminants due to trypsin autolysis. [d] The relatively high recoveries may be due to inaccuracy in the measurement of the amount of $CAPP_1$–calmodulin subjected to HPLC. [e] Sum of peptides 38–74 and 38–71. [f] ND, not identified. [g] Sum of peptides 75–86 and 78–86 in the case of calmodulin. $CAPP_1$– and $CAPP_2$–calmodulin digests contained only peptide 78–86.

10 peptides and 1 lysyl residue are expected. As shown in Figure 5A all the expected peptides were accounted for with the exception of peptides containing Lys 75. The tripeptide 75–77 and dipeptide 76–77 (cleavage at Lys 75 is usually slow), which are expected to elute at the void volume (Manalan & Klee, 1987), would not be detected because of their low absorbance. The presence of a small amount of peptide 75–86 indicated that cleavage at Lys 77 was also incomplete.[4] In agreement with Watterson et al. (1980), anomalous cleavage at Met 71 was observed. Two peptides (RT 44.5 and 47.5 min) were identified by their amino acid composition as fragments 38–71. Their different retention times may be due to partial oxidation of methionines or to deamidation. The relative amounts of peptides 38–74 and 38–71 varied from digest to digest. Peptide 72–74 (result of partial cleavage at Met 71) should elute early and would not be detected. The good recoveries in the $CAPP_1$–calmodulin digest of tryptic peptides 1–13, 14–30, and 78–86 indicate that trypsin cleavage at Lys 13, 30, and 77 is not impaired and that these lysines are therefore not substituted (Table II). Lys 21, 94, and 148, His 107, and Tyr 99 and 138, located in peptides 14–30, 91–106, 107–126, and 127–148 (all recovered in excellent yield), are also not involved in complex formation (Table II). The only lysine that was not monitored by the tryptic peptide map is Lys 75. Substitution of Lys 75 should prevent trypsin cleavage at this site and yield a labeled peptide 75–77 (N^{ϵ}-CAPP-Lys-Met-Lys) and possibly a labeled peptide 75–86 if the digestion at Lys 77 were incomplete. As shown in Figure 5B two new UV-absorbing peaks were present in the digest of $CAPP_1$–calmodulin (RT 41.5 and 53.5 min). Both absorbed at 280 nm and were radioactive as expected for CAPP-substituted peptides. The minor peak eluting at 53.5 min contained 20% of the radioactivity and coeluted with intact $CAPP_1$–calmodulin. The major radioactive peptide (RT 41.5 min) contained 79% of the radioactivity applied to the column. It was isolated from a digest of 3 mg of $CAPP_1$–calmodulin and further purified by two additional HPLC steps as illus-

[4] The lack of methionine in fragment 78–86 rules out contamination by peptide 76–86, which coelutes with peptide 78–86.

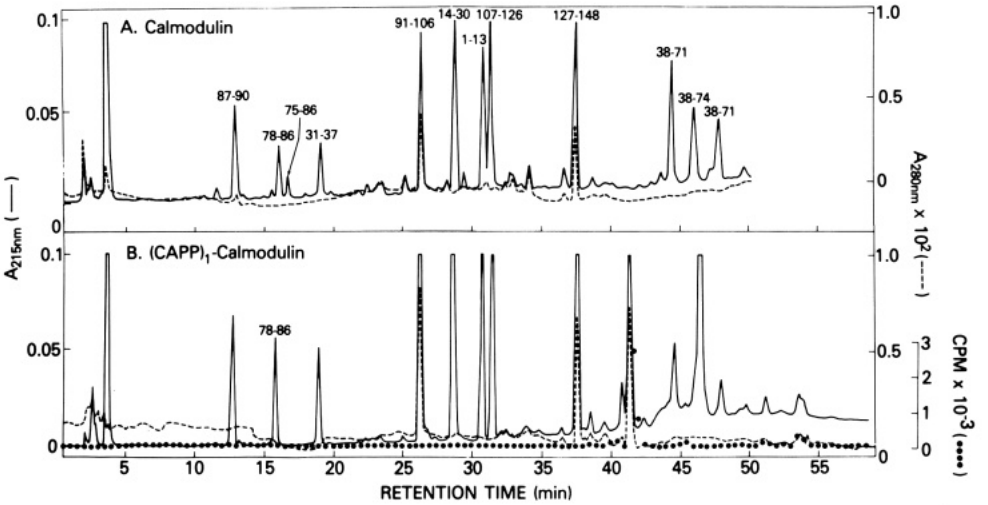

FIGURE 5: HPLC peptide maps of the tryptic digests of calmodulin and $CAPP_1$–calmodulin. Calmodulin (2.8 mg/mL) and [^{3}H]$CAPP_1$–calmodulin (2.0 mg/mL) were digested with trypsin as described under Materials and Methods. Samples of the digests (3.78 nmol of calmodulin and 3.31 nmol of $CAPP_1$–calmodulin) were analyzed by HPLC on C18 column as described under Materials and Methods. The low rate of cleavage at Met 71 in the $CAPP_1$–calmodulin digest resulted in a better recovery of peptide 38–74 (RT 46.5 min) than in the calmodulin digest. The total recovery of radioactivity was 99%.

Table III: Amino Acid Composition of Radiolabeled Tryptic Peptide of $CAPP_1$–Calmodulin[a]

	fraction 27 (nmol/mL)	fraction 28 (nmol/mL)
Asp	−0.2 ± 0.4	−0.1 ± 0.4
Glu	−0.4 ± 0.4	0 ± 0.4
Ser	0 ± 1.0	0.4 ± 1.0
Gly	−1.5 ± 1.0	0 ± 2.0
His	0	0
Arg	−0.2 ± 0.3	0.1 ± 0.3
Thr	0 ± 0.3	0.2 ± 0.6
Ala	−0.5 ± 0.6	0.1 ± 0.6
Pro	−0.2 ± 0.3	0.1 ± 0.3
Tyr[b]	ND	ND
Val	0.2 ± 0.6	0.6 ± 0.9
Met	3.6 ± 0.2	2.7 ± 0.6
Ile	−0.6 ± 0.4	−0.3 ± 0.7
Leu	0	0
Phe	0	0
Lys	4.7 ± 0.2	6.2 ± 0.2
Lys/Met	1.3	2.3

[a] Ten-microliter aliquots of fractions 27 and 28 (Figure 6, panel 3) were hydrolyzed and analyzed by using the Pico Tag prederivatization system. The values reported in the table are the average plus or minus the range of duplicate determinations. They are corrected for blank values obtained by duplicate amino acid analysis of similar aliquots of fractions 25 and 31. [b] ND, not determined. The presence of a side product of the derivatization, eluting 0.15–0.2 min after PTC tyrosine, prevented quantitation of low levels of tyrosine.

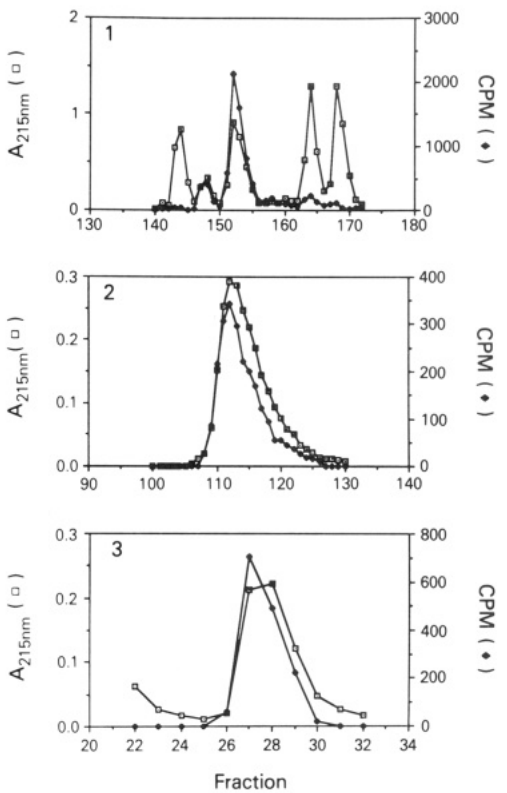

FIGURE 6: Purification of radiolabeled tryptic peptide of $CAPP_1$–calmodulin. (Panel 1) Preparative HPLC of a $CAPP_1$–calmodulin tryptic digest on a C18 column as described under Materials and Methods. (Panel 2) Fractions 151–155 from step 1 were pooled and rechromatographed on a new C18 column eluted with a 25–35% linear gradient of CH_3CN in 0.1% PO_4H_3 over 50 min followed by a 35–50% CH_3CN gradient in the same solvent (5 min). (Panel 3) Fractions 109–116 from column 2 were pooled, and 56% of the pooled material was applied to the same column using a 15–50% gradient of CH_3CN in 0.1% CF_3COOH (60 min) followed by a 10-min 50–65% gradient of CH_3CN. The flow rate was 1.5 mL/min, and fractions were collected every 18 s. Radioactivity was measured on duplicate 15- and 25-μL aliquots for columns 2 and 3, respectively.

trated in Figure 6. In this digest, performed with a more extensively modified protein, 61% of the total radioactivity was associated with the peak (Figure 6, panel 1). Fractions 150–156 were pooled and rechromatographed on a new C18 column using a shallow gradient of CH_3CN (Figure 6, panel 2). Loss of radioactivity (42%) during this process suggested some modification due to photolysis. Because of the lack of coincidence between radioactivity and UV absorbance, the peptide was subjected to a third HPLC step (Figure 6, panel 3). Minor contaminants were removed, but the A_{215nm} and radioactivity profiles remained slightly displaced. The amino acid compositions of the two peak tubes (27 and 28) are shown in Table III. Methionine and lysine are the major components of these two fractions with lysine to methionine ratios of 1.3 and 2.3 for tubes 27 and 28, respectively. The specific activities of the two fractions expressed in counts per minute per nanomole of methionine were similar, 3300 and 3400, respectively. The amino acid compositions suggested that the labeled peptides may be fragment 75–77 (Lys-Met-Lys) in fraction 28 and fragment 76–77 (Met-Lys) or 75–76 (Lys-Met) in

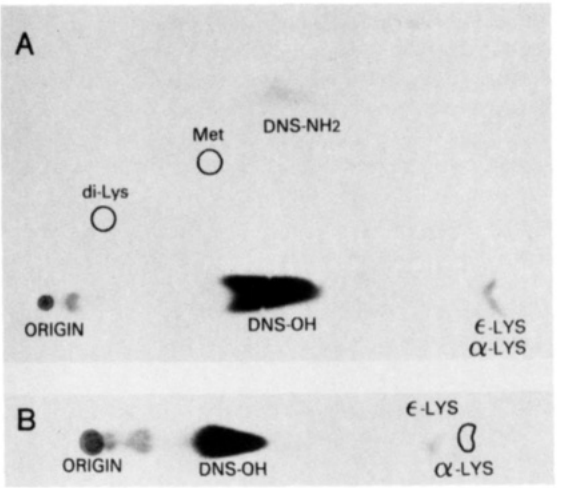

FIGURE 7: End group analysis of the radiolabeled tryptic peptide. (A) Radiolabeled peptide obtained as described in the legend to Figure 6C (fraction 27) dansylated with [^{14}C]dansyl chloride and acid hydrolyzed. The dansylated amino acids were resolved by two-dimensional chromatography and visualized by autoradiography. (B) Autoradiogram of the one-dimensional chromatographic pattern of the dansylated peptide (fraction 27, Figure 6C) performed in the presence of $CuSO_4$ to resolve the α- and ϵ-DNS-Lys (similar patterns were obtained with fraction 28). The positions of dansylated amino acid standards (Met, dansyl methionine; dilys, didansyllysine; ϵ-Lys, N^{ϵ}-dansyllysine; α-Lys, N^{α}-dansyllysine) are indicated on the autoradiograms.

fraction 27. Abnormal cleavage at Met 76 could yield a Lys-Met fragment.

End group analysis was used to distinguish between these possibilities. As shown in Figure 7A monodansyllysine is the only end group found in fraction 27. When the α and ϵ derivatives were resolved by thin-layer chromatography in the presence of Cu^{2+}, N^{ϵ}-dansyllysine was the major radioactive component (Figure 7B), confirming the carboxyl-terminal position of a free lysine corresponding to Lys 77 and ruling out the presence of peptide 75–76 (Lys-Met). The lack of dansylated methionine is in agreement with the central position of Met 76 and rules out the presence of peptide 76–77 (Met-Lys). The absence of didansylated lysine indicates that the ϵ amino group of the amino-terminal lysine (Lys 75) is blocked. The expected N^{α}-dansyllysine was not detected on the autoradiogram, but when radioactivity was measured by scintillation counting of the spots corresponding to α- and ϵ-dansyllysine, 90% of the total radioactivity was found associated with ϵ-dansyllysine and 10% with α-dansyllysine. This low recovery of α-dansyllysine can be explained either by the instability of the ϵ-thiocarbamyl derivative of lysine or by the incomplete removal of the CAPP–thiocarbamyl group upon acid hydrolysis of ϵ-acylated lysine (Konigsberg, 1967). End-group analyses of fraction 28 gave similar results (data not shown). Thus, the only modified peptide is apparently N^{ϵ}-CAPP-Lys-Met-Lys. The presence of contaminants may explain the difference in absorbance at 215 nm and lysine to methionine ratio.

$CAPP_2$–calmodulin was also digested with trypsin. The recovery of fragment 127–148 was only 10 mol %, and we were unable to identify peptides 38–74 and 38–71 (Table II). All other peptides were recovered in excellent yields. The HPLC pattern revealed new UV-absorbing peaks and a complete loss of identifiable radioactive peptides (21% of the radioactivity applied to the column was eluted at the void volume, and the remaining 78% was spread throughout the entire elution profile), thus preventing definitive identification of the attachment sites in $CAPP_2$–calmodulin. The selective loss of fragments 38–74 and 127–148 and the destruction of the radiolabeled peptide contrast with the good preservation of the rest of the molecule and suggest that Lys 148, the only lysine present in peptide 127–148, is the probable site of attachment for the second molecule of CAPP.

Table IV: Tryptic Peptides of UV-Irradiated Calmodulin and $CAPP_1$–Calmodulin[a]

peptide	calmodulin (mol %)	$CAPP_1$–calmodulin (mol %)	ratio[b]
1–13	98 ± 5	106 ± 9	1.1
14–30	118 ± 6	108 ± 9	0.9
31–37	79 ± 1	52 ± 6	0.7
38–74[c]	74 ± 2	37 ± 2	0.5
75–86	126 ± 6	108 ± 8	0.9
87–90	96 ± 3	94 ± 5	1.0
91–106	84 ± 3	87 ± 9	1.0
107–126	80 ± 4	54 ± 1	0.7
127–148	91 ± 2	39 ± 1	0.4

[a] Tryptic digests of UV-irradiated calmodulin and $CAPP_1$–calmodulin were analyzed as described in legend to Table II. The results are the average of three determinations. [b] Recovery of peptide in the $CAPP_1$–calmodulin digest over recovery of the same peptide in the calmodulin digest. [c] Sum of peptides 38–74 and 38–71.

Identification of CAPP-Binding Site(s). Having characterized the attachment site of the side chain of CAPP to calmodulin, we tried to identify the regions of calmodulin responsible for CAPP binding. For this purpose $CAPP_1$–calmodulin was irradiated at 4 °C at wavelengths above 310 nm. Only those amino acids close to the phenothiazine ring would be expected to be modified by such irradiation. The recovery of peptides after trypsin digestion of radiolabeled $CAPP_1$–calmodulin and calmodulin is shown in Table IV. Irradiation of native calmodulin did not affect the recovery of any of the tryptic peptides. Four peptides were found in diminished yield after digestion of irradiated $CAPP_1$–calmodulin. The yields of fragments 38–74 and 127–148, located at the carboxyl ends of domains II and IV, were reduced to 50 and 40% of those of the same peptides in the calmodulin digest. The yields of adjacent peptides 31–37 and 107–126 were also significantly decreased to 70% each. Irradiation was accompanied by a loss of radioactivity, reflecting photodestruction of the ligand. Thus, isolation of the labeled peptides was not possible after photolysis.

Discussion

$CAPP_1$–calmodulin, the covalent adduct of calmodulin with norchlorpromazine isothiocyanate, was used to characterize the phenothiazine-binding site(s) of calmodulin. Several lines of evidence suggest that, in $CAPP_1$–calmodulin, CAPP is bound to a specific site: (1) the 1:1 stoichiometry; (2) the inability of CAPP to bind calmodulin once it is linked to calmodulin; (3) the inability of $CAPP_1$–calmodulin to activate some calmodulin-regulated enzymes (Newton & Klee, 1984; Newton et al., 1985); and (4) the high affinity of $CAPP_1$–calmodulin for Ca^{2+}. $CAPP_1$–calmodulin retains 2 mol of Ca^{2+}/mol under conditions that allow almost complete decalcification of calmodulin (data not shown).

The characterization of $CAPP_1$–calmodulin described in this paper provides additional evidence for the specific modification of calmodulin by CAPP-NCS. The isothiocyanate group reacts with a single lysyl residue of calmodulin, Lys 75. The high reactivity of Lys 75 (Giedroc et al., 1985) may favor attachment at this particular site. In spite of the presence of two drug-binding sites on calmodulin, the covalent attachment of a second molecule of CAPP proceeds slowly. There is no definitive evidence for a difference in affinity between the two sites (Levin & Weiss, 1978; Marshak et al., 1985); on the

contrary, a positive cooperativity between them has been suggested (Johnson et al., 1983; Newton et al., 1983). The slow reaction rate may be due to the lack of a reactive residue near the isothiocyanate group of the second CAPP molecule. Although the second attachment site was not definitively identified, the loss of fragment 127–148 (containing a single lysine, Lys 148) and the good recovery of the peptides containing the other lysines (except Lys 75) of $CAPP_2$–calmodulin suggest that in this case both Lys 75 and 148 are acylated. Further identification was prevented by the presumably photolytic loss of the radiolabel during the handling. These observations are in good agreement with the report of a phenothiazine inhibition of the Ca^{2+}-dependent acylation of calmodulin with a fluorenyl-based spin label reagent that occurs preferentially at Lys 148 and 75, with Lys 75 being the most reactive (Jackson & Puett, 1984). A different phenothiazine derivative, 10-(3-propionyloxysuccinimido)-2-(trifluoromethyl)phenothiazine, is preferentially linked to Lys 148 (Faust et al., 1987). Thus, different drugs may interact with calmodulin in different ways, as do the calmodulin-regulated enzymes (Newton et al., 1984; Klevit & Vanaman, 1984; Putkey et al., 1986; Jarrett, 1986; Craig et al., 1987). Distinct attachment and binding sites for CAPP are expected on the basis of the structural requirements exhibited by calmodulin antagonists. It has been proposed that the amino-terminal helices of the first and third Ca^{2+} sites and the F helices of the second and fourth Ca^{2+}-binding domains contain recognition sites for the hydrophobic rings (Krebs & Carafoli, 1982; Dalgarno et al., 1984). The side chains of the drugs, bearing the positive charges important for high affinity (Prozialeck & Weiss, 1982), lie close to the stretch of acidic residues in the central helix, near Lys 75 and 148 (Strynadka & James, 1988), thus explaining the protection against acetylation of Lys 75 and 148 by phenothiazine (Giedroc et al., 1985). Lys 75 does not appear directly involved in the interaction since its acylation by CAPP-NCS does not prevent calmodulin binding to phenothiazine. Lys 75 is also not required for interaction with or activation of target enzymes because the affinity of enzymes like myosin light chain kinase and calcineurin [which also protect Lys 75 against acetylation (Jackson et al., 1987; Manalan & Klee, 1987)] is only slightly decreased by acylation of Lys 75 by CAPP-NCS.

The Ca^{2+}-dependent acylation of Lys 75 and Lys 148 by CAPP-NCS must follow binding of CAPP to one of the two hydrophobic patches induced by Ca^{2+} (LaPorte et al., 1980; Tanaka et al., 1980). Thus, irradiation of $CAPP_1$–calmodulin at wavelengths above 300 nm may result in the modification of residues located near the bound phenothiazine and the subsequent loss of peptides containing these amino acid residues. In similar experiments performed with mixtures of phenothiazine and calmodulin, the high concentrations of phenothiazine required to support sufficient site occupancy may lead to large amounts of nonspecific binding and the partial loss of most peptides. UV irradiation of $CAPP_1$–calmodulin resulted in the selective loss of 50% of two sets of peptides corresponding to the F helix of the first and both E and F helices of the second Ca^{2+}-binding domain (residues 31–74) and the corresponding parts of the third and fourth Ca^{2+}-binding domain (residues 107–148). The almost complete loss of fragments 38–74 and 127–148 from the digest of $CAPP_2$–calmodulin may reflect spontaneous photolysis of the doubly modified protein. The peptides lost upon UV irradiation are not necessarily the original targets of photolysis that contain the residues forming the phenothiazine-binding sites. However, the fact that these peptides contain five of the eight residues that have been proposed to be in the proximity of the phenothiazine rings by NMR studies (Dalgarno et al., 1984) and computer modeling of the structure of the calmodulin–trifluoperazine complex (Strynadka & James, 1988) is consistent with the proposed identification of the phenothiazine-binding sites. Similar locations for the drug-binding sites of calmodulin were proposed by Lukas et al. (1985), who labeled peptides 38–75, 107–126, and 127–148 by modification with phenoxybenzamine.

The crystal structure of calmodulin shows the molecule as an elongated dumbbell with large hydrophobic patches near each end separated by a long stretch of α helix (Babu et al., 1985; Kretsinger et al., 1986). Lys 75 is located in the central helix near its amino-terminal end. Computer modeling, using the program Chem-X, indicates that whereas it is easy to fit the hydrophobic rings of the covalently bound norchlorpromazine molecule to the amino-terminal site, it is not possible to reach the carboxyl-terminal site. Because acylation of Lys 75 by CAPP-NCS results in the almost equal sensitization to photolysis of both the amino-terminal and carboxyl-terminal hydrophobic patches, it is likely that, even in the presence of Ca^{2+}, the central helix is highly flexible in solution. Possibly, binding of 1 mol of CAPP induces a conformational change in the central helix allowing interaction at either one of the two sites. There is also evidence that in solution the central helix of Ca^{2+}-free calmodulin is not fully extended (Seaton et al., 1985; Heidorn & Trewhella, 1988; Small & Anderson, 1988). The cross-linking of a calmodulin derivative in which Gln 3 and Thr 146 have been replaced by cysteines led Persechini and Kretsinger (1988) to propose that the central helix of calmodulin in solution is bent, bringing the halves of the molecule closer to each other. Thus, the crystal structure of calmodulin may not be completely representative of the structure of the protein in solution at pH 7.5.

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Registry No. Lys, 56-87-1; phenothiazine, 92-84-2.

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Orientation of Type VI Collagen Monomers in Molecular Aggregates†

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ABSTRACT: Type VI collagen, prepared from guanidine extracts of human amnion, contains very little monomeric material, the major forms being dimers and tetramers. In order to study the orientation of the molecules in these aggregates, they were digested with pepsin followed by bacterial collagenase. Two fragments were isolated, one containing part of the inner globular domain still attached to part of the triple helix and the other containing large fragments of the outer globular domain. Each fraction was further analyzed; peptides were isolated and their amino-terminal amino acid sequences determined. By comparing the determined sequences with published data, it was found that the outer globular domain contained sequences derived from the amino-terminal domain of all three chains of type VI collagen whereas the inner globular domain contained sequences from the carboxy-terminal domain. This provided direct chemical evidence that dimers and tetramers of type VI collagen are formed by overlapping carboxy-terminal regions of the monomers.

Type VI collagen chains were first isolated from the pepsin-solubilized intimal layer of human blood vessel (Chung et al., 1976). Since then, native pepsin-solubilized type VI collagen has been isolated and characterized from a variety of tissues including placenta, cornea, and ligamentum nuchae [for a review, see Timpl and Engel (1987)]. A structural model for type VI collagen filaments was proposed by Furthmayr et al. (1983) based upon electron microscope studies of rotary-shadowed native and the partially reduced pepsin-solubilized type VI collagen. Type VI collagen monomers contain three different polypeptide chains (Jander et al., 1981, 1983) which form a central 105-nm-long triple-helical region with globular domains at each end. Both intrachain and interchain disulfide bonds are present within the

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