Chemical and Functional Characterization of a Fragment of $C\bar{1}s$ Containing the Epidermal Growth Factor Homology Region†

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ABSTRACT: $C\bar{1}s$, one of the three subcomponents of $C\bar{1}$, the first component of complement, is a serine protease comprising two disulfide-linked chains, the B chain, containing the catalytic site, and the A chain, involved in Ca^{2+} binding and Ca^{2+}-dependent interaction(s) with the other $C\bar{1}$ subcomponents. In an attempt to identify the regions responsible for the latter functions, $C\bar{1}s$ was submitted to limited proteolysis with plasmin, a treatment that split the A chain into three major fragments, $\alpha 1$, $\alpha 2$, and γ. Fragment $\alpha 2$, which comprised the epidermal growth factor-like (EGF-like) region of $C\bar{1}s$, was heterogeneous, starting at serine 97 or phenylalanine 105 and ending at lysine 195. This fragment was reduced and alkylated and then digested with elastase, and three peptides covering positions 131–135, 131–139, and 131–140 were characterized by amino acid analysis, Edman degradation, and mass spectrometry, showing that position 134 of $C\bar{1}s$ is occupied partly by an asparagine (47%) and partly by an *erythro*-β-hydroxyasparagine, in contrast with the homologous position (150) of $C\bar{1}r$ which only contains *erythro*-β-hydroxyasparagine. As measured by equilibrium dialysis, native $\alpha 2$, like the other plasmin-cleavage fragments, did not retain the ability of intact $C\bar{1}s$ to bind Ca^{2+}. In the same way, plasmin cleavage abolished the ability of $C\bar{1}s$ to dimerize or to associate with $C\bar{1}r$ in the presence of Ca^{2+}. In contrast, both $\alpha 2$ and the N-terminal $\alpha 1$ fragment, starting at serine 24 of the A chain, were able to compete significantly with intact $C\bar{1}s$ for the formation of the Ca^{2+}-dependent $C\bar{1}s$-$C\bar{1}r$-$C\bar{1}r$-$C\bar{1}s$ tetramer. These data indicate that the EGF homologous region of $C\bar{1}s$ does not contain all the structural elements required for Ca^{2+} binding and Ca^{2+}-dependent protein–protein interaction but suggest that these elements are contributed by both the $\alpha 1$ and $\alpha 2$ regions.

The first component of the classical pathway of complement, C1, is a complex enzyme comprising a noncatalytic protein (C1q) and two homologous serine proteases, C1r and C1s.[1] C1q mediates the binding of C1 to activating substances, triggering the sequential conversion of proenzyme C1r, then of proenzyme C1s, to their active forms, designated $C\bar{1}r$ and $C\bar{1}s$ [reviewed by Cooper (1985), Schumaker et al. (1987), and Arlaud et al. (1987c)]. This double activation process occurs within a Ca^{2+}-dependent tetrameric complex, C1s-C1r-C1r-C1s, and involves in each case the cleavage of a single Arg–Ile bond (Arlaud & Gagnon, 1985; Spycher et al., 1986). Thus, proenzyme C1s is a single-chain glycoprotein, and $C\bar{1}s$ comprises two disulfide-linked chains, A (N-terminal) and B (C-terminal), of known sequence (Mackinnon et al., 1987; Tosi et al., 1987). The B chain (251 residues) contains the active site serine, whereas the A chain (422 residues) comprises five structural units (regions), including two pairs of internal repeats (I/III and IV/V) and a single EGF-like segment (II).

Treatment of $C\bar{1}s$ with plasmin removes the N-terminal two-thirds of the A chain, ending in a truncated protein comprised of the remaining C-terminal portion of the A chain (γ) disulfide linked to the B chain. This γ-B derivative is still catalytically active but fails to participate in the Ca^{2+}-dependent associations characteristic of the native protein (Villiers et al., 1985). In contrast, trypsin cleavage of $C\bar{1}s$ in the presence of Ca^{2+} produces a fragment ($C\bar{1}s$-A) lacking most of the B chain, which is therefore catalytically inactive, but retains the interaction properties of the parent protein (Busby & Ingham, 1988). These elements, and the fact that $C\bar{1}s$ exhibits distinct temperature transitions (Busby & Ingham, 1988), strongly support the hypothesis that $C\bar{1}s$ comprises at least two domains: a catalytic domain (γ-B), and a region, corresponding roughly to the N-terminal half of the A chain, containing the sites(s) responsible for Ca^{2+} binding, Ca^{2+}-dependent $C\bar{1}s$ dimerization, and formation of the Ca^{2+}-dependent $C\bar{1}s$-$C\bar{1}r$-$C\bar{1}r$-$C\bar{1}s$ tetramer.

The objective of the present study, based on the use of limited proteolysis with plasmin, was to isolate and characterize fragments from this region, with the view to test their ability to mediate these interactions. On the basis of indirect evidence (Aude et al., 1988) and by analogy with other proteins (Öhlin et al., 1988a; Huang et al., 1989), it seemed reasonable to hypothesize that the EGF-like segment of $C\bar{1}s$ would represent the minimal structure required for Ca^{2+} binding. Another unanswered question was that of the presence, in this segment, of hydroxyasparagine, a modified amino acid identified and localized within the homologous region of $C\bar{1}r$ (Arlaud et al.,

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; iPr_2P-F, diisopropyl phosphorofluoridate; LSIMS, liquid secondary ion mass spectrometry; MCA, multichannel analyzer; m/z, mass to charge ratio; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tos-Phe-CH_2Cl, 1-chloro-4-phenyl-3-(L-tosylamino)butan-2-one. The nomenclature of complement components is that recommended by the World Health Organization; activated components are indicated by an overhead bar, e.g., $C\bar{1}s$.

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1987b). Our data show that hydroxylation of the critical asparagine residue is only partial in $C\bar{1}s$. Furthermore, the EGF homology region is not able, per se, to mediate Ca^{2+} binding and Ca^{2+}-dependent functions, which likely require other structural elements located, in particular, within the N-terminal region I of the A chain.

Experimental Procedures

Materials

Human plasmin was obtained from Kabi Vitrum, Stockholm, Sweden; porcine elastase (type IV), trypsin (treated with Tos-Phe-CH_2Cl), and thermolysin (protease type X) were from Sigma. Iodo[1-^{14}C]acetic acid (52 Ci/mol) was from the Commissariat à l'Energie Atomique, Saclay, France, and [1,3-^{3}H]iPr_2P-F (6.5 Ci/mmol) and $^{45}CaCl_2$ (22 Ci/g of calcium) were purchased from Radiochemical Centre, Amersham. *erythro*-β-Hydroxyaspartic acid was made by starting from fumaric acid according to Okai et al. (1967). Chelex 100 (100–200 mesh, Na^+ form) was obtained from Bio-Rad.

$C\bar{1}r$ and $C\bar{1}s$ were isolated from human plasma as described previously (Arlaud et al., 1979). The concentrations of purified $C\bar{1}r$ and $C\bar{1}s$ were measured by using values of $A_{280,1\%} = 12.4$ and 14.5, respectively, determined experimentally by amino acid analysis of protein samples of known absorbance at 280 nm. The molecular weights of $C\bar{1}r$ (86 300) and $C\bar{1}s$ (78 900) were calculated from the amino acid sequences (Arlaud & Gagnon, 1983; Leytus et al., 1986; Journet & Tosi, 1986; Arlaud et al., 1987a; Mackinnon et al., 1987; Tosi et al., 1987), plus 2000 for each carbohydrate moiety. The concentrations of the α2 and α1 fragments were determined by using values of $A_{280,1\%} = 7.1$ and 13.0, respectively, calculated by the method of Edelhoch (1967) from the number of Trp, Tyr, and disulfides (Tosi et al., 1987), with molecular weights of 13 300 and 8400, respectively. The $C\bar{1}s$–Sepharose and $C\bar{1}r$–Sepharose columns were prepared by coupling purified $C\bar{1}s$ or $C\bar{1}r$ to CNBr-activated Sepharose 4B according to the method of March et al. (1974).

The A chain containing fragment of $C\bar{1}s$ ($C\bar{1}s$-A) was produced by incubation of $C\bar{1}s$ with trypsin for 60 min at 30 °C as described by Busby and Ingham (1988), except that the trypsin:$C\bar{1}s$ ratio was 1:100 (w/w) instead of 1:400 (w/w).

Methods

Isolation of the EGF Homology Region of Human $C\bar{1}s$. Human $C\bar{1}s$ (1.0 mg/mL) in 145 mM NaCl/50 mM triethanolamine hydrochloride (pH 7.4) was incubated with plasmin (5% w/w) at 37 °C for 90 min. The reaction was stopped by addition of iPr_2P-F to a final concentration of 5 mM, and the plasmin digest was dialyzed against 0.1 M Na_2HPO_4 (pH 7.4). Purification of the fragments was realized by high-pressure hydrophobic interaction chromatography on a TSK-Phenyl 5PW column (7.5 × 75 mm) (LKB). The dialyzed material was loaded onto the column equilibrated in 0.1 M Na_2HPO_4/1.5 M $(NH_4)_2SO_4$ (pH 7.4), and elution was carried out by decreasing the concentration of $(NH_4)_2SO_4$ from 1.5 to 0 M in 30 min (flow rate = 1 mL/min). The different fragments were detected from their absorbance at 230 nm and analyzed by SDS–PAGE. The isolated fragments were concentrated by ultrafiltration on a YM5 membrane (Amicon Corp.) and dialyzed against 145 mM NaCl/50 mM triethanolamine hydrochloride (pH 7.4) with Spectrapor 6 dialysis tubing (M_r cutoff = 1000).

SDS–PAGE Analysis. Proteins and fragments were analyzed on 12.5% polyacrylamide gels as described by Laemmli (1970). Protein staining was performed with Coomassie Blue, and carbohydrate-containing bands wre identified by specific staining as described by Kapitani and Zebrowski (1973). Phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100), and α-lactalbumin (M_r 14 400) were used as molecular weight markers.

Cleavage of the α2 Fragment by Elastase. The isolated α2 fragment (~100 nmol) was reduced and radioalkylated by iodo[1-^{14}C]acetic acid as described previously (Arlaud et al., 1982). The reduced and S-^{14}C-carboxymethylated α2 fragment was dissolved in 0.1 M NH_4HCO_3 and incubated with elastase (2% w/w) for 3 h at 37 °C. Peptide separation was performed by reversed-phase HPLC on a Nova-PAK C_{18} column (Waters Associates) with solvent system 1 consisting of 0.1% NH_4HCO_3 and acetonitrile. The peptides were then further purified on the same column with solvent system 2 consisting of 0.1% trifluoroacetic acid and acetonitrile. Peptides were detected from absorbance at 215 nm.

Amino Acid Analysis and N-Terminal Sequence Determination. Samples were hydrolyzed for 24 h under reduced pressure at 110 °C in constant-boiling HCl containing 1% (w/v) phenol and 1% (v/v) 2-mercaptoethanol. Analyses were performed by reversed-phase HPLC of amino acid phenylthiocarbamyl derivatives with a Pico-Tag amino acid analysis system (Waters Associates) using the standard procedure recommended by the manufacturer. The phenylthiocarbamyl derivative of *erythro*-β-hydroxyaspartic acid was resolved from that of glutamic acid by lowering the pH of the starting buffer from 6.4 to 5.3. Automated Edman degradation was performed in a Applied Biosystems Model 470A gas-phase protein sequencer, and amino acid phenylthiohydantoin derivatives were identified according to the protocol recommended by the manufacturer on a Model 120A HPLC system.

Liquid Secondary Ion Mass Spectrometry (LSIMS). Positive ion mass spectra were obtained on a VG Analytical ZAB-2SE double-focusing mass spectrometer (mass range 15 kDa at 8-keV energy) and recorded on a VG 11-250 data system (VG Analytical Ltd., Manchester, England). Ionization was performed with the standard cesium gun (1 mA of 30 keV energy cesium ions) normally fitted on the mass spectrometer. Resolution was about 1400.

Peptides were dissolved in 5% (v/v) acetic acid, and thioglycerol was used as the matrix. Peptide 15b was recorded in the classical way, each scan generating a bar graph spectrum. Due to the low amount available, the spectrum of peptide 21c was recorded by operating the data system as a multichannel analyzer (MCA), scanning a narrow zone of ~400 masses covering two consecutive cesium iodide clusters (912.3352 and 1172.1451), in order to increase the sensitivity. Processing of these data and calibration were then performed as described by Barber and Green (1987).

^{45}Ca Overlay Technique. Proteins and fragments were electrophoresed on 12.5% polyacrylamide gels according to Laemmli (1970) and electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell) (pore size 0.45 μm) as described by Towbin et al. (1979). The nitrocellulose membrane was then labeled with $^{45}CaCl_2$ (4.5 μM) according to the procedure of Maruyama et al. (1984) and autoradiographed. After autoradiography the membrane was stained with Coomassie Blue R-250.

Equilibrium Dialysis. Protein samples were dialyzed once against 145 mM NaCl/50 mM triethanolamine hydrochloride (pH 7.4) containing 0.1% Chelex 100 and then twice against the same buffer passed through a Chelex 100 column ("Ca^{2+}-free" buffer). Ca^{2+} binding was measured with a Dianorm equilibrium dialyzer equipped with 250-μL microcells

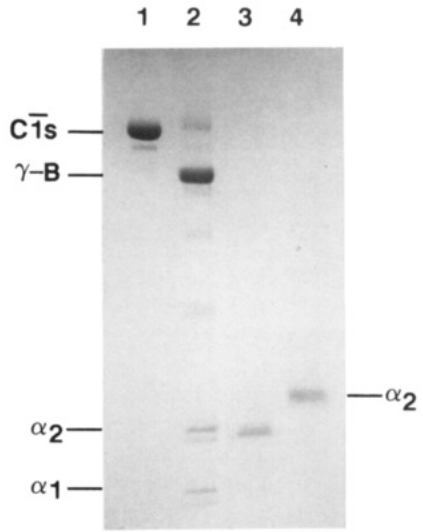

FIGURE 1: SDS–PAGE analysis of C1̄s, the C1̄s plasmin digest, and the isolated α2 fragment. Limited proteolysis of C1̄s by plasmin and isolation of fragment α2 were performed as described under Methods. (lane 1) Intact C1̄s, unreduced; (lane 2) whole plasmin digest, unreduced; (lanes 3 and 4) isolated α2 fragment, unreduced and reduced, respectively.

and Spectrapor 3 membranes (M_r cutoff 3500). Dialysis was carried out at 4 °C for 29 h under continuous rotation. One of the cell compartments was filled with the protein solution and the other with Ca^{2+}-free buffer containing varying amounts of $^{45}CaCl_2$. Ca^{2+} binding was determined by measuring the Ca^{2+} concentration in both compartments, on the basis of the initial specific ^{45}Ca radioactivity. Protein concentration was determined as described by Bradford (1976) with the appropriate purified protein as a standard.

High-Pressure Gel Permeation. Native C1̄s and the isolated α2 fragment were submitted to high-pressure gel permeation on a TSK-G3000 SW column (7.5 × 600 mm) (LKB) equilibrated in 145 mM NaCl/50 mM triethanolamine hydrochloride (pH 7.4) containing either 2 mM EDTA or 2 mM $CaCl_2$ and run at 1.0 mL/min.

Sucrose Density Gradient Ultracentrifugation. The C1̄s active site serine residue was labeled with [1,3-^{3}H]iPr_2P-F as previously described (Arlaud et al., 1986). As already shown (Arlaud et al., 1980), this treatment did not modify the Ca^{2+}-dependent interaction properties of C1̄s. [1,3-^{3}H]-iPr_2P-C1̄s (1.3–8.0 μg), alone or mixed with equimolar amount of C1̄r, was preincubated with purified α1 or α2 fragments [α1(α2):C1̄s molar ratio 1.8–103] in 150 μL of 145 mM NaCl/2.5 mM $CaCl_2$/50 mM triethanolamine hydrochloride (pH 7.4) for 6 h at 0 °C. Samples were applied to 5–20% (v/v) linear sucrose gradients prepared in the same buffer and containing uniform amounts of α1 or α2, at the same concentration as in the initial sample. Runs were performed on a SW60 rotor in a Beckman L8M ultracentrifuge at 43 000 revolutions/min for 15 h at 4 °C. After centrifugation, fractions were collected from the top of the gradients and assayed for ^{3}H radioactivity. Yeast alcohol dehydrogenase (7.6 S), beef liver catalase (11.4 S), and horse spleen apoferritin (17.6 S) were used as standards for estimation of $s_{20,w}$.

Results

Isolation and Chemical Characterization of α2, a Fragment of C1̄s Containing the EGF Homology Region. The monomeric form of C1̄s was submitted to limited proteolysis with plasmin as described under Methods, and the digest was analyzed by SDS–PAGE (Figure 1). Under nonreducing

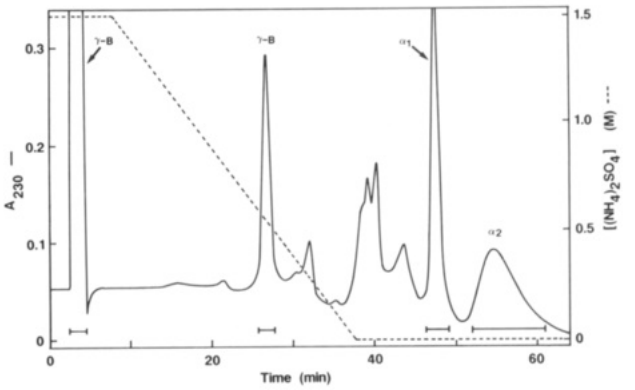

FIGURE 2: Fractionation of the C1̄s plasmin digest by high-pressure hydrophobic interaction chromatography. The C1̄s plasmin digest (~1 mg) was dialyzed against 0.1 M Na_2HPO_4 (pH 7.4) and loaded onto the TSK-Phenyl 5PW column equilibrated in the same buffer containing 1.5 M $(NH_4)_2SO_4$. Elution was realized by decreasing the $(NH_4)_2SO_4$ concentration from 1.5 to 0 M in 30 min. Pools corresponding to the γ-B, α1, and α2 fragments were made as indicated by bars.

conditions, three major cleavage products, γ-B (M_r ~54 000), α2 (M_r ~15 000), and α1 (M_r ~10 000), were separated, corresponding to the fragments previously described by Villiers et al. (1985). With the view to purify these fragments, the digest was fractionated by high-pressure hydrophobic interaction chromatography, as illustrated in Figure 2. Under the conditions used (see Methods), most of the γ-B fragment was not retained on the column, and only a small fraction was eluted during the descending $(NH_4)_2SO_4$ gradient, preceding several peaks containing partially cleaved material. Fragments α1 and α2 were eluted in that order after the end of the gradient, the former as a sharp peak and the latter as a broad, asymmetrical peak. SDS–PAGE analysis of the purified α2 fragment (Figure 1) showed that it migrated as a diffuse band, in both its unreduced and its reduced forms, suggesting heterogeneity. This was more evident with analyses performed on high amounts of material (20–30 μg), where α2 was clearly resolved into four bands, with apparent M_r ranging from 14 000 to 16 000. As indicated by specific staining (not shown), each band contained carbohydrate, under both reducing and nonreducing conditions. In contrast, α1 migrated as a sharp band on electrophoresis.

In order to locate the cleavage sites at the molecular level, each purified fragment was submitted to Edman degradation. Analysis of fragment α2 yielded two sequences, Ser-Asp-Phe-Ser-Asn-Glu-Glu-Arg-Phe-Thr-... and Phe-Thr-Gly-Phe-Ala-Ala-Tyr-Tyr-Val-Ala-..., obtained in a 3:1 relative proportion, indicating that α2 originated from cleavage of both the Lys–Ser and the Arg–Phe bonds at positions 96 and 104 of the C1̄s A chain. Fragment α1 yielded a single sequence, Ser-Trp-Asp-Ile-Glu-Val-Pro-Glu-Gly-Tyr-..., resulting from cleavage of the Lys^{23}–Ser^{24} bond. This fragment was therefore clearly derived from the N-terminal end of the C1̄s A chain, as proposed previously (Villiers et al., 1985), but lacked the first 23 amino acid residues of this chain. In addition to the sequence corresponding to the N-terminus of the B chain, the γ-B fragment yielded a second sequence, Leu-Arg-Tyr-His-Gly-Asp-Pro-Met-Pro-..., indicating that γ originated from cleavage of the Lys–Leu bond at position 269 of the C1̄s A chain. These two sequences were obtained in equimolar amounts and accounted for ~95% of the residues identified at each cycle.

With the view to complete the chemical characterization of fragment α2 and to identify the residue at position 134 of the C1̄s A chain, α2 was reduced and S-^{14}C-carboxy-

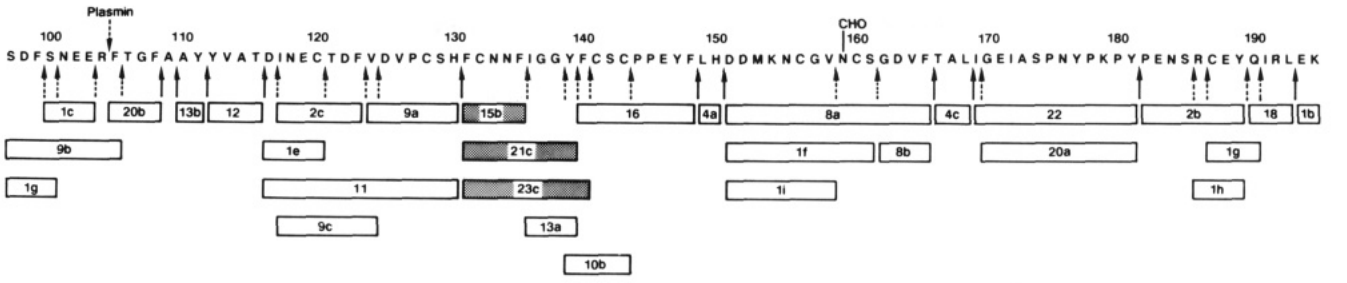

FIGURE 3: Diagrammatic representation of the peptides generated by cleavage of fragment $\alpha 2$ with elastase. Dotted arrows indicate sites of partial cleavage. Peptides are numbered according to their elution order on HPLC (the number indicates the elution position of the peptide in the first separation with solvent system 1, and the letter refers to the second separation in solvent system 2) and positioned in the vertical direction according to their relative yields. Stippling indicates the peptides covering position 134 of the $C\bar{1}s$ A chain. –CHO denotes the carbohydrate attachment site.

Peptide	131 134
15b	Phe-Cmc-Asn-Asn-Phe
21c	Phe-Cmc-Asn-Xaa-Phe-Ile-Gly-Gly-Tyr
23c	Phe-Cmc-Asn-Xaa-Phe-Ile-Gly-Gly-Tyr-Phe

FIGURE 4: N-Terminal sequences of peptides covering position 134 of the $C\bar{1}s$ A chain.

methylated and then digested with elastase as described under Methods. Initial fractionation of the cleavage peptides was realized by reversed-phase HPLC using solvent system 1. Twenty-three pools were collected, freeze-dried, and further purified by reversed-phase HPLC using solvent system 2. As summarized in Figure 3, 30 major peptides were recovered, identified by amino acid analysis, and replaced within the sequence of fragment $\alpha 2$. These resulted from cleavage of seryl, alanyl, glycyl, leucyl, and isoleucyl bonds, in agreement with the known specificity of elastase (Shotton, 1970), as well as from unexpected cleavage of phenylalanyl, tyrosyl, and histidyl bonds, indicating that the elastase preparation used was contaminated by a chymotrypsin-like protease, as already reported (Sampath Narayanan & Anwar, 1969). Identification of peptides 1b and 18 allowed us to locate the COOH-terminal residue of $\alpha 2$ at lysine 195 of the $C\bar{1}s$ A chain, whereas identification of peptides 9b and 20b confirmed the results obtained by N-terminal sequence analysis.

Three peptides containing the residue at position 134 were obtained, covering positions 131–135 (peptide 15b), 131–139 (peptide 21c), and 131–140 (peptide 23c). Amino acid analysis of acid hydrolysates from peptides 21c and 23c indicated in each case a single aspartic acid residue per mole and the presence of *erythro*-β-hydroxyaspartic acid, estimated as 0.9 and 0.7 mol/mol of peptide, respectively (Table I). In contrast, peptide 15b yielded two aspartic acid residues per mole and no detectable *erythro*-β-hydroxyaspartic acid. N-Terminal sequence analysis was performed on peptides 15b, 21c, and 23c and showed unambiguously that position 3 of each peptide and position 4 of peptide 15b were occupied by an asparagine (Figure 4). In contrast, peptides 21c and 23c each yielded at cycle 4 three peaks obtained in equivalent proportions, a peak corresponding to the phenylthiohydantoin derivative of asparagine (elution time 6.75 min) and two unidentified peaks eluting at 6.48 and 7.07 min. On the basis of these data, it became clear that position 134 of the $C\bar{1}s$ A chain was occupied partly by an asparagine and partly by a modified amino acid converted upon acid hydrolysis to *erythro*-β-hydroxyaspartic acid, in the relative proportions of 47% and 53%, as estimated from the yields of peptides 15b, 21c, and 23c (Table I).

Table I: Amino Acid Compositions[a] of Peptides Covering Position 134 of the $C\bar{1}s$ A Chain

		mol of amino acid/mol of peptide		
	peptide:	15b	21c	23c
amino acid	position in sequence: yield (nmol)[b]	131–135 7.5	131–139 6.0	131–140 2.5
β-OH-Asx			0.93 (1)	0.70 (1)
Asx		2.07 (2)	1.08 (1)	0.90 (1)
Thr				
Ser				
Glx				
Pro				
Gly			1.87 (2)	1.98 (2)
Ala				
Val				
$^1/_2$-Cys[c]		1.00 (1)	0.94 (1)	0.70 (1)
Ile			1.12 (1)	1.12 (1)
Leu				
Tyr			1.05 (1)	1.21 (1)
Phe		1.89 (2)	2.00 (2)	2.88 (3)
His				
Lys				
Arg				

[a] Calculated from 24-h HCl hydrolysates. Values derived from the sequence are indicated in parentheses. [b] Estimated from the amounts recovered after the first HPLC separation (see Methods). [c] Estimated as *S*-(carboxymethyl)cysteine.

Analysis of Peptides 15b and 21c by Mass Spectrometry. With the view to verify the above findings and to identify the modified amino acid at position 134, peptides 15b and 21c were submitted to mass spectrometry. The LSIMS spectrum obtained from peptide 15b (Figure 5A) showed two small peaks separated by 22 m/z units, at m/z 702.3 and 724.2. These can be interpreted respectively as the protonated $[(M + H)^+]$ and the cationized $[(M + Na)^+]$ pseudo-molecular ions of a peptide of molecular mass 701.2 Da, a value that is in good agreement with that derived from the sequence Phe-Cmc-Asn-Asn-Phe (701.25 Da). Peptide 21c was first analyzed in the classical (non-MCA) mode and yielded a spectrum with two small peaks emerging from the background at m/z 1108.4 and 1130.4 (Figure 5B). These values are close to those expected for the $(M + H)^+$ and $(M + Na)^+$ pseudo-molecular ions of the peptide Phe-Cmc-Asn-Asn(OH)-Phe-Ile-Gly-Gly-Tyr (calculated molecular mass = 1107.44 Da). In order to improve the sensitivity, a second analysis of peptide 21c was performed in the MCA mode. As shown on Figure 5C, the monoisotopic peaks were then clearly and accurately detected, at m/z 1108.39 for $(M + H)^+$ (calculated 1108.44) and at m/z 1130.37 for $(M + Na)^+$ (calculated 1130.42). Interestingly, two minor peaks were detected at m/z 1092.42 and 1114.36, likely corresponding to the $(M + H)^+$ and $(M + Na)^+$ pseudo-molecular ions of the major peptide with as-

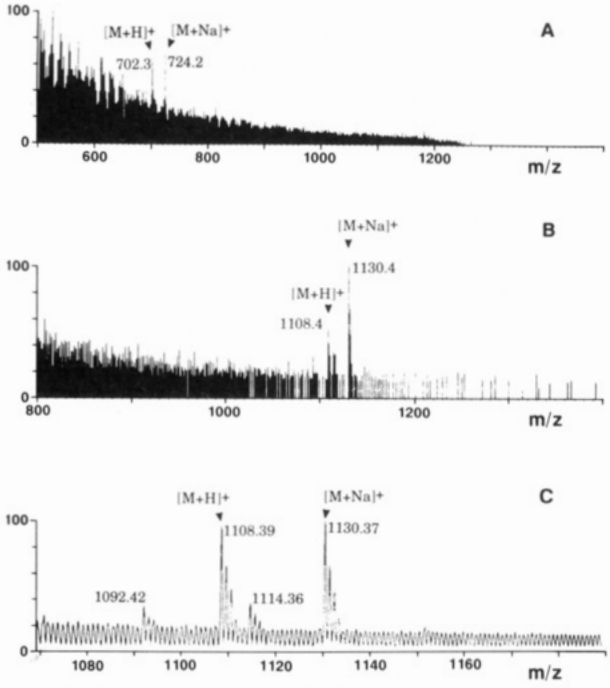

FIGURE 5: Mass spectrometry analysis of peptides 15b and 21c. (A and B) Analysis of peptides 15b and 21c (classical mode); (C) analysis of peptide 21c (MCA mode).

Table II: Calcium Binding Ability of C$\bar{1}$s and Its Fragments

protein or fragments	concn (μM)[a]	fraction of Ca^{2+} bound[b]
native C$\bar{1}$s	12.2	0.318
C$\bar{1}$s + trypsin (30 °C, 60 min)	6.9	0.300
C$\bar{1}$s (30 °C, 60 min)	11.8	0.324
C$\bar{1}$s + plasmin (37 °C, 90 min)	5.5	ND[c]
C$\bar{1}$s (37 °C, 90 min)	10.1	0.223
fragment α2	10.7	0.004

[a] Protein concentration was estimated after equilibrium dialysis by the method of Bradford (1976), using native C$\bar{1}$s as a standard for all samples except fragment α2. [b] The initial Ca^{2+} concentration was 20 μM. [c] Not detectable.

paragine instead of hydroxyasparagine at position 3. Taken together with the data obtained by amino acid analysis and Edman degradation, these data thus clearly demonstrated that position 134 of the C$\bar{1}$s A chain was occupied either by an *erythro*-β-hydroxyasparagine or by an unmodified asparagine.

Calcium Binding Ability of Fragment α2. The Ca^{2+} binding ability of native fragment α2 was initially investigated with the ^{45}Ca overlay technique described by Maruyama et al. (1984). By this method, binding of ^{45}Ca to α2 bound to nitrocellulose sheets was observed, suggesting the presence of a potential Ca^{2+} binding site. However, further investigation indicated that α2 retained this apparent binding ability after reduction and alkylation. Similarly, the unreduced γ-B fragment and the reduced and alkylated γ fragment were also found to bind ^{45}Ca by this technique. Considering that fragments α2 and γ each contain one of the two asparagine-linked oligosaccharide units of C1s (Spycher et al., 1986; Mackinnon et al., 1987; Tosi et al., 1987; Aude et al., 1988) and the presence of sialic acid in these units (Sim, 1981), the question of the specificity of the observed binding was raised, given the known property of sialic acid residues to bind Ca^{2+} (Jaques et al., 1977; Schauer, 1982).

The Ca^{2+} binding ability of fragment α2 was therefore tested by the equilibrium dialysis technique and compared with that of intact C$\bar{1}$s (Table II). In the 5–60 μM $CaCl_2$ concentration

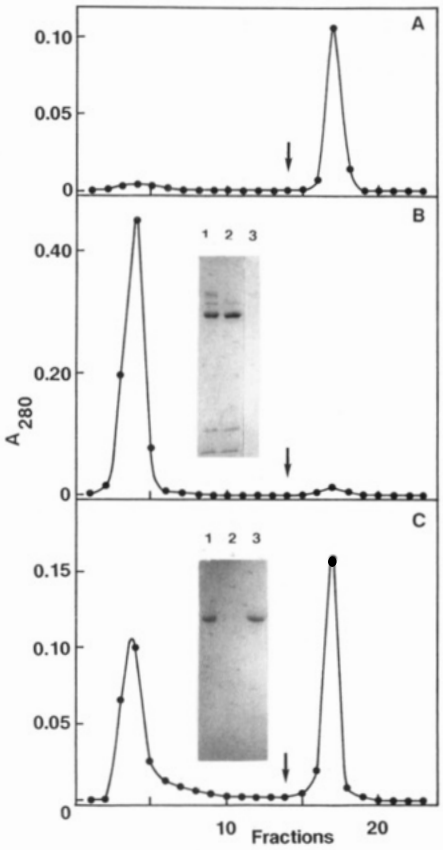

FIGURE 6: Ca^{2+}-dependent interaction of C$\bar{1}$s and its fragments with C$\bar{1}$s–Sepharose. Intact C$\bar{1}$s (0.4 mg), the whole C$\bar{1}$s plasmin digest (2 mg), or the whole C$\bar{1}$s tryptic digest (1 mg) was loaded onto a C$\bar{1}$s–Sepharose column equilibrated in 145 mM NaCl/2.5 mM $CaCl_2$/50 mM triethanolamine hydrochloride (pH 7.4). Elution of the bound material was realized in each case with the same buffer containing 5 mM EDTA instead of $CaCl_2$, as indicated by the arrows. Inserts show SDS–PAGE analyses in nonreducing conditions of (1) the material applied to the column, (2) the peak flowing through, and (3) the peak eluted with EDTA. (A) Intact C$\bar{1}$s; (B) C$\bar{1}$s plasmin digest; (C) C$\bar{1}$s tryptic digest.

range, intact C$\bar{1}$s was found to bind Ca^{2+}, as already demonstrated by the flow dialysis technique (Villiers et al., 1980). Under the same conditions and at comparable protein concentration, the isolated α2 fragment showed no significant Ca^{2+} binding ability, and the same negative result was obtained with the whole plasmin digest (containing fragments γ-B, α2, and α1). A significant decrease ($\sim$30%) in Ca^{2+} binding ability occurred upon incubation of C$\bar{1}$s without plasmin, but this did not account for the complete loss observed in the presence of protease. It became clear, therefore, that this loss was the direct consequence of the fragmentation of the A chain by plasmin. In contrast, trypsin cleavage of C$\bar{1}$s, which leads to extensive fragmentation of the C-terminal B chain into small polypeptides but preserves the integrity of the A chain (Busby & Ingham, 1988), did not significantly affect the initial calcium binding ability of the protein. It appeared clearly, therefore, that none of the fragments generated by plasmin cleavage of C$\bar{1}$s retained the ability to bind Ca^{2+}, confirming that the apparent fixation observed by the ^{45}Ca overlay technique was nonspecific.

Ca^{2+}-Dependent Interaction Properties of C$\bar{1}$s Fragments. The ability of C$\bar{1}$s fragments to mediate Ca^{2+}-dependent interaction with intact C$\bar{1}$s was tested with a C$\bar{1}$s–Sepharose column. As expected, native C$\bar{1}$s bound to the column in the presence of Ca^{2+} and was eluted with EDTA (Figure 6, panel A). In contrast, when the whole C$\bar{1}$s plasmin digest was loaded

onto the column, most of the material was not retained (Figure 6, panel B). SDS–PAGE analysis indicated that the large peak flowing through contained fragments γ-B, α2, and α1, as well as a fragment of apparent M_r 72000 corresponding to C$\bar{1}$s I (Villiers et al., 1985), an intermediate cleavage product lacking the N-terminal α1 region. The small peak eluting with EDTA only contained residual uncleaved C$\bar{1}$s. Identical results were obtained when a C$\bar{1}$r–Sepharose column was used, indicating that, in agreement with Ca^{2+} binding experiments, none of the C$\bar{1}$s plasmin-cleavage fragments was able to form a stable complex with C$\bar{1}$s or C$\bar{1}$r in the presence of Ca^{2+} and that removal of the α1 region alone was sufficient to abolish this ability. On the other hand, when the whole C$\bar{1}$s trypsin digest was loaded onto C$\bar{1}$s–Sepharose (Figure 6, panel C), only the small B chain fragments (not visible on SDS–PAGE analysis) passed through the column, whereas the C$\bar{1}$s-A fragment (containing the complete A chain disulfide linked to a small residual piece of the B chain) was fully retained and then subsequently eluted with EDTA.

As expected from the above observations, analysis by high-pressure gel permeation also showed that, in contrast with native C$\bar{1}$s, which exhibited a large shift toward the void volume when chromatographed in the presence of Ca^{2+}, the elution behavior of α2 was virtually identical in the presence of Ca^{2+} or EDTA. Thus, unlike the parent protein (Valet & Cooper, 1974; Arlaud et al., 1980; Busby & Ingham, 1988), this fragment was not able to dimerize in the presence of Ca^{2+}.

Although fragments α1 and α2 were clearly unable to form stable Ca^{2+}-dependent complexes with C$\bar{1}$r or C$\bar{1}$s, the question arose whether these could act individually as competitors of native C$\bar{1}$s for Ca^{2+}-dependent dimerization or heteroassociation with C$\bar{1}$r. To address this question, [1,3-^{3}H]iPr_2P-C$\bar{1}$s was incubated in the presence of Ca^{2+} with equimolar amounts of unlabeled C$\bar{1}$r and varying amounts of purified α1 or α2. Formation of the C$\bar{1}$s-C$\bar{1}$r-C$\bar{1}$r-C$\bar{1}$s complex was estimated from the radioactivity incorporated into the 8.7S tetramer (Arlaud et al., 1980), after sedimentation of each sample through sucrose gradients containing the same concentration of α1 or α2 as in the sample. Increasing the concentration of either α1 or α2 led to a progressive decrease in the amount of radiolabeled C$\bar{1}$s incorporated into the C$\bar{1}$s-C$\bar{1}$r-C$\bar{1}$r-C$\bar{1}$s tetramer. This correlated with an increase of the peak corresponding to the C$\bar{1}$s-C$\bar{1}$s dimer, sedimenting at 6.0 S (Arlaud et al., 1977). As shown in Table III, fragments α1 and α2 appeared to be equally efficient, but the competition effect was limited, as a 103-fold molar excess of α1 over C$\bar{1}$s only produced ~25% inhibition of the formation of the tetramer. Similar experiments performed in the absence of C$\bar{1}$r confirmed that α1 and α2 did not interfere with the formation of the C$\bar{1}$s-C$\bar{1}$s dimer and therefore that their inhibitory effect was restricted to the C$\bar{1}$s–C$\bar{1}$r interaction.

Discussion

The data obtained in this study provide detailed information on the location of the peptide bonds cleaved upon limited proteolysis of C$\bar{1}$s with plasmin, as well as on the chemical structure of the major fragments generated by this protease, thereby allowing further insights at the molecular level into the domain structure of C$\bar{1}$s, as summarized in Figure 7. In agreement with a previous report (Villiers et al., 1985), the α1 fragment obtained in our study was clearly derived from the N-terminal end of the A chain. However, in contrast with the same report, it was found to lack the first 23 amino acid residues of this chain. The origin of this discrepancy is not known and does not seem to be attributable to the extrinsic protease, given that both studies were conducted under the same conditions, with human plasmin obtained from the same source. Although the C-terminal residue of α1 was not formally identified, it appears likely, given its high apparent M_r (10000) and the location of Lys and Arg residues in this region (Mackinnon et al., 1987; Tosi et al., 1987), that this fragment ends at Lys 96 or Lys 90 and covers therefore the major part of structural unit I of the A chain (Figure 7). In any case, α1 contains half-cystine residues at positions 50 and 68, which can therefore be assumed to form an internal disulfide loop, in agreement with current hypotheses (Mackinnon et al., 1987; Aude et al., 1988).

N-Terminal sequence analysis also indicates that fragment γ originates, for the most part, from cleavage of the Lys–Leu bond at position 269 in the sequence ...-Gly-Trp-Lys-Leu-Arg-Tyr-... and comprises therefore structural units IV and V of the A chain (Figure 7). Interestingly, an identical stretch of sequence is also found in C$\bar{1}$r (Arlaud et al., 1987a), and plasmin cleavage occurs at exactly the same site (Lys 282–Leu 283) (Arlaud et al., 1986), which suggests that these particular regions of C$\bar{1}$r and C$\bar{1}$s have comparable structural organizations.

Detailed investigation of the chemical structure of fragment α2 indicates that it extends from Ser 97 to Lys 195, with a single minor cleavage at arginyl bond 104 (Figure 3). Thus, α2 contains not only the EGF-like region II of C$\bar{1}$s but also the N-terminal end of the following region III. Again, this is consistent with current concepts of the disulfide bridge pattern of C$\bar{1}$s, as schematized in Figure 7. The occurrence of two cleavages at the N-terminus of α2, as well as possible microheterogeneities in the oligosaccharide chain linked to Asn 159, may explain in part the apparent heterogeneity of α2 on SDS–PAGE analysis. As judged from the various analyses performed on peptides 15b, 21c, and 23c, position 134 of the C$\bar{1}$s A chain, which is coded as asparagine (Mackinnon et al., 1987; Tosi et al., 1987), is occupied partly by an *erythro*-β-hydroxyasparagine and partly by an unmodified asparagine, in the approximate relative proportions of 1:1. The key question, therefore, is to know whether this results from incomplete enzymic hydroxylation of Asn 134 in vivo or from partial chemical dehydroxylation occurring during preparation of fragment α2 in vitro. As judged from mass spectrometry analysis of peptide 21c and from Edman degradation of peptides 21c and 23c (see Results), it appears likely that some dehydroxylation may occur, although to a limited extent, during peptide handling. However, it should be mentioned that cleavage of the reduced and alkylated fragment α2 with thermolysin (Thielens, Gagnon, and Arlaud, unpublished data) also indicates that position 134 of the C$\bar{1}$s A chain is occupied in part by an unmodified asparagine. Furthermore, our data agree entirely with those obtained by Przysiecki et al. (1987), who found that acid hydrolysis of C$\bar{1}$s yields 0.47 mol of *erythro*-β-hydroxyaspartic acid/mol of protein. It can therefore be concluded that, in contrast with the homologous residue in C$\bar{1}$r (Arlaud et al., 1987a,b), the asparagine residue at position 134 of the C$\bar{1}$s A chain is only partially hydroxylated in vivo.

Other cases of EGF-like domains containing partially hydroxylated asparagine or aspartic acid residues have been reported, particularly in human factor IX (Fernlund & Stenflo, 1983), bovine protein S (Stenflo et al., 1987a), bovine factor VII (Takeya et al., 1988), and bovine low-density lipoprotein receptor and bovine thrombomodulin (Stenflo et al., 1987b). All these domains, like other EGF-like domains containing completely hydroxylated Asp/Asn, possess the consensus sequence (...-Cys-Xaa-Asp/Asn-$(Xaa)_4$-Phe/Tyr-Xaa-Cys-

Table III: Competitive Inhibition of the Ca^{2+}-Dependent $C\bar{1}s$–$C\bar{1}r$ Interaction by $C\bar{1}s$ Fragments $\alpha 1$ and $\alpha 2$

		$\alpha 2$ or $\alpha 1/C\bar{1}s$ molar ratio			
		0	1.8	18.0	103
formation of the Ca^{2+}-dependent $C\bar{1}s$-$C\bar{1}r$-$C\bar{1}r$-$C\bar{1}s$ tetramer (%)[a]	$\alpha 1$	100	92.1	83.3	74.2
	$\alpha 2$	100	90.8	85.8	ND[b]

[a] Formation of the tetramer was estimated as described under Methods. Each value is expressed relative to that determined in the control experiment performed in the absence of $\alpha 1$ and $\alpha 2$. [b] Not determined.

FIGURE 7: Schematic representation of the linear structure of $C\bar{1}s$ (a) and of the fragments generated by limited proteolysis of $C\bar{1}s$ with plasmin (b) and trypsin (c). I–V represent the structural units of the A chain and α, β, and γ the regions defined on the basis of limited proteolysis (Villiers et al., 1985). The positions of the Asn-linked carbohydrates (♦), the active site serine residue (S), and the partially hydroxylated asparagine residue (N) are indicated (Spycher et al., 1986; Mackinnon et al., 1987; Tosi et al., 1987; Aude et al., 1988). The disulfide bridge pattern is based on homology with serine proteases and other proteins (Aude et al., 1988). The arrows indicate the sites of plasmin cleavage identified in this study. The structure of the $C\bar{1}s$ A fragment obtained by limited tryptic cleavage (Busby & Ingham, 1988) is shown for comparison.

Xaa-Cys-...) that was proposed to represent the structural requirements of the enzyme(s) responsible for hydroxylation (Stenflo et al., 1987a). Also, this sequence is present in both C1r and C1s, but hydroxylation is complete in the former protein and incomplete in the latter. This strongly suggests that the specificity of the hydroxylase(s) could involve higher structural requirements. As already mentioned by Stenflo et al. (1987b), this also raises the question of a possible regulatory function of this (these) enzyme(s). In the case of C1s, it should be emphasized that the low degree of hydroxylation is not due to a sequence heterogeneity in the surrounding $\alpha 2$ region, as this contains a single sequence (see Results), in contrast with the homologous region of C1r, where a Ser/Leu polymorphism occurs at position 135 (Arlaud et al., 1987a,b).

From a structural point of view, it should be mentioned finally that no major fragment corresponding to the β region of $C\bar{1}s$ could be recovered from plasmin cleavage (Figure 7). In this respect, it is worth pointing out that this sequence segment, which covers positions 196–269, contains several arginyl bonds (at positions 204, 205, and 230) and lysyl bonds (at positions 249, 265, and 266) (Mackinnon et al., 1987; Tosi et al., 1987) and is therefore probably fragmented into small pieces by plasmin.

From a functional point of view, it can be concluded from our data that none of the fragments generated by plasmin cleavage of $C\bar{1}s$ retains the ability to bind Ca^{2+} or to form stable complexes with $C\bar{1}r$ or $C\bar{1}s$ in the presence of Ca^{2+}. With respect to Ca^{2+} binding, it is most likely that the positive results obtained by the ^{45}Ca overlay technique with fragments $\alpha 2$, γ-B, and γ arise from nonspecific Ca^{2+} fixation to clusters of negative charges artificially created on the nitrocellulose surface. The presence of sialic acid in the oligosaccharide moieties attached to $\alpha 2$ and γ (see Figure 7), the intrinsic acidic character of both sequence segments (Tosi et al., 1987), and the fact that the apparent binding persisted after reduction and alkylation of fragments $\alpha 2$ and γ all favor this hypothesis. Equilibrium dialysis, on the other hand, shows unambiguously that plasmin cleavage abolishes the ability of $C\bar{1}s$ to bind Ca^{2+} in solution. As $C\bar{1}s$ itself, incubated under the conditions used for cleavage (37 °C, 90 min), retains its initial Ca^{2+} binding potential, it can be concluded that the complete loss observed with plasmin is not due to a temperature effect but is the direct consequence of the cleavage process itself. This is consistent with the data obtained by Busby and Ingham (1988), as these authors found that the irreversible low-temperature transition (midpoint ~31 °C) that occurs in the A chain of $C\bar{1}s$, likely in the α region, has no apparent consequence on the interaction properties of this protein.

Consistent with the loss of Ca^{2+} binding ability discussed above, plasmin cleavage of $C\bar{1}s$ also abolishes its ability to dimerize or to form stable complexes with $C\bar{1}r$ in the presence of Ca^{2+}. In this respect, the observation that the removal of $\alpha 1$ only is sufficient (see Figure 6B) is important, for it indicates that this region of the molecule is directly involved in its Ca^{2+}-dependent functions. This conclusion is strengthened by competition experiments (see Table III), which show that fragments $\alpha 1$ and $\alpha 2$ are equally able, at high concentrations, to produce partial inhibition of the formation of the Ca^{2+}-dependent $C\bar{1}s$-$C\bar{1}r$-$C\bar{1}r$-$C\bar{1}s$ tetramer. The reason why this competition effect is restricted to the heterologous $C\bar{1}s$–$C\bar{1}r$ interaction, and does not apply to the homologous $C\bar{1}s$–$C\bar{1}s$ interaction, is unknown. Yet, these data strongly suggest that the site(s) of $C\bar{1}s$ responsible for Ca^{2+}-dependent interaction with $C\bar{1}r$ (and probably Ca^{2+} binding) involve(s) amino acid residues that are located in both the $\alpha 1$ and $\alpha 2$ regions. This hypothesis is consistent with the data obtained by Busby and Ingham (1988), showing that the low-temperature transition already mentioned above, which is stabilized by Ca^{2+}, is correlated with an increase in the intrinsic fluorescence of Trp 25, in the $\alpha 1$ region. In the same way, the functional implication of the $\alpha 2$ region of $C\bar{1}s$ has also already been suggested on the basis of the observation that the oligosaccharide chain linked to Asn 159, at the C-terminal end of the EGF-like region II (see Figure 7), is protected from enzymic hydrolysis upon homologous ($C\bar{1}s$–$C\bar{1}s$) or heterologous ($C\bar{1}s$–$C\bar{1}r$) Ca^{2+}-dependent interactions (Aude et al., 1988).

A number of EGF-like domains containing a β-hydroxyaspartic acid or β-hydroxyasparagine residue have been reported in various proteins, where their presence can be correlated with a high-affinity Ca^{2+} binding site (K_d 10–100 μM) [reviewed in Rees et al. (1988)]. Several studies suggest that this type of structure could indeed be responsible for Ca^{2+} binding (McMullen et al., 1983; Morita et al., 1984; Sugo et al., 1984; Öhlin & Stenflo, 1987; Rees et al., 1988), and it was demonstrated recently that *isolated fragments* corresponding to the first EGF-like domain of human factor IX and bovine factor X (Huang et al., 1989; Persson et al. 1989) and to the first and second EGF-like domains of bovine protein C (Öhlin et al., 1988a) have the ability to bind Ca^{2+}, with dissociation constants ranging from 0.1 to 2–5 mM. This contrasts, therefore, with our data which, although suggesting

that the EGF homology region of $C\bar{1}s$ could *participate* in Ca^{2+} binding, show clearly that this region is not able, per se, to bind Ca^{2+} with an affinity comparable to that of the intact protein. It cannot be excluded that the isolated $\alpha 2$ fragment retains a weak affinity for Ca^{2+} that would not be detected in the experimental conditions used in our study. It should be emphasized, however, that $C\bar{1}s$, like $C\bar{1}r$, contains a single EGF-like domain, whereas at least two copies of this sequence element are found in all other proteins of this type reported so far. In $C\bar{1}r$ and $C\bar{1}s$, also, the hydroxylated residue is an asparagine, whereas in all other known cases (and particularly in factors IX and X and protein C) the first EGF-like domain always contains a β-hydroxyaspartic acid (Rees et al., 1988). In addition, for factor IX and protein C, experimental evidence has been obtained that the β-hydroxyaspartic acid plays a crucial role in Ca^{2+} binding (Öhlin et al., 1988b; Huang et al., 1989). In this respect, it should be kept in mind that β-hydroxylation of aspartic acid decreases the pK of the side-chain carboxyl group, thereby increasing its reactivity, whereas the consequence of such a modification on an asparagine residue is not clear. It is possible, therefore, that in $C\bar{1}s$ (and probably in $C\bar{1}r$) Ca^{2+} binding involves mechanisms that are slightly different (not necessarily involving β-hydroxyasparagine) and possibly more complex than those found, for instance, in factors IX and X and protein C. Further studies on the structure–function relationships in the various proteins of this type should help clarify this question. With respect to $C\bar{1}r$ and $C\bar{1}s$, work is in progress to isolate and characterize fragments containing all the structural elements required for Ca^{2+} binding and Ca^{2+}-dependent interactions.

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Registry No. $C\bar{1}s$, 80295-70-1; EGF, 62229-50-9; $C\bar{1}r$, 80295-69-8; Ca, 7440-70-2.

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Aluminum Fluoride Inhibition of Glucocorticoid Receptor Inactivation and Transformation†

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ABSTRACT: Fluoride, in the presence of aluminum ions, reversibly inhibits the temperature-mediated inactivation of unoccupied glucocorticoid receptors in cytosol preparations from mouse L cells. The effect is concentration-dependent, with virtually complete stabilization of specific glucocorticoid-binding capacity at 2 mM fluoride and 100 μM aluminum. These concentrations of aluminum and fluoride are ineffective when used separately. Aluminum fluoride also stabilizes receptors toward inactivation by gel filtration and ammonium sulfate precipitation. Aluminum fluoride prevents temperature-dependent transformation of steroid-receptor complexes to the DNA-binding state. Aluminum fluoride does not inhibit calf intestine alkaline phosphatase, and unoccupied receptors inactivated by this enzyme in the presence of aluminum fluoride can be completely reactivated by dithiothreitol. The effects of aluminum fluoride are due to stabilization of the complex between the glucocorticoid receptor and the 90-kDa mammalian heat-shock protein *hsp*90, which suggests that aluminum fluoride interacts directly with the receptor. Endogenous thermal inactivation of receptors in cytosol is not accompanied by receptor dephosphorylation. However, inactivation is correlated with dissociation of *hsp*90 from the unoccupied receptor. These results support the proposal that *hsp*90 is required for the receptor to bind steroid and dissociation of *hsp*90 is sufficient to inactivate the unoccupied receptor.

The untransformed[1] mouse glucocorticoid receptor (GR)[2] in cytosol preparations exists as an 8S–9S heteromeric complex containing the steroid-binding phosphoprotein and a 90-kDa non-steroid-binding phosphoprotein (Housley & Pratt, 1983; Housley et al., 1985; Mendel et al., 1986; Sanchez et al., 1987a). The steroid-binding phosphoprotein contains 783 amino acids with a predicted mass of 86 kDa (Danielsen et al., 1986), although it exhibits anomalous electrophoretic mobility on SDS–polyacrylamide gels with an apparent mass of ~100 kDa (Housley et al., 1985; Mendel et al., 1986). The 90-kDa protein has been identified as the mammalian heat-shock protein *hsp*90 (Sanchez et al., 1985). *hsp*90, or a similar 90-kDa protein, is also found as a common component of untransformed progesterone, estrogen, androgen, mineralocorticoid, and aryl hydrocarbon receptors (Joab et al., 1984; Schuh et al., 1985; Catelli et al., 1985; Perdew, 1988; Denis et al., 1988; Rafestin-Oblin et al., 1989). Transformation of the steroid-bound GR to the DNA-binding state is accompanied by dissociation of *hsp*90 from the GR (Sanchez et al., 1985; Mendel et al., 1986), concomitant with a reduction in the size of the GR from an apparent mass of ~300 kDa to an apparent mass of ~100 kDa (Holbrook et al., 1983; Vedeckis, 1983).

The possibility that receptors are dephosphorylated during transformation has been inferred from the ability of phosphatase inhibitors to prevent steroid receptor transformation. The group VIA transition metal oxyanion molybdate is an effective inhibitor of transformation (Toft & Nishigori, 1979; Leach et al., 1979), and molybdate has been widely used in the purification of untransformed steroid receptors. Although F^- was initially reported to be ineffective at inhibiting transformation of steroid receptors (Leach et al., 1979; Nishigori & Toft, 1980), more recent studies suggest that F^- can partially inhibit transformation of the steroid-bound GR induced by dialysis of AtT-20 cell cytosol (Reker et al., 1987). On the basis of this and other indirect evidence obtained in both intact cells and cytosols, several groups have suggested that transformation may require dephosphorylation of the GR or some other cytosol component (Munck & Leung, 1977; Sando et al., 1979; Barnett et al., 1980; Reker et al., 1987). However, the effects of molybdate are not mediated by phosphatase inhibition but rather are due to a direct interaction with steroid

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[1] The term "transformation" is used to describe the process whereby the steroid-bound receptor is converted to a form that binds to nuclei, DNA–cellulose, etc. The terms "inactivation" and "reactivation" are used to describe the loss and restoration of specific steroid-binding capacity, respectively.

[2] Abbreviations and trivial names: GR, glucocorticoid receptor; *hsp*90, 90-kDa heat-shock protein; triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; DEAE, diethylaminoethyl; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid; GTP, guanosine 5′-triphosphate.

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