Effect of Lactoperoxidase-Catalyzed Iodination on the Ca^{2+}-Dependent Interactions of Human $\bar{C}1s$. Location of the Iodination Sites

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ABSTRACT: $\bar{C}1s$, one of the two serine proteases of $\bar{C}1$, the first component of complement, has the ability to mediate heterologous ($\bar{C}1r$–$\bar{C}1s$) as well as homologous ($\bar{C}1s$–$\bar{C}1s$) Ca^{2+}-dependent interactions, both involving the NH_2-terminal α region of its A chain. Lactoperoxidase-catalyzed iodination of $\bar{C}1s$ in its monomeric form was found to abolish its ability to form Ca^{2+}-dependent homodimers, without impairing its ability to mediate $\bar{C}1r$–$\bar{C}1s$ heteroassociation. $\bar{C}1s$ iodinated in its dimeric form, in contrast, fully retained the ability to self-associate. With a view to identify the tyrosine residues iodinated in each case, $\bar{C}1s$ was radioiodinated in its monomeric and dimeric forms, and comparative tryptic mapping was performed on the resulting ^{125}I-labeled A chains. Most of the tyrosine residues either were not iodinated or were equivalently iodinated in both monomeric and dimeric $\bar{C}1s$. In contrast, Tyr-33 and Tyr-38 were iodinated in the monomer but not in the dimer. Conversely, Tyr-52 and Tyr-147 were iodinated only in the dimer. These results provide further evidence that the structural determinants of $\bar{C}1s$ required for Ca^{2+} binding and Ca^{2+}-dependent protein–protein interactions are contributed by both the NH_2-terminal motif I (positions 1–110) and the epidermal growth factor like motif II (positions 111–159) of the α region. On the basis of available information, tentative models of the $\bar{C}1s$–$\bar{C}1s$ and $\bar{C}1r$–$\bar{C}1s$ Ca^{2+}-dependent interactions are proposed.

The first component of complement, C1, is a complex enzyme comprising two homologous multidomain serine proteases, C1r and C1s, that are sequentially activated upon binding of C1 to various activators [reviewed by Cooper (1985), Schumaker et al. (1987), and Arlaud et al. (1987a)]. Activation results in the conversion of single-chain proenzymes (C1r, C1s) into active proteases ($\bar{C}1r$, $\bar{C}1s$)[1] comprising two disulfide-linked chains. The COOH-terminal B chains are trypsin-like serine protease domains, whereas the NH_2-terminal A chains are homologous mosaic-like polypeptides, each composed of five structural units, including two pairs of internal repeats (I/III and IV/V) and a single β-hydroxyasparagine-containing EGF-like motif II (Leytus et al., 1986; Arlaud et al., 1987b,c; Mackinnon et al., 1987; Tosi et al., 1987; Thielens et al., 1990a).

A fundamental feature of $\bar{C}1r$ and $\bar{C}1s$ is their ability to mediate the heterologous ($\bar{C}1r$–$\bar{C}1s$) Ca^{2+}-dependent interaction that is involved in the assembly of $\bar{C}1s$–$\bar{C}1r$–$\bar{C}1r$–$\bar{C}1s$, the tetrameric catalytic subunit of $\bar{C}1$. Interestingly, in the absence of $\bar{C}1r$, $\bar{C}1s$ also has the ability to form Ca^{2+}-dependent dimers. Both interactions involve the α region of $\bar{C}1s$ that, like its counterpart in $\bar{C}1r$, corresponds to the NH_2-terminal half of the A chain, consisting of structural units I, II, and part of the following unit III (Busby & Ingham, 1990; Thielens et al., 1990b). However, while the $\bar{C}1r$–$\bar{C}1s$ heteroassociation coincides with the binding of two Ca^{2+} atoms (i.e., one per α region), the homologous ($\bar{C}1s$–$\bar{C}1s$) interaction provides one additional Ca^{2+} binding site (Thielens et al., 1990b). Other data (Thielens et al., 1990a) suggest that the structural determinants of $\bar{C}1s$ required for Ca^{2+} binding and Ca^{2+}-dependent interactions are contributed by both the NH_2-terminal motif I and the EGF-like motif II of the α region, but no precise information has been obtained with regard to the nature and location of these determinants.

Early studies (Arlaud et al., 1980) had shown that lactoperoxidase-catalyzed iodination of $\bar{C}1s$ in its monomeric form inhibits its ability to form Ca^{2+}-dependent dimers. The purpose of the present study was to reinvestigate this effect at the molecular level, with a view to identify the iodination sites, and possibly to define more precisely the portions of the α region of $\bar{C}1s$ that are important for Ca^{2+}-dependent interactions.

Experimental Procedures

Materials

Lactoperoxidase (purified grade) was obtained from Calbiochem. Trypsin (sequencing grade) was from Boehringer Mannheim. Bio-Gel P-10 (fine) was purchased from Bio-Rad Laboratories and Sephadex G-50 (fine) from Pharmacia Fine Chemicals. $Na^{125}I$ (17.4 mCi/μg of iodine) and $^{45}CaCl_2$ (36 Ci/g of calcium) were from the Radiochemical Centre, Amersham. Monoiodotyrosine and diiodotyrosine were purchased from Sigma. Human $\bar{C}1r$ and $\bar{C}1s$ were isolated from plasma as described previously (Arlaud et al., 1979). The concentrations of purified $\bar{C}1r$ and $\bar{C}1s$ were estimated from their absorbance at 280 nm by use of $E(1\%, 1\text{ cm}) = 12.4$ and 14.5 and molecular weights of 86 300 and 78 900, respectively (Thielens, et al., 1990b).

Methods

Iodination of $\bar{C}1s$. $\bar{C}1s$, in its monomeric and dimeric forms, was labeled with ^{125}I in the presence of lactoperoxidase according to a further modification of the method described by

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; HPLC, high-pressure liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The nomenclature of complement components is that recommended by the World Health Organization; activated components are indicated by an overhead bar, e.g., $\bar{C}1s$.

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Heusser et al. (1973) and modified by Arlaud et al. (1980). Briefly, $C\bar{1}s$ (0.125–1.0 mg/mL) in 145 mM NaCl/50 mM triethanolamine–HCl (pH 7.4) containing either 5 mM EDTA or 5 mM $CaCl_2$ was cooled to 4 °C, and the following reagents were added successively: 30–310 μM KI, 16 pM $Na^{125}I$ (2.18 Ci/μmol), 31 μg/mL lactoperoxidase, and 2.2 μM H_2O_2 (all final concentrations). After incubation for 50 min at 4 °C with stirring, the reaction was stopped by addition of 1% (w/v) sodium azide. Free $Na^{125}I$ was removed by centrifugation on a Sephadex G-50 fine column equilibrated in 145 mM NaCl/50 mM triethanolamine–HCl (pH 7.4) as described by Penefsky (1977). The amount of iodine incorporated into each protein sample was calculated from measurement of ^{125}I radioactivity and from protein concentration determined by the method of Bradford (1976).

SDS-PAGE Analysis. ^{125}I-Labeled $C\bar{1}s$ and its isolated A and B chains were analyzed on 12.5% polyacrylamide gels as described by Laemmli (1970). Protein staining was performed with Coomassie blue, and phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soybean trypsin inhibitor (M_r 20100) and α-lactalbumin (M_r 14400) were used as molecular weight markers. The distribution of ^{125}I radioactivity between the A and B chains of $C\bar{1}s$ was determined from the dried gels using a Berthold TLC-linear analyzer.

Sucrose Density Gradient Ultracentrifugation. Samples were sedimented in 5–20% (w/v) linear sucrose gradients prepared in 145 mM NaCl/50 mM triethanolamine–HCl (pH 7.4) containing either 5 mM EDTA or 5 mM $CaCl_2$. Runs were performed on a SW60 rotor in a Beckman L8M ultracentrifuge at 43000 rpm for 15 h at 4 °C. After centrifugation, fractions were collected from the top of the gradients and assayed for ^{125}I radioactivity. Protein was determined by the method of Bradford (1976). Yeast alcohol dehydrogenase (7.6 S), beef liver catalase (11.4 S), and horse spleen apoferritin (17.6 S) were used as standards for measurements of $s_{20,w}$.

Equilibrium Dialysis. $C\bar{1}s$ samples were dialyzed once against 145 mM NaCl/50 mM triethanolamine–HCl (pH 7.4) containing 0.1% Chelex 100 and then twice against the same buffer passed through a Chelex 100 column. Ca^{2+} binding was measured with a Dianorm equilibrium dialyzer, as described by Thielens et al. (1990b).

Isolation of the A Chain of ^{125}I-Labeled $C\bar{1}s$. $C\bar{1}s$ (50 nmol), either in its monomeric or dimeric form, was iodinated as described above, with a concentration of KI of 310 μM. After four successive dialyses against 1% (v/v) acetic acid, ^{125}I-labeled $C\bar{1}s$ was freeze-dried and then reduced with dithiothreitol and alkylated by iodoacetic acid as described by Arlaud et al. (1982). Reduced and alkylated, ^{125}I-labeled $C\bar{1}s$ was dissolved in 6 M urea/0.2 M formic acid (700 μL), and the A and B chains were isolated by high-pressure gel permeation chromatography on a 7.5 × 600 mm TSK G-3000 SW column (LKB), as described previously by Arlaud and Gagnon (1981). The isolated chains were thoroughly dialyzed at 4 °C against 1% (v/v) acetic acid and freeze-dried.

Tryptic Cleavage of ^{125}I-Labeled $C\bar{1}s$ A Chain and Purification of the Tryptic Peptides. Reduced and alkylated, ^{125}I-labeled $C\bar{1}s$ A-chain (37 nmol) was dissolved in 0.1 M NH_4HCO_3/1 mM $CaCl_2$ (pH 7.8) (2.5 mL) and incubated with trypsin (4%, w/w) for 2 h at 37 °C. After the addition of 2 mM diisopropyl phosphorofluoridate, the mixture was applied to a column (2.5 × 90 cm) of Bio-Gel P-10 equilibrated with 0.1 M NH_4HCO_3 (pH 7.8) and run at a flow rate of 15 mL/h. Eight pools were collected and freeze-dried, and peptides were further purified by reversed-phase HPLC with use of a Waters NovaPak C-18 column (3.9 mm × 150 mm) equilibrated with a mixture composed of 0.1% (w/v) NH_4HCO_3 and CH_3CN in the ratio 95:5 (v/v). Elution was carried out at a flow rate of 1 mL/min with a 30-min linear gradient to give a final ratio of 40:60. Peptides were detected from the absorbance at 215 nm and from measurement of ^{125}I radioactivity.

Peptide Nomenclature. Tryptic peptides obtained from the A chain of $C\bar{1}s$ iodinated in its monomeric and dimeric forms are designated M and D, respectively. The next number indicates the elution position of the peptide on the Bio-Gel P-10 column, followed by a letter indicating its elution position on the reversed-phase HPLC column.

Amino Acid Analysis and NH_2-Terminal Sequence Determination. Peptides 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 phenyl thiocarbamyl derivatives with a Picotag amino acid analysis system (Waters Associates), with use of the standard procedure recommended by the manufacturer.

Automated Edman degradation of ^{125}I-labeled tryptic peptides was performed in an Applied Biosystems Model 477 A gas-phase protein sequencer, and amino acid phenylthiohydantoin derivatives were identified by reversed-phase HPLC according to the protocol recommended by the manufacturer on a Model 120 A HPLC system. Phenylthiohydantoin derivatives released at each cycle were collected and counted for ^{125}I radioactivity. Monoiodotyrosine was identified from the elution position of its phenylthiohydantoin derivative by comparison with the corresponding standard, which was obtained by performing, in the sequencer, one cycle of Edman degradation on synthetic monoiodotyrosine. No characteristic peak could be ascribed to the phenylthiohydantoin derivative of diiodotyrosine in the chromatographic system used, and diiodotyrosine was only tentatively identified on the basis of (i) the absence of the peak characteristic of monoiodotyrosine and (ii) the relative amount of ^{125}I radioactivity measured at the corresponding cycle.

Results

Iodination of $C\bar{1}s$ in Its Monomeric and Dimeric Forms. It was shown previously that lactoperoxidase-catalyzed iodination of monomeric $C\bar{1}s$ inhibits its ability to form Ca^{2+}-dependent dimers (Arlaud et al., 1980). Further investigations using the same iodination method indicated weak incorporation of iodine (<0.1 iodine atom/mol of $C\bar{1}s$). The experimental conditions were therefore modified with a view to increase the amount of iodine incorporated into $C\bar{1}s$ and to obtain quantitative inhibition of $C\bar{1}s$ dimerization. As shown in Figure 1, increasing the concentration of KI in the reaction mixture from 30 to 310 μM resulted in a linear increase in the amount of iodine incorporated into monomeric $C\bar{1}s$, and no saturation was observed within this range. Parallel analysis of each sample by sucrose density gradient ultracentrifugation indicated that increasing iodination induced a proportional decrease of the ability of $C\bar{1}s$ to form Ca^{2+}-dependent dimers (Figure 1). Complete inhibition of $C\bar{1}s$ dimerization was obtained at a KI concentration of approximately 240 μM. Under these conditions, $C\bar{1}s$ incorporated 8–10 iodine atoms/mol and sedimented on sucrose gradients in the presence of Ca^{2+} with a 4.5 S coefficient corresponding to the monomer (Arlaud et al., 1980). In contrast, $C\bar{1}s$ iodinated under the same conditions but in its dimeric form (in the presence of Ca^{2+}) only incorporated approximately 7–8 iodine atoms/mol

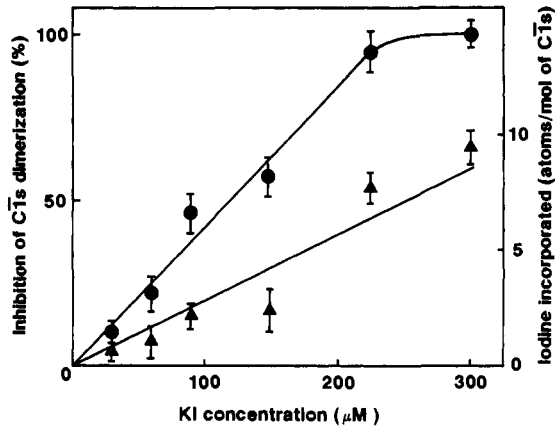

FIGURE 1: Iodination of monomeric C1s: correlation between the extent of iodine incorporation and the inhibition of C1s Ca^{2+}-dependent dimerization. C1s (125 μg/mL) was iodinated as described under Methods, in the presence of increasing concentrations of KI (30–310 μM) and $Na^{125}I$ as a tracer, in 145 mM NaCl/50 mM triethanolamine–HCl (pH 7.4) containing 5 mM EDTA. The extent of iodine incorporation was determined on each sample from measurement of protein and ^{125}I radioactivity, and the ability of ^{125}I-labeled C1s to form Ca^{2+}-dependent dimers was estimated by ultracentrifugation on sucrose gradients containing 5 mM $CaCl_2$, as described under Methods: (▲) iodine incorporated, (●) inhibition of C1s dimerization.

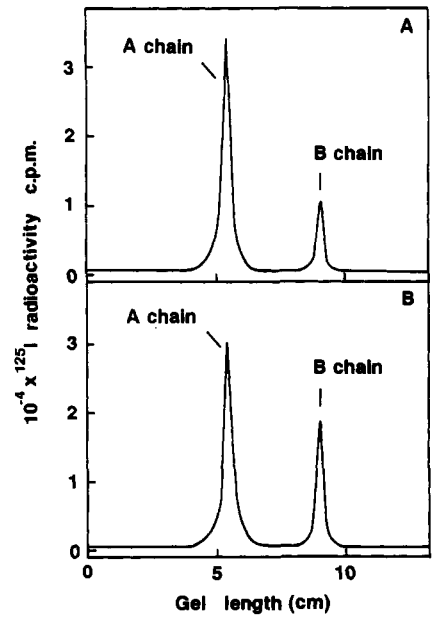

FIGURE 2: Distribution of radioactivity in the A and B chains of ^{125}I-labeled C1s. Effect of Ca^{2+}-dependent C1s dimerization. C1s (1.0 mg/mL) was iodinated as described under Methods, in the presence of 310 μM KI and 16 pM $Na^{125}I$, in 145 mM NaCl/50 mM triethanolamine–HCl (pH 7.4) containing either 5 mM EDTA (A) or 5 mM $CaCl_2$ (B). After reduction and alkylation, ^{125}I-labeled C1s was analyzed by SDS-PAGE as described under Methods.

and fully retained the ability to form Ca^{2+}-dependent dimers sedimenting on sucrose gradients with a coefficient of 5.8 S, close to the value previously determined for native C1s dimers (Arlaud et al., 1977).

With a view to further study the effect of C1s iodination on its functional properties, C1s was iodinated in the presence of 310 μM KI, either in its monomeric or dimeric form. Repeated equilibrium dialysis experiments showed unambiguously that, in both cases, C1s was still able to bind Ca^{2+} in the 10–100 μM $CaCl_2$ concentration range. Both iodinated species, however, bound significantly less Ca^{2+} than native C1s but, due to the high dispersion of experimental values, Scatchard analysis of the binding data did not allow us to discriminate between a decrease in affinity and a loss of Ca^{2+} binding site(s). Further studies by sucrose gradient ultracentrifugation also showed that C1s iodinated in its monomeric or dimeric form fully retained the ability to interact with native C1r to form Ca^{2+}-dependent C1s–C1r–C1r–C1s complexes sedimenting with a coefficient of 8.7 S, identical with that determined for the native tetramer (Arlaud et al., 1980). It became clear, therefore, that lactoperoxidase-catalyzed iodination specifically abolished the ability of C1s to self-associate without impairing its ability to mediate C1r–C1s heteroassociation.

In order to study the relative incorporation of iodine into its A and B chains, C1s was iodinated as described above in the presence of trace amounts of $Na^{125}I$ and then analyzed by SDS–PAGE after reduction and alkylation. As shown in Figure 2, C1s iodinated in its monomeric form incorporated 85% of ^{125}I radioactivity in the A chain and only 15% in the B chain. In agreement with previous observations (Arlaud et al., 1980), the Ca^{2+}-dependent C1s dimerization significantly modified this distribution, by both decreasing the radioactivity incorporated in the A chain (65%) and increasing the radioactivity incorporated in the B chain (35%).

Tryptic Mapping of the A Chain of ^{125}I-Labeled C1s. The data reported above strongly suggested that the observed inhibition of C1s ability to form Ca^{2+}-dependent dimers was a consequence of the iodination of tyrosine residues accessible in the monomer, but protected in the dimer, and therefore located within or near the region involved in C1s–C1s interaction. Considering that this region is located in the NH_2-terminal A chain of C1s (Busby & Ingham, 1988, 1990; Thielens et al., 1990b), we performed a comparative study of the iodination sites within this chain after radioiodination of C1s in its monomeric and dimeric forms.

To this end, C1s (50 nmol), either in its monomeric or dimeric form, was submitted to lactoperoxidase-catalyzed iodination in the presence of trace amounts of $Na^{125}I$, under conditions (see Methods) where monomeric C1s loses completely its ability to dimerize in the presence of Ca^{2+}. Both ^{125}I-labeled C1s samples were reduced and alkylated, and their A chains were separated from the B chains and from contaminating lactoperoxidase by high-pressure gel permeation, as described under Methods. Each type of reduced and alkylated ^{125}I-labeled C1s A chain was then submitted to full tryptic cleavage, and initial fractionation of the digests was performed by gel permeation on a Bio-Gel P-10 column. As shown in Figure 3, both digests yielded comparable elution profiles, and eight pools were made in each case. The specific radioactivity of pool 3, however, was significantly higher in the case of the A chain obtained from C1s labeled in its monomeric form (Figure 3A). The early fractions, corresponding to pools 1 and 2, contained partially cleaved material and were not further analyzed. Peptides contained in pools 3–8 were further purified by reversed-phase HPLC as described under Methods and identified by amino acid analysis.

From the number of arginine and lysine residues present in C1s A chain (Tosi et al., 1987; Mackinnon et al., 1987), 32 peptides were expected from tryptic cleavage, among which were 11 peptides containing at least one tyrosine residue. At least two susceptible bonds (Arg 205–Glu 206 and Lys 354–Val 355) were found resistant to tryptic cleavage, and no significant cleavage could be detected at residues other than arginine and lysine. As summarized in Table I, two tyrosine-containing peptides, extending from Tyr-296 to Arg-299, and from Tyr-369 to Lys-405, could not be recovered. The other nine tyrosine-containing peptides were identified, most of them being resolved into several peaks by reversed-phase

Table I: Various Forms of the Tyrosine-Containing Peptides Obtained from Tryptic Cleavage of the A Chain of CĪs Iodinated in Its Monomeric and Dimeric Forms

tyrosine-containing peptide[a]	monomeric CĪs		dimeric CĪs	
	unlabeled form	labeled forms	unlabeled form	labeled forms
(1)EPTMYGEILSPNYPQAYPSEVEK(23)	M5b	M5c, M5d	D5b	D5c, D5d, D5e, D5f
(24)SWDIEVPEGYGIHLYFTHLDIELSENCAYDSVQIISGDTEEGR(66)		M3f		D3d
(72)SSNNPHSPIVEEFQVPYNK(90)	M6c	M6e, M6f	D6c	D6e, D6f
(105)FTGFAAYYVATDINECTDFVDVPCSHFCNNFIGGYFCSCPPEYFLHDDMK(154)		M3f		D3d
(155)NCGVNCSGDVFTALIGEIASPNYPKPYPENSR(186)	M3e		D3e	
(187)CEYQIR(192)		M8a		D8a
(231)QFGPYCGHGFPGPLNIETK(249)		M6h		D6h
(272)YHGDPMPCPK(281)	M7a		D7a	
(296)YVFR(299)	NR[b]	NR	NR	NR
(317)VGATSFYSTCQSNGK(331)	M7b	M7d, M7f	D7b	D7b, D7f
(369)YTCEEPYYYMENGGGGEYHCAGNGSWVNEVLGPELPK(405)	NR	NR	NR	NR

[a] Positions in the sequence of the first and the last amino acid of each peptide are indicated in parentheses. [b] NR, not recovered.

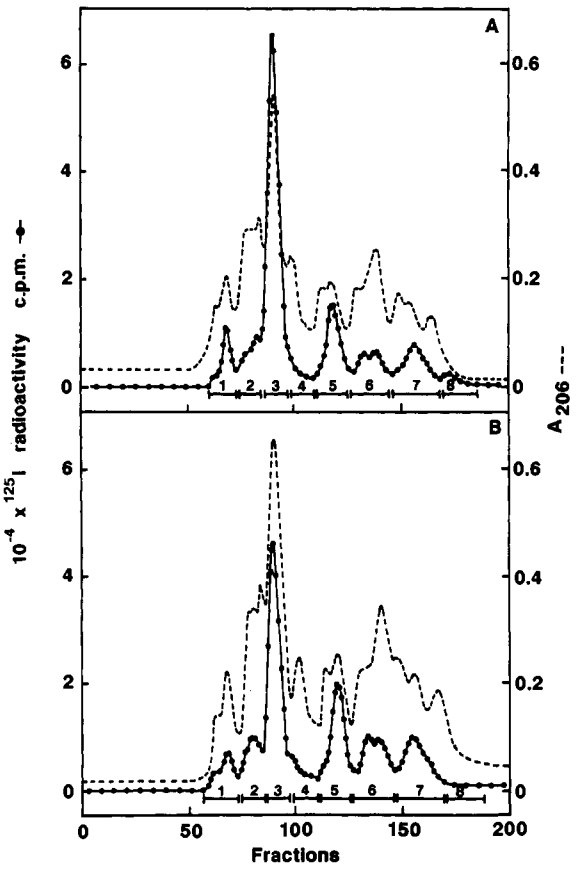

FIGURE 3: Initial fractionation by gel permeation of the tryptic peptides from the A chain of ^{125}I-labeled CĪs. CĪs (50 nmol), either in its monomeric or dimeric form, was submitted to lactoperoxidase-catalyzed radioiodination. Both samples were reduced and alkylated, and their A chains were purified by high-pressure gel permeation and then submitted to full tryptic cleavage. Each digest was fractionated on a Bio-Gel P-10 column as described under Methods. Fractions of 2.5 mL were collected. Pools were made as indicated by bars: (A) peptides obtained from the A chain of CĪs iodinated in its monomeric form, (B) peptides obtained from the A chain of CĪs iodinated in its dimeric form.

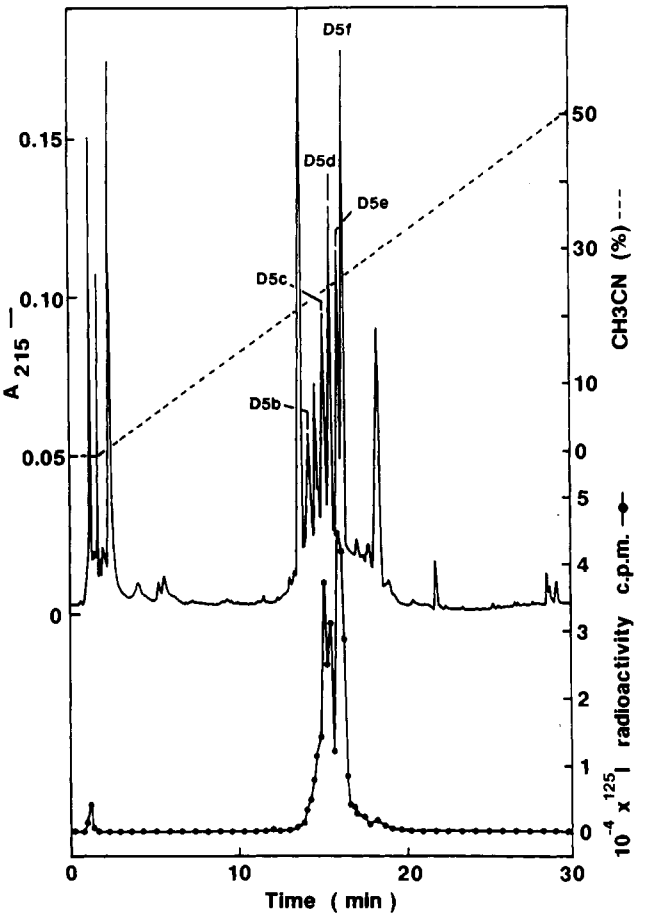

FIGURE 4: Purification by reversed-phase HPLC of the peptides from pool D5. Peptides contained in pool D5 from Bio-Gel P-10 (see Figure 3B) were separated by reversed-phase HPLC on a NovaPak C-18 column as described under Methods. Peptides were detected by absorbance at 215 nm, and fractions were collected and counted for ^{125}I radioactivity.

HPLC. This was expected, since it is known that the incorporation of an iodine atom into a tyrosine-containing peptide can significantly alter its elution position under reversed-phase chromatographic conditions (Bishop et al., 1981; Stanton & Hearn, 1987). For example, as detailed below, the NH_2-terminal peptide of the A chain obtained from CĪs iodinated in its dimeric form was found to be resolved into five forms of identical amino acid compositions, corresponding to the unmodified peptide (D5b) and to various iodinated forms (D5c, D5d, D5e, D5f) (Table I; Figure 4).

Identification of the Iodination Sites. Radioactive peptides containing a single tyrosine residue (see Table I) were assumed to be iodinated at this residue and were not further analyzed. Those containing two or more tyrosine residues were submitted to NH_2-terminal sequencing, with a view to identify the iodination site(s) and to measure the relative amounts of ^{125}I radioactivity at each cycle. Only tyrosine residues were found to be iodinated, and tentative assignment of the mono- and diiodotyrosine forms was done as described under Methods. As is commonly observed during sequencing of radiolabeled peptides, a broad "carry-over" of radioactivity was observed after each iodinated tyrosine, as illustrated in Figure 5.

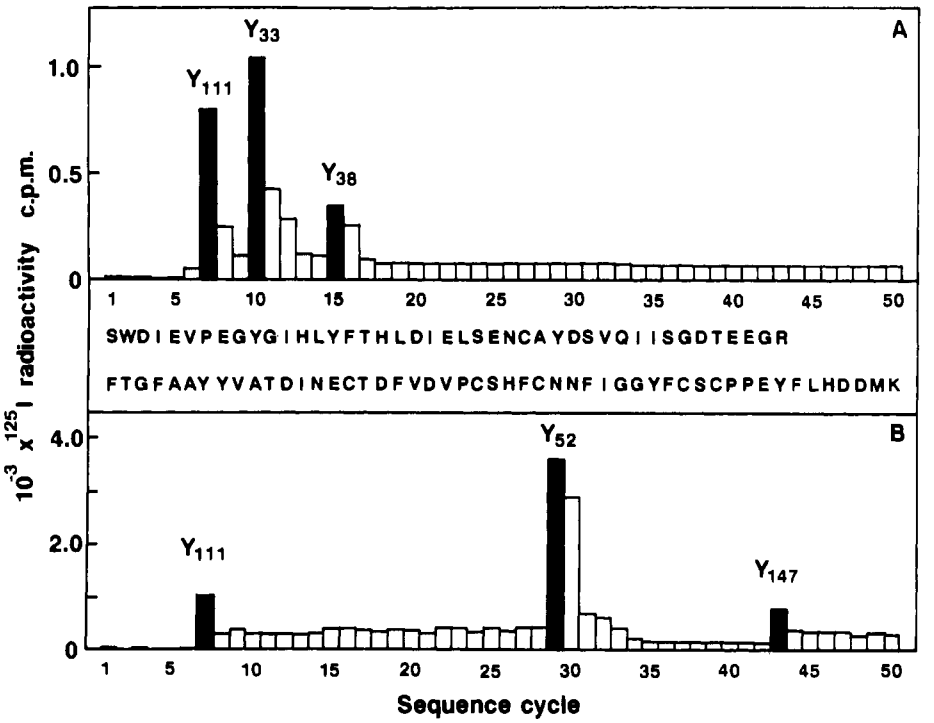

FIGURE 5: N-Terminal sequence analysis of the peptides contained in pools M3f and D3d. Pools M3f and D3d were obtained respectively by fractionation of pools M3 (see Figure 3A) and D3 (see Figure 3B) by reversed-phase HPLC. Each pool contained two peptides, extending from Ser-24 to Arg-66 and from Phe-105 to Lys-154, in the respective amounts 114 and 43 pmol (pool M3f) and 851 and 393 pmol (pool D3d). Automated Edman degradation was performed as described under Methods, and phenylthiohydantoin derivatives released at each sequence cycle were also counted for ^{125}I radioactivity. The tyrosine residues at positions 111, 38, and 147 were identified as the monoiodotyrosine derivatives, and those at positions 33 and 52 were assigned as the diiodotyrosine derivatives: (A) pool M3f, (B) pool D3d.

NH_2-terminal sequence analysis of the various iodinated forms of the NH_2-terminal peptide of the A chain (see Table I) indicated that each form was iodinated at Tyr-5, whereas Tyr-13 and Tyr-17 were never modified. When C1s was iodinated in its monomeric form, two labeled forms, M5c and M5d, were obtained, which contained respectively the diiodotyrosine and the monoiodotyrosine derivative of Tyr-5. The corresponding peptides (D5c and D5d) were also obtained when iodination was performed on dimeric C1s, but two additional forms, D5e and D5f, again containing the diiodotyrosine and the monoiodotyrosine derivatives, respectively, of Tyr-5, were also recovered (Table I; Figure 4). This behavior was presumably due to the partial oxidation of the methionine residue at position 4 or to cis/trans isomerization at a proline residue.

Most of the peptides were satisfactorily purified by reversed-phase HPLC by using the NH_4HCO_3/CH_3CN system defined under Methods and yielded single NH_2-terminal amino acid sequences. However, two peptides, extending from Ser-24 to Arg-66 and from Phe-105 to Lys-154, could not be separated in this system, and further attempts using other systems proved unsuccessful. NH_2-terminal sequence analysis was therefore performed on pools M3f and D3d, which both contained a mixture of these two peptides (Table I; Figure 5).

The analyses performed on the tryptic peptides and the relative quantitation of the various forms of each peptide enabled us to estimate the extent of iodination of the tyrosine residues of the A chain within the monomeric and dimeric forms of C1s, as summarized in Table II. In both forms, no significant iodination of the tyrosine residues at positions 13, 17, 112, 139, 177, 181, and 272 was observed. Conversely, the tyrosine residues at positions 5, 88, 111, 189, 235, and 323 were all found to be partially (50–95%) or completely iodinated in monomeric and dimeric C1s, and the extent of iodination of each of these residues was similar in both cases. In contrast,

Table II: Extent of Iodination of the Tyrosine Residues of C1s A Chain

position of tyrosine residue in sequence	extent of iodination (%)[a]	
	monomeric C1s	dimeric C1s
5	80 (mono, di)[b]	95 (mono, di)
13	–[c]	–
17	–	–
33	100 (di)	–
38	100 (mono)	–
52	–	100 (di)
88	50	60
111	100 (mono)	100 (mono)
112	–	–
139	–	–
147	–	100 (mono)
177	–	–
181	–	–
189	100	100
235	100	100
272	–	–
296	ND[d]	ND
323	70	70
369, 375, 376, 377, 386	ND	ND

[a] The extent of iodination of a given tyrosine residue is calculated from the recovery of all the peptides containing that tyrosine, on the basis of amino acid analysis. [b] Mono, monoiodotyrosine; di, diiodotyrosine. [c] A dash indicates that no significant iodination was observed. [d] ND, not determined.

differential iodination was observed in the case of the tyrosine residues at positions 33, 38, 52, and 147, as illustrated in Figure 5. Tyr-33 and Tyr-38, on one hand, were found to be iodinated in the monomer (Figure 5A) but were not modified in the dimer (Figure 5B). On the other hand, Tyr-52 and Tyr-147 were not modified in the monomer (Figure 5A) but were iodinated in the dimer (Figure 5B). Due to the loss of the corresponding peptides, no information was obtained on the extent of iodination of the tyrosine residues at positions 296,

369, 375, 376, 377, and 386.

Discussion

In agreement with earlier observations (Arlaud et al., 1980), the data obtained in this study indicate that lactoperoxidase-catalyzed iodination of C$\bar{1}$s in its monomeric form quantitatively and specifically inhibits its ability to dimerize in the presence of Ca^{2+}. Interestingly, iodinated C$\bar{1}$s retains the ability to mediate C$\bar{1}$s–C$\bar{1}$r heteroassociation. In agreement with recent findings (Thielens et al., 1990b), the latter observation provides further evidence that the two types of interaction (C$\bar{1}$s–C$\bar{1}$s and C$\bar{1}$r–C$\bar{1}$s), although both mediated by the NH_2-terminal α region of C$\bar{1}$s A chain, do not involve strictly identical sites and/or mechanisms. Thus, iodination of C$\bar{1}$s could alter a site in region α that is essential for C$\bar{1}$s dimerization but not for C$\bar{1}$r–C$\bar{1}$s heteroassociation. Given that C$\bar{1}$s iodinated in its dimeric form incorporates less iodine in its A chain and fully retains the ability to dimerize, it was tempting to hypothesize that the observed inhibition was the consequence of the specific iodination of tyrosine residues reactive in the monomer but protected in the dimer and therefore located within or near the site(s) critical for C$\bar{1}$s dimerization.

With a view to test this hypothesis, C$\bar{1}$s was radioiodinated in its monomeric and dimeric forms, and comparative tryptic mapping was performed on both types of ^{125}I-labeled A chains, in order to identify the tyrosine residues iodinated in each case. The extent of iodination was estimated for 17 of the 23 tyrosine residues contained in C$\bar{1}$s A chain and, in the case of 13 of these residues, no significant difference was observed between monomeric and dimeric C$\bar{1}$s. It should be mentioned that no obvious correlation was found between the reactivity of these tyrosine residues and their hydrophilic character, predicted according to the method of Kyte and Doolittle (1982). Thus, Tyr-13 and Tyr-17, both expected to be located in hydrophilic regions, were not iodinated, neither in monomeric nor in dimeric C$\bar{1}$s. In contrast, Tyr-111, although predicted to be located in a hydrophobic environment, was reactive in both cases. In agreement with studies on the mechanism of lactoperoxidase-catalyzed iodination of tyrosine (Huber et al., 1989), this suggests that surface accessibility is not an absolute prerequisite for iodination of tyrosine residues in proteins and that the reactivity of a particular tyrosine residue depends primarily on its ionization and other factors related to its microenvironment.

Our data indicate that the reactivity of at least four tyrosine residues of C$\bar{1}$s A chain is strictly dependent on the association state of the protein. It should be emphasized that these residues, namely, Tyr-33, Tyr-38, Tyr-52, and Tyr-147, are all located within the NH_2-terminal α region of C$\bar{1}$s A chain (Figure 6), known to be responsible for Ca^{2+}-dependent C$\bar{1}$s dimerization (Thielens et al., 1990b; Busby & Ingham, 1990). Tyr-33 and Tyr-38 are both fully iodinated in monomeric C$\bar{1}$s but are not reactive in dimeric C$\bar{1}$s. It is tempting to hypothesize that iodination of these residues, or of one of them, alters the three-dimensional structure of the α interaction region of C$\bar{1}$s in such a way that subsequent C$\bar{1}$s–C$\bar{1}$s interaction in the presence of Ca^{2+} is prevented. That both residues are protected from iodination within the dimer suggests that they are concealed by the interaction. Another possibility is that the Ca^{2+}-dependent interaction, or Ca^{2+} binding itself, induces a conformational rearrangement in the α region that decreases their reactivity. The fact that Tyr-52 and Tyr-147, which are not iodinated in monomeric C$\bar{1}$s, both become reactive in the dimer also strongly supports the hypothesis of a conformational change that, in the case of these latter tyrosine residues, results in an increased reactivity toward iodination. Evidence for a comparable conformational change has been obtained in the case of protein C, where binding of Ca^{2+} to the EGF-like domain influences the structure of the neighboring NH_2-terminal region of the protein (Öhlin et al., 1988, 1990; Orthner et al., 1989).

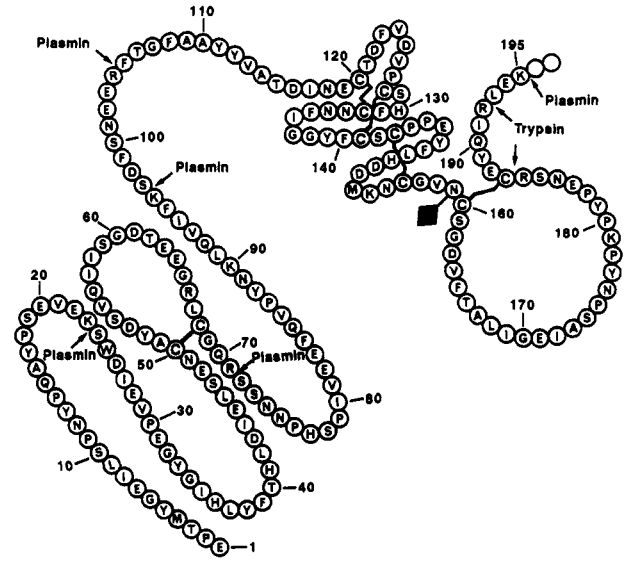

FIGURE 6: Schematic representation of the NH_2-terminal α region of human C$\bar{1}$s. The secondary structure of the EGF-like motif II (positions 111–159) is inferred from the solution structure of human EGF (Cooke et al., 1987), whereas that of motif I (positions 1–110) and of the NH_2-terminal part of motif III (positions 160–195) is not known. Known sites of limited cleavage by plasmin and trypsin (Thielens et al., 1990a,b) are indicated by arrows. The asparagine residue at position 134 is partially hydroxylated; ♦ indicates an Asn-linked carbohydrate.

Three of the above-mentioned tyrosine residues (Tyr-33, Tyr-38, and Tyr-52) are located in the NH_2-terminal motif I of the α region (positions 1–110), and one (Tyr-147) is contained in the following EGF-like motif II (position 111–159) (see Figure 6). These locations confirm, therefore, previous data indicating that the structural determinants required for Ca^{2+} binding and Ca^{2+}-dependent protein–protein interactions are contributed by both motifs I and II of C$\bar{1}$s A chain (Thielens et al., 1990a). We have recently shown that the α regions of C$\bar{1}$r and C$\bar{1}$s each contain one intrinsic high-affinity Ca^{2+} binding site ($K_d \sim 19\ \mu M$) and that this 1:1 stoichiometry is maintained upon C$\bar{1}$r–C$\bar{1}$s heteroassociation (Thielens et al., 1990b). In contrast, the C$\bar{1}$s–C$\bar{1}$s homodimer binds 3 Ca^{2+} atoms/dimer, indicating that one additional Ca^{2+} binding site, likely contributed in part by each C$\bar{1}$s α region, is formed upon C$\bar{1}$s dimerization. Recent studies by Dahlbäck et al. (1990) indicate that a fragment of protein S comprising two contiguous β-hydroxyasparagine-containing EGF-like domains retains high-affinity Ca^{2+} binding sites. On the basis of a sequence comparison of 48 domains of this type, these authors have defined a consensus sequence and determined the distribution of negatively charged amino acids that may be important for Ca^{2+} binding. Although hydroxylation is only partial in C$\bar{1}$s, the EGF-like motifs of C$\bar{1}$r and C$\bar{1}$s both contain β-hydroxyasparagine (Arlaud et al., 1987c; Thielens et al., 1990a), and they both conform to the proposed consensus sequence and charge distribution. These motifs appear therefore as the best candidates for the *intrinsic* Ca^{2+} binding site found in each of the α regions of C$\bar{1}$r and C$\bar{1}$s. We propose that, in contrast, the *extra* Ca^{2+} binding site provided by the homologous C$\bar{1}$s–C$\bar{1}$s interaction is contributed mainly by the NH_2-terminal motifs I of each C$\bar{1}$s monomer. As already

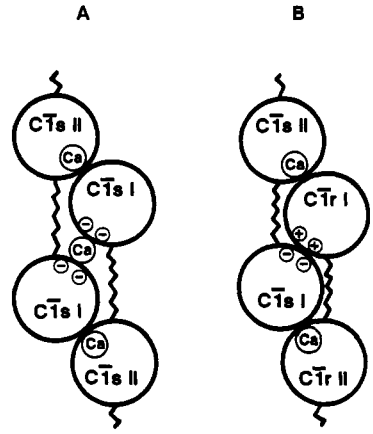

FIGURE 7: Models of (A) the homologous (C1s–C1s) and (B) the heterologous (C1r–C1s) Ca^{2+}-dependent interactions. The domain structure of the NH_2-terminal α regions of C1r and C1s (including the NH_2-terminal motif I and the EGF-like motif II) is adapted from Arlaud et al. (1987a). The COOH-terminal part of the A chains (motifs III, IV, and V) and the B chains are not represented. The conformational changes that probably occur in regions I and II of C1s upon C1s–C1s interaction are not depicted.

noticed by Tosi et al. (1987), motif I of C1s differs strikingly from the homologous motif of C1r with regard to the contents of charged residues. Thus, glutamic or aspartic acid residues at positions 1, 20, 26, 28, 31, 61, 63, 64, and 82 of C1s A chain (see Figure 6) have no equivalent in C1r, whereas, conversely, a number of lysine or arginine residues contained in motif I of C1r are not present in C1s. It is noteworthy that most of the above-mentioned acidic residues are located between positions 20 and 64, i.e., in the sequence stretch that also contains Tyr-33, Tyr-38, and Tyr-52. We suggest that the C1s–C1s interaction involves a linkage between the corresponding regions of motif I of each C1s monomer, through a Ca^{2+} ion bridge (Figure 7A). Similarly, it can be hypothesized that the heterologous C1r–C1s interaction also involves ionic bonds between motifs I of C1r and C1s, although in that case, due to the complementarity of the charges in these motifs, a Ca^{2+} bridge would not be required (Figure 7B). In this hypothesis, iodination of monomeric C1s (likely through modification of Tyr-33 and/or Tyr-38) would destroy the extra Ca^{2+} binding site and thereby inhibit subsequent C1s dimerization while the structures involved in C1r–C1s interaction would be preserved.

It should be considered, however, that none of the plasmin fragments α1 and α2 (covering positions 24–71 and 97–195 of C1s A chain, respectively; see Figure 6) retain the ability to bind Ca^{2+} with an affinity comparable to that of intact C1s ($K_d \sim 19$ μM) or its α fragment ($K_d \sim 38$ μM) (Thielens et al., 1990a,b). In the same way, Scatchard analysis of the binding of Ca^{2+} by the C1s–C1s and C1sα–C1sα dimers provides no evidence for the presence of binding sites with different affinities (Thielens et al., 1990b). As discussed previously (Thielens et al., 1990b), a likely explanation is that the proposed Ca^{2+} binding sites are not self-sufficient, but interdependent. Thus, the intrinsic Ca^{2+} binding site mainly contributed by the EGF-like motif II would also involve structural determinants in motif I, whereas Ca^{2+} binding to this site would, in turn, condition the formation of the Ca^{2+} ion bridge between motifs I. The schematic representations of the C1s–C1s and C1r–C1s interactions shown in Figure 7 account for the data and hypotheses discussed above and are compatible with available information. Additional work will be required to test these models and to identify the amino acids involved in Ca^{2+} binding and Ca^{2+}-dependent interactions.

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Registry No. C1s, 80295-70-1; C1r, 80295-69-8; C1s–C1s, 131384-37-7; C1r–C1s, 131384-35-5; Ca, 7440-70-2; tyrosine, 60-18-4.

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