of the inhibitory complex between enolase–Mg^{2+}–tartronate semialdehyde phosphate (TSP). In TSP, the hydroxyl is replaced by the aldehydic oxygen atom which cannot donate a hydrogen bond (Spring & Wold, 1971).

The structure of the ternary complex enolase–Zn^{2+}–phosphoglycolate shows another mode of inhibitory ligand binding. The nonproductive binding is in agreement with the data showing that yeast enolase does not catalyze the exchange of protons of phosphoglycolate while rabbit muscle enolase does (Stubbe & Abeles, 1980).

The most interesting observation is that despite the presence of 20 mM Zn^{2+} there is no indication of the presence of the metal ion bound in the catalytic site. It has been shown that the catalytic metal ion (Mg^{2+}) binds to the enzyme at pH 7.8 in the presence of PG (Brewer, 1971). Such binding is associated with changes in fluorescence and absorption of the enzyme (Brewer, 1971). Admittedly, the high ionic strength in the crystals and the low pH (6 rather than 7.8) could weaken binding, but under the same conditions, if PGA is used instead of PG, the crystals shatter immediately. We think, therefore, that the conformational transition, the onset of which was observed in the precatalytic ternary complex and which is not observed in the two inhibitory complexes, is crucial for the formation of the catalytic metal ion binding site.

ACKNOWLEDGMENTS

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Identification of the Disulfide Bonds of Human Complement C1s†

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ABSTRACT: C1s, one of the three subcomponents of C1, the first component of the complement system, is a complex serine protease. To determine the disulfide-bonding pattern, fragments of C1s were generated by cleavage with pepsin, thermolysin, or subtilisin. Disulfide bonds have been identified by several methods, for example, direct observation of the phenylthiohydantoin derivative of cystine during Edman degradation of isolated peptides and placement in the known cDNA sequence. All of the 26 half-cystines are linked in disulfide bonds occurring at positions 50–68, 120–132, 128–141, 143–156, 160–187, 219–236, 279–326, 306–339, 344–388, 371–406, 410–534, 580–603, and 613–644. All of the disulfide bonds of the earlier described substructures of C1s, the EGF-homologous part, the two SCR units, and the two domains typical for C1s and C1r are localized within these domains.

The first component of the classical complement pathway, C1,[1] is a complex proteinase with C1q as the recognition unit and a catalytic unit consisting of a Ca^{2+}-dependent tetrameric complex of two homologous serine proteases, C1r and C1s. Binding of C1 to various immunocomplexes or nonimmune activators is mediated by C1q and leads to activation of the catalytic subunit by a two-step process. Initially the active form of C1r is generated by an autocatalytic process, which then activates C1s to $\overline{C1s}$, the proteolytic enzyme that finally triggers the classical pathway [reviewed by Cooper (1985) and Schumaker et al. (1987)].

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[1] Abbreviations: RP-HPLC, reversed-phase high-performance liquid chromatography; ABI-sequencer, Applied Biosystems protein sequencer 477A; SBD-F, ammonium 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonate; TFA, trifluoroacetic acid; Cys, half-cystine. The nomenclature of complement components is used as recommended by the World Health Organization; activated components are indicated by a superscripted bar.

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The activation of C1r and also of C1s occurs by cleavage of a single Arg–Ile bond that leads to an active two-chain proteinase consisting of a heavy chain (A) and a light chain (B) linked by a disulfide bridge (Arlaud & Gagnon, 1985; Spycher et al., 1986). The activation process of C1 and the proteolytic activity of $\overline{C1}$ are both controlled by the C1 inhibitor, a member of the serine protease inhibitor gene family [reviewed by Müller-Eberhard (1988)].

The complete sequence of C1s has been determined by cDNA sequencing (Mackinnon et al., 1987; Tosi et al., 1987) and is confirmed by protein sequencing data (Carter et al., 1984; Spycher et al., 1986). C1s contains 673 amino acids; 26 of them are half-cystines, and all of them are engaged in disulfide bonds. Half-cystines occur at positions 50, 68, 120, 128, 132, 141, 143, 156, 160, 187, 219, 236, 279, 306, 326, 339, 344, 371, 388, 406, 410, 534, 580, 603, 613, and 644. The B-chain, representing the carboxy-terminal portion of the proenzyme, is homologous to the catalytic chain of other mammalian serine proteinases (Carter et al., 1984). The A-chain, the amino-terminal part of the zymogen, is directly involved in the formation of the C1s–C1r–C1r–C1s complex (Schumaker et al., 1987; Arlaud et al., 1987). The heavy chain can be divided into five structural domains: one epidermal growth factor like segment, two pairs of internal repeats, and two 60-residue repeating sequences found also in a number of other complement proteins such as C2, factor B, C4-binding protein, factor H, and the CR1 receptor for C3b/C4b as well as in a number of other proteins that are not related to the complement system, such as β-2-glycoprotein I and coagulation factor XIII. A potential disulfide bridge arrangement of C1s has been published by Mackinnon et al. (1987).

The aim of this study was to determine experimentally the arrangement of disulfide bridges in this molecule. For that purpose we used techniques of proteolytic fragmentation and sequence analysis. With sequence evidence for 11 pairs of peptides connected by one disulfide bridge and one fragment containing two disulfide bridges, it was possible to identify all 13 disulfide bridges within C1s.

Experimental Procedures

Materials

Pepsin (EC 3.4.23.1) was from Sigma, thermolysin (EC 3.4.24.4) and subtilisin (EC 3.4.21.14) were from Serva, and SBD-F was from Dojin, Kumamoto, Japan. The reagents used for N-terminal sequence analysis were from Applied Biosystems, and the reagents used for RP-HPLC were lichrosolv or uvasol grade supplied by Merck. All other chemicals were of analytical reagent grade, purchased either from Fluka, Merck, or Sigma.

Methods

C1s was isolated from human serum by a rapid two-step method involving affinity chromatography on IgG–Sepharose followed by ion-exchange chromatography on DEAE–Sephacel as previously described (Spycher et al., 1986). The C1s-containing fractions eluted from the DEAE column were pooled, dialyzed, and concentrated to 0.25 mg/mL with Bio-Gel concentrator resin in 1 mM HCl.

SDS–polyacrylamide gel electrophoresis was performed as described by Laemmli and Favre (1973).

Cleavage with pepsin was carried out at an enzyme:substrate ratio of 1:50 (w/w) for 24 h at 37 °C in 0.01 M HCl, pH 2.

Subdigestions of the peptic fragments with thermolysin were performed at a molar ratio of 1:100 for 5 h at 50 °C in 1 mM borate buffer, pH 6.5, containing 2 mM $CaCl_2$ and 1 mM iodoacetamide to minimize disulfide interchange.

Subdigestion with subtilisin was carried out at a molar ratio of 1:50 for 40 h at 37 °C in 0.1 M NH_4HCO_3, pH 8.0, containing 1 mM iodoacetamide.

Mixtures of peptides were usually separated first by RP-HPLC on a Bakerbond C4 column (4.6 × 250 mm, wide pore, 33 nm, 5 μm; J. T. Baker Chemicals, Deventer, The Netherlands) in a Hewlett-Packard 1090 liquid chromatograph. The acetonitrile gradient systems used are described in the figures of the corresponding chromatograms. For further purification, a Bakerbond diphenyl column (4.6 × 250 mm, wide pore, 33 nm, 5 μm; J. T. Baker Chemicals, Deventer, The Netherlands) or a butyl Aquapore column (100 × 2.1 mm, 7 μm; Brownlee, Santa Clara, CA) was used in the same acetonitrile systems.

Cys-containing peptides were detected according to the procedure of Sueyoshi et al. (1985). The fluorescence intensities were measured in a Perkin-Elmer LS-5B luminescence spectrophotometer with excitation at 385 nm and emission at 515 nm.

For amino acid analysis, the samples were hydrolyzed in the gas phase with 6 M hydrochloric acid containing 0.1% (v/v) phenol and 0.05% (v/v) 2-mercaptoethanol for 22 h at 115 °C under vacuum. Liberated amino acids were reacted with phenylisothiocyanate and the resulting phenylthiocarbamyl amino acids were analyzed by RP-HPLC on a Nova Pak C18 column (4 μm, 3.9 × 150 mm; Waters, Milford, MA) in a Hewlett-Packard 1090 liquid chromatograph with automatic injection system (Hewlett-Packard, Waldbronn, FRG) according to Bidlingmeyer et al. (1984). The 0.14 M sodium acetate buffer, pH 6.4, was replaced by the corresponding ammonium acetate buffer.

Disulfide-linked peptides were analyzed in an Applied Biosystems 477A sequencer by using a program adapted from Hunkapiller et al. (1983) and with a Model 120A PTH Analyzer [reviewed by Hunkapiller (1987)]. PTH-cystine was identified after its release in the corresponding cycle as previously reported by Lu et al. (1987) and Marti et al. (1987). Since C1s had to be analyzed with intact disulfide bonds, the first half-cystine of a disulfide-linked pair is not released in its degradation cycle and is therefore denoted by X. Thus, this symbol stands for a half-cystine residue expected from the known sequence, but one which is not detected in the sequence analysis. It is not before the second half-cystine is cleaved that the corresponding product, di-PTH-cystine is identified in the sequencer.

Results

Purified C1s was isolated in a final yield of approximately 10 mg/L of serum. The protein was identified and characterized by its N-terminal sequence and by SDS–polyacrylamide gel electrophoresis as previously described by Spycher et al. (1986). Both criteria indicated that the preparation consisted essentially of the single-chain proenzyme.

Limited Cleavage of C1s with Pepsin. Upon RP-HPLC on a C4 column (Figure 1) the peptic digest of unreduced C1s yielded 61 fractions. Aliquots of 50–200 pmol of each fraction were treated with SBDF to identify cystine-containing peptides. The fluorescent fraction P28 was rechromatographed by RP-HPLC on a C4 column. Edman degradation of P28 revealed two sequences in approximately equimolar amounts. N-Terminal and compositional analyses indicated the presence of a chain extending from Ser_{47} to Gln_{56}, containing Cys_{50}, and a second chain comprising Gly_{65} to Glu_{83}, thus including Cys_{68}.

Cleavage with Thermolysin. Thermolysinolysis of the pooled peptide fractions P52 and P53 generated nine pairs of

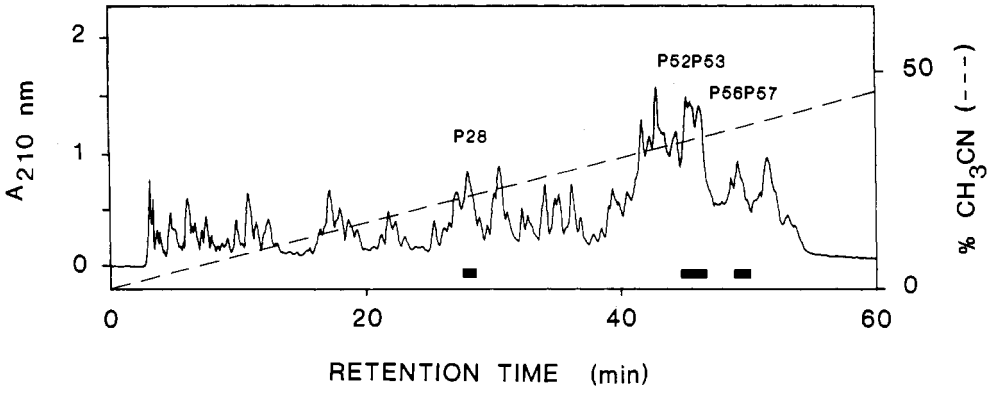

FIGURE 1: Fractionation of the C1s pepsin digest. RP-HPLC on Bakerbond C4 (wide pore, 330 Å, 5 μm, 4.6 × 250 mm) using a linear acetonitrile gradient was performed. Solution A, 0.1% TFA in H_2O; solution B, 0.1% TFA in 80% acetonitrile; flow rate, 1 mL/min; detection, 210 nm. Pools corresponding to P28, P52P53, and P56P57 are indicated by bars.

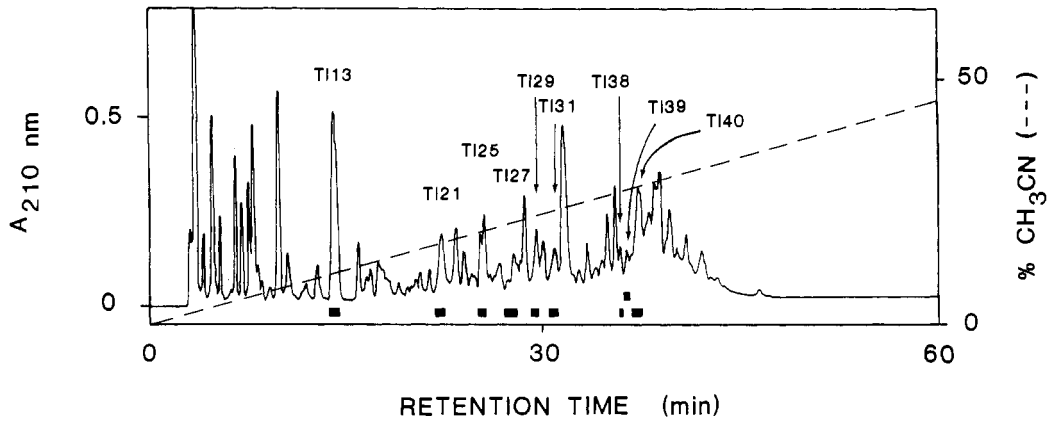

FIGURE 2: Partial separation of the thermolytic digest of P52P53 (Figure 1). RP-HPLC on Bakerbond C4 (wide pore, 330 Å, 5 μm, 4.6 × 250 mm) using a linear acetonitrile gradient was performed. Chromatographic conditions are as described in Figure 1. Pools corresponding to TI13, TI21, TI25, TI27, TI29, TI31, TI38, TI39, and TI40 are indicated by bars.

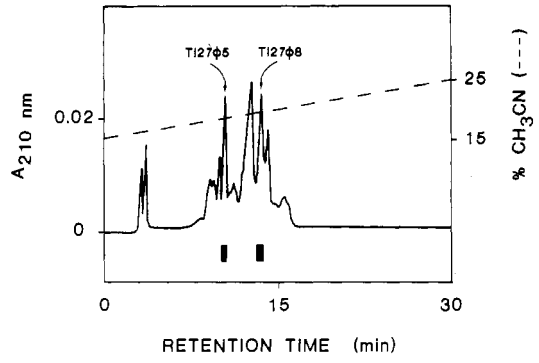

FIGURE 3: Final purification of TI27 (peak 27, Figure 2). RP-HPLC on Bakerbond diphenyl (wide pore, 330 Å, 5 μm, 4.6 × 250 mm) using a linear acetonitrile gradient was performed. Chromatographic conditions are as described in Figure 1. Pools corresponding to TI27φ5 and TI27φ8 are indicated by bars.

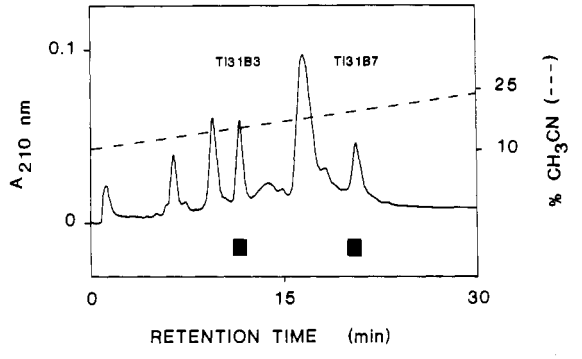

FIGURE 4: Final purification of TI31 (peak 31, Figure 2). RP-HPLC on Aquapore butyl (2.1 mm × 100 mm, 7 μm, Brownlee Labs) using a linear acetonitrile gradient was performed. Solution A, 0.1% TFA in H_2O; solution B, 0.1% TFA in 80% acetonitrile; flow rate, 0.3 mL/min; detection, 210 nm. Pools corresponding to TI31B3 and TI31B7 are indicated by bars.

peptides, each of them containing only one disulfide and one fragment with three disulfides. The digest was separated by RP-HPLC on a C4 column (Figure 2). The fluorescent fractions TI13, TI21, TI25, TI27, TI29, TI31, TI38, TI39, and TI40 were further purified on a Bakerbond diphenyl column and a butyl Aquapore column, respectively. Fractions TI27 and TI31 consisted of two disulfide-paired peptides each. They were separated by rechromatography on a diphenyl or a butyl Aquapore column, respectively (Figures 3 and 4). Edman degradation of the fragments TI13, TI21, TI25, TI27φ5, TI27φ8, TI29, TI31B3, TI31B7, TI38, and TI39 revealed in each case two sequences present in approximately equimolar amounts (Table I).

The sequences of TI13 were interpreted as Leu-Arg-Lys-Cys- and Ile-X-Ala-Gly-. N-Terminal and compositional analyses indicated the presence of a chain extending from Leu_{577} to Glu_{582}, containing Cys_{580}, and a second chain comprising residues Ile_{602} to Gly_{609}, thus including Cys_{603}. N-Terminal and compositional analyses of fragment TI21 indicated the presence of two chains, one extending from Met_{610} to Ala_{620}, containing Cys_{613}, and the other extending from Trp_{640} to Gly_{645}, including Cys_{644}. The fragment TI25 consisted of the two peptides Ala_{212} to Cys_{219} and Phe_{232} to Cys_{236}. Two cystine-containing fragments were obtained from pool TI27 (Figure 3). The sequences of TI27φ5 were interpreted as Ile_{117}-Asn-Glu-Cys_{120} and Phe_{131}-X-Asn-?-Phe_{135} (? indicates

Table I: Edman Degradation Data and Identification of Disulfide Bonds in Thermolytic Fragments

Peptide	Structural Data[a]	Disulfide Bond Cys-Cys
T127ϕ5	117 I N E C / F X N ? F 131	120-132
T131B3	116 D I N E X T D / F X N N F 131 ?	120-132
T125	212 A A D S A G N C / F G P Y X 232	219-236
T127ϕ8 [b]	273 h G d P M P X p K E* / Y s T X Q s N G* 323	279-326
T129	304 I T C L / L K C Q P 337	306-339
T131B7 [c]	342 V D X G I P E S / CHO Y M E N G G G G E Y H X A G(N)G S 377	344-388
T139	370 T X E E P Y Y / P E L P K X V P 401	371-406
T138	409 V X G V P R E P / P I C L P G T S S d 532	410-534
T113	577 L R K C K E / I X A G G E K G 602	580-603
TL21	610 M D S X K G D S G G A / W G P Q C G 640	613-644

[a] Sequences in capital letters indicate the results of Edman degradation of unreduced peptides; amino acids in lower case letters are not unambiguously identified. X denotes a Cys residue expected from the known sequence but not observed. C denotes the di-PTH of cystine. ? indicates a PTH derivative not corresponding to anyone of the known 20 amino acids. [b] Asterisks indicate chain ends that have not been rigorously identified. [c] Asn_{391} could not be identified by Edman degradation due to its carbohydrate moiety.

a PTH derivative not corresponding to any one of the known 20 amino acids). The fragment T127ϕ8 consisted of the two peptides His_{273}-Glu_{282} and Tyr_{323}-Gly_{330}, connected by Cys_{279} and Cys_{326}. N-Terminal and compositional analyses of T129 gave evidence for the presence of the tetrapeptide Ile_{304}-Leu_{307} and the pentapeptide Leu_{337}-Pro_{341}, connected by Cys_{306} and Cys_{339}. Two cystine-containing fragments were isolated from pool T131 (Figure 4). The sequences of T131B3 were interpreted as Asp-Ile-Asn-Glu- and Phe-X-Asn-Asn-. N-Terminal and compositional analyses showed the presence of a chain extending from Asp_{116} to Asp_{122}, containing Cys_{120}, and a second chain comprising residues Phe_{131} to Phe_{135}, therefore including Cys_{132}. Analysis of T131B7 indicated the presence of the chain Val-Asp-X-Gly- extending from Val_{342} to Ser_{349}, containing Cys_{344}, and a second chain extending from Tyr_{377} to Ser_{393}, thus including Cys_{388}. N-Terminal and compositional analyses of T138 yielded the peptide Val_{409} to Pro_{416} and a second one extending from Pro_{532} to Asp_{541}, connected by Cys_{410} and Cys_{534}. The fragment T139 consisted of the peptide Thr_{370} to Tyr_{376}, containing Cys_{371}, and a second one extending from Pro_{401} to Pro_{408}, with Cys_{406}. Fragment T140 revealed four sequences present in approximately equimolar amounts. Their N-terminal and compositional analyses were indicative for the following four chains: Val_{124} to (His_{130}), Phe_{140} to Tyr_{147}, Leu_{149} to (Phe_{165}), and Ile_{172} to (Gln_{190}), containing the half-cystines 128, 141, 143, 156, 160, and 187, respectively (parentheses indicate chain ends that have not been rigorously identified).

Table II: Edman Degradation Data and Identification of Disulfide Bonds in Fragments Obtained by Cleavage With Subtilisin

Peptide	Structural Data[a]	Disulfide Bond Cys-Cys
S7 [b]	157 CHO G V(N)C S / R X E Y Q 186	160-187
S21	123 F V D V P C / 155 N X / 140 F X S C P P E Y / 153 (M K N C)	128-141; 143-156

[a] Sequences in capital letters indicate the results of Edman degradation of unreduced peptides. X denotes a Cys residue expected from the known sequence but not observed. C denotes the di-PTH of cystine. [b] Asn_{159} could not be identified by Edman degradation due to its carbohydrate moiety. The amino acid analysis of fragment S7 shows Asp and GlcNAc.

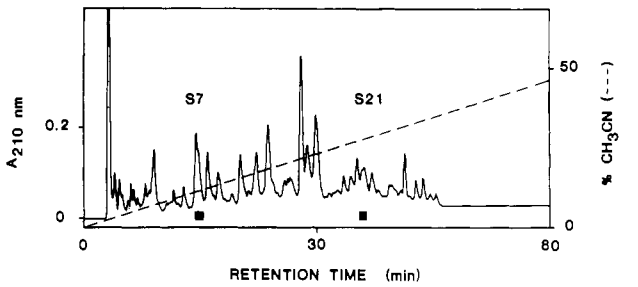

FIGURE 5: Separation of the subtilisin digest of P56P57. RP-HPLC on Bakerbond C4 (wide pore, 330 Å, 5 μm, 4.6 × 250 mm) using a linear acetonitrile gradient was performed. Chromatographic conditions are as described in Figure 1. Pools corresponding to S7 and S21 are indicated by bars.

Cleavage with Subtilisin. Up to this point, the pairing of the six half-cystines 128, 141, 143, 156, 160, and 187 was not possible due to incomplete cleavage of the protein by pepsin or of peptic fragments by thermolysin. For this reason, an extensive digestion of the peptic fragments P56 and P57 with subtilisin was performed. This enabled the isolation of one pair of peptides with the two half-cystines 160 and 187 and the isolation of a fragment with the half-cystines 128, 141, 143, and 156 (Table II). Separation was achieved by RP-HPLC on a C4 column (Figure 5) and by further purification on a diphenyl column. Fragments S7 and S21, which contained

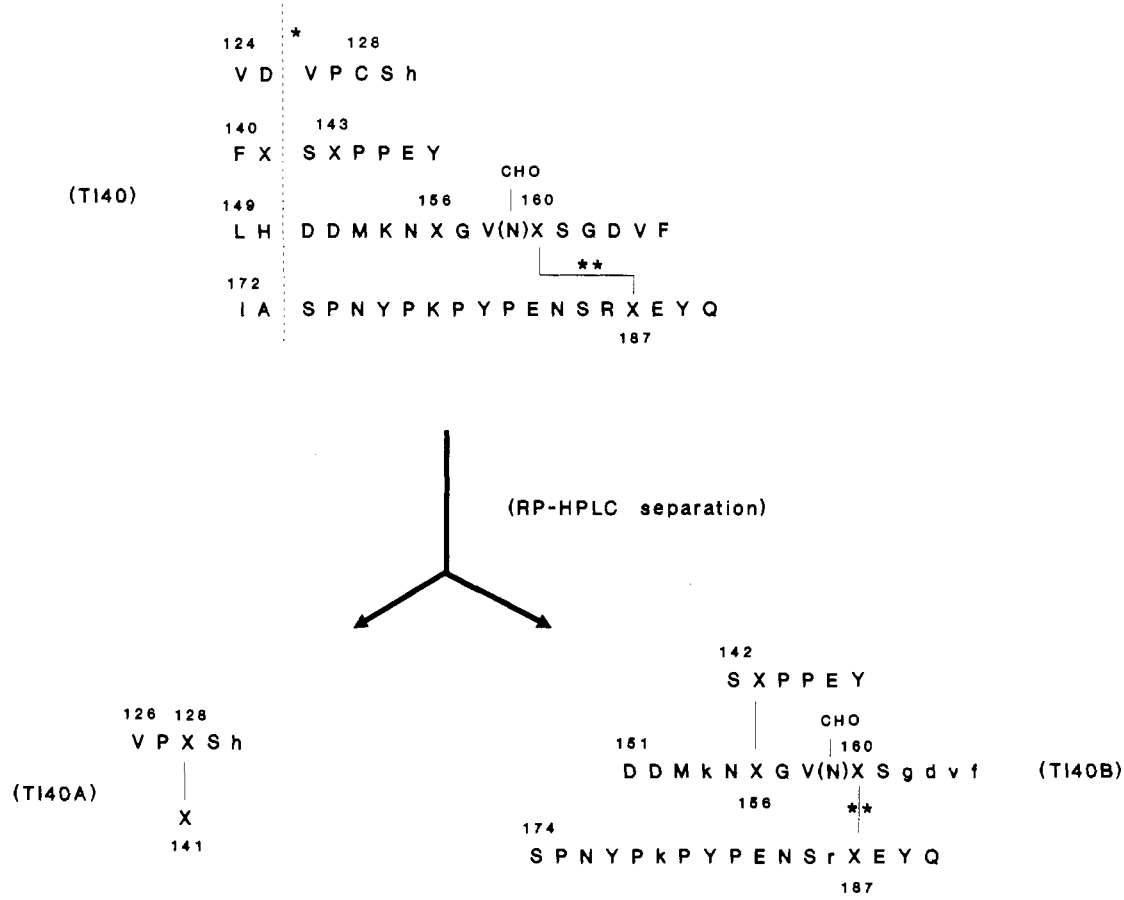

FIGURE 6: Polypeptide chains within fragment T140. (*) The dotted line indicates the situation after two Edman degradation steps. (**) The disulfide bridge (Cys_{160}–Cys_{187}) shown was previously described with fragment S7 (Table II). Amino acids in lower case letters are not unambiguously identified.

the undetermined half-cystines, were selectively identified in the HPLC fractions by SBDF treatment and by compositional and sequence analysis. Fragment S7 revealed two sequences in about equimolar amounts: Gly_{157}-Val-Asx-Cys-Ser and Arg_{186}-X-Glu-Tyr-Gln. Asx_{159} represents one of two N-glycosylated positions and was therefore determined as Asp only by compositional analysis. Fragment S21 contained the following four peptides: Phe_{123}-(Cys_{128}), Phe_{140}-X-Ser-Cys-...-Tyr_{147}, Met_{153}-Lys-Asn-Cys, and Asn_{155}-X in a relative ratio of about 3:3:1:2. The latter peptide apparently originates from an incomplete cleavage of the Lys_{154}–Asn_{155} bond. Since sequence analysis yielded no di-PTH-cystine in degradation step two, Cys_{156} cannot be linked to Cys_{141}. Cys_{156} must therefore be connected with Cys_{143} and Cys_{128} with Cys_{141}. This observation was crucial for an additional experiment. Fragment T140, isolated from the thermolytic pool (Figure 2), was subjected to two degradation cycles in the sequencer. The sequences Val-Asp-, Phe-X-, Leu-His-, and Ile-Ala- were detected in about equimolar amounts. The remaining fragment (T140 minus 2 positions) was recovered from the sequencer by elution of the glass fiber support with aqueous TFA and acetonitrile. The extract was separated on a butyl Aquapore column into fragments T140A and T140B. T140A yielded a single pentapeptide sequence from Val_{126} to (His_{130}), while fragment T140B contained three sequences that were indicative for the peptides Ser_{142} to Tyr_{147}, Asp_{151} to Phe_{165}, and Ser_{174} to Gln_{190} (Figure 6). Since the pairing of Cys_{160} with Cys_{187} had already been established with fragment S7, it follows that Cys_{143} must be linked to Cys_{156}.

Discussion

The large size and the multivalent character of the C1s molecule offer a challenge in attempts to locate specific domains responsible for its various functions.

In the present work, proteolytic fragments of C1s were generated with either pepsin, thermolysin, or subtilisin. Each fragment was characterized with regard to its location in the primary structure. All of the 13 disulfide bonds have been assigned (Figure 7). The result obtained is in agreement with the putative assignment by Mackinnon et al. (1987). Sequence comparison leads to a six-domain model of C1s, in which the heavy chain consists of domains I–V and the light chain represents the last one of the six domains (Mackinnon et al., 1987; Tosi et al., 1987). Such domains have also been described for C1r (Leytus et al., 1986; Journet & Tosi, 1986).

Domains I and III are specific structural motifs of C1s and C1r. They are homologous to each other and contain two and four half-cystines, respectively (Mackinnon et al., 1987). The connection of Cys_{50} with Cys_{68} of domain I was proven with the peptic fragment P28. The fragment T125, isolated after additional digestion of C1s with thermolysin, gave evidence for a disulfide bridge between the third and the fourth half-cystines of domain III. The two disulfides correspond to each other, whereas the disulfide, existing between the first and the second half-cystines of domain III, has no counterpart in domain I.

Gagnon and Arlaud (1985) described an autocatalytic degradation product of $C\bar{1}r$ that contains only the third and the fourth half-cystines of domain III. This so-called β-fragment is released from the $C\bar{1}r$ heavy chain without reduction. This observation underlines the adjoinment of the two homologous half-cystines in domain III of C1s and, due to the internal homology, also of those in domain I. Leytus

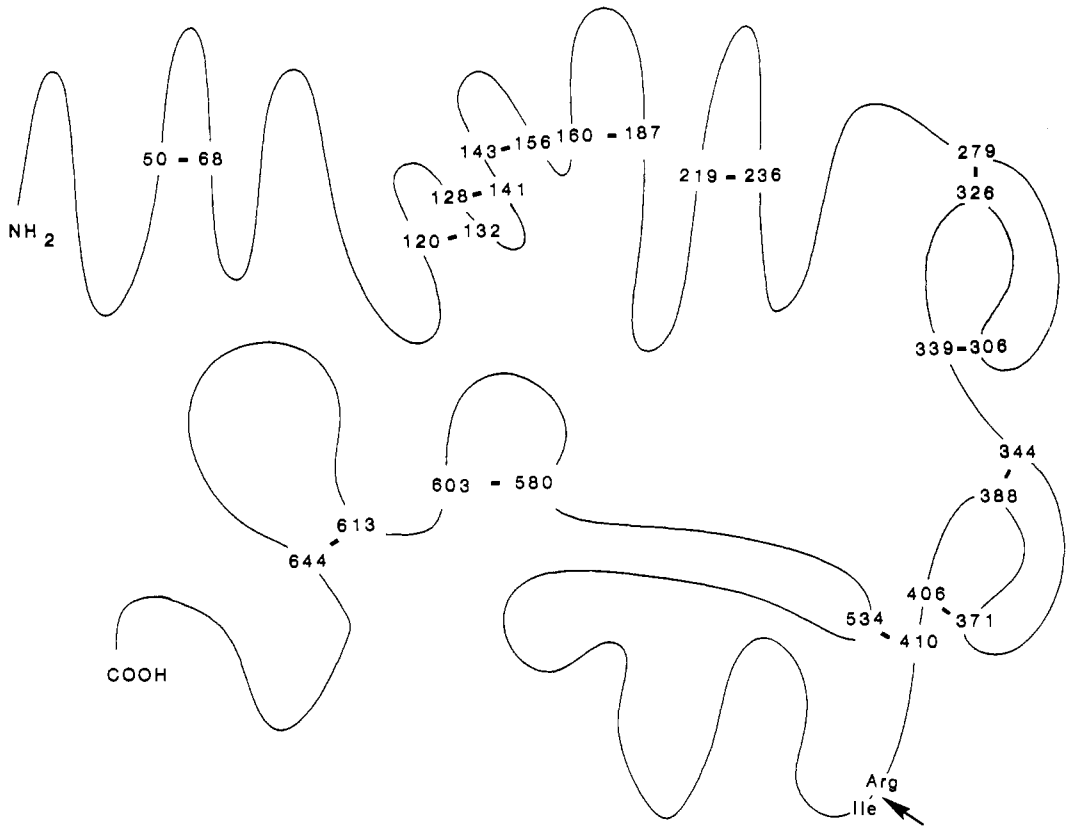

FIGURE 7: Disulfide bond pairing of C1s. Numbers denote Cys residue numbers in the C1s sequence (Mackinnon et al., 1987; Tosi et al., 1987). Disulfide bridges are symbolized by bars. The arrow indicates the Arg_{422}–Ile_{423} bond cleaved during the activation of C1s to $\overline{C1s}$.

et al. (1986) also predicted the disulfide bridge of the first and second half-cystines of domain III. The present study provides the experimental evidence that in C1s the disulfides of the third domain indeed exist in a 1–2, 3–4 pattern and therefore create a relatively independent domain.

Domains IV and V are two other sequence regions with internal homology between each other. These domains are generally designated as short consensus repeats (SCR's) and are widely found in complement proteins as well as in proteins not related to the complement system [reviewed by Reid and Day (1989)]. They consist of a framework of four conserved half-cystine residues and several other highly conserved residues, which include proline, tryptophan, and glycine.

These 60 amino acid residue repeating units were first recognized in β_2-glycoprotein I (Lozier et al., 1984). Recently, Janatova et al. (1989) pointed out that in C4b-binding protein the four half-cystines of each SCR unit are connected in a 1–3, 2–4 pattern. The present study shows that the disulfide bonds of the two SCR units within C1s exist also in a 1–3, 2–4 pattern. They are therefore responsible for two relatively independent domains. Calorimetric investigations by Medved et al. (1989) with a relatively thermostable fragment consisting of the two SCR units and a short segment of the B-chain, isolated after limited proteolysis with plasmin, indicated that the two SCR's melt independently. This implies the independent folding of the two domains IV and V.

The connection of the five half-cystines of the catalytically active light chain was determined with three pairs of peptides containing only one disulfide each. The first half-cystine ($\underline{Cys}_{534}$) is involved in the only interchain disulfide bridge of $\overline{C1s}$. It is connected to Cys_{410}, the last half-cystine of the A-chain. Mackinnon et al. (1987) reported the sequence evidence for a pair of peptides including Cys_{580} and Cys_{603}, the second and third half-cystines of the light chain. This disulfide bridge was confirmed in the present study. The pairing of these two half-cystines is homologous to the known linkage of Cys_{168} to Cys_{182} in chymotrypsinogen, whereas the disulfide bridge between Cys_{191} and Cys_{220} of chymotrypsinogen (Brown & Hartley, 1966) is conserved in the half-cystine pair (Cys_{613}-$\underline{Cys}_{644}$) of the $\overline{C1s}$ light chain. As in $\overline{C1r}$ the light chain of $\overline{C1s}$ also lacks the cystine responsible for the formation of the so-called histidine loop, which is otherwise widely found in serine proteases (Carter et al., 1984). The remaining half-cystines are connected in a manner typical for serine proteases.

Domain II, the epidermal growth factor (EGF) domain, is resistant toward proteolytic digestion. After sequential digestion with pepsin and thermolysin, only one of the three disulfides could be isolated in a pair of peptides. The connection of Cys_{120} with Cys_{132} was proven and is consistent with the known linkage of Cys_6 with Cys_{20} in EGF (Savage et al., 1973). Two different fragments, each containing Cys_{120} and Cys_{132}, were isolated. In one fragment amino acid 134 could clearly be detected as asparagine, whereas in the other fragment the PTH derivative of amino acid 134 did not correspond to any one of the known amino acids. Its retention time was identical with that of PTH-erythro-β-hydroxyasparagine as reported by Thielens et al. (1990).

A fragment containing the other two disulfides of the EGF domain was isolated after extensive cleavage of a peptic fragment with subtilisin. The length of the peptides allowed a direct assignment of the four half-cystines to two disulfide bridges by Edman degradation. This result was confirmed by chemical cleavage of the Cys_{141}–Ser_{142} peptide bond by two Edman degradation cycles followed by separation and analysis of the fragment generated. The six half-cystines are linked in a 1–3, 2–4, 5–6 pattern as described for epidermal growth

factor (Savage et al., 1973). It has to be mentioned that this is the first time that the assignment of the six half-cystines of the EGF-homologous domain within a complex protein was experimentally proven.

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