Molecular Characterization of the Catalytic Domains of Human Complement Serine Protease C1r†

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Received February 20, 1986

ABSTRACT: Limited cleavages of human C1r by extrinsic proteases of various specificity (plasmin, elastase, chymotrypsin, thermolysin) yield dimeric associations of two globular domains, each comprised of the intact B chain disulfide linked to γ, the C-terminal fragment of the A chain. These $(\gamma\text{–B})_2$ domains, which are homologous to those obtained from C1r by autolytic cleavage [Villiers, C. L., Arlaud, G. J., & Colomb, M. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4477–4481], represent the core of the C1r molecule and are associated with the catalytic properties of the serine active site. V8 protease also yields $(\gamma\text{–B})_2$ associations, although additional cleavages occur in the B chain. Sequence analysis shows that all cleavages generating the γ fragments occur within a 13-residue sequence extending from positions 274 to 286 of the C1r A chain. Chemical cross-linking with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide of the $(\gamma\text{–B})_2$ catalytic domains obtained from C1r autolytic cleavage indicates that each γ–B domain interacts with its neighbor in a "head to tail" configuration, the γ region of one domain interacting with the B chain of the other domain, and conversely. No evidence is found of γ–γ or B–B interactions. Such a head to tail configuration, placed in the context of the model proposed for the C1s–C1r–C1r–C1s catalytic subunit of C1 [Colomb, M. G., Arlaud, G. J., & Villiers, C. L. (1984) *Philos. Trans. R. Soc. London, B 306*, 283–292], is compatible with autolytic activation of C1r through an intramolecular cross-mechanism and with subsequent activation of C1s by activated C1r.

The first component of the classical pathway of complement, C1, is a macromolecular assembly of three glycoproteins, C1q, C1r, and C1s, arranged in two structurally and functionally distinct entities, C1q and a calcium-dependent tetrameric complex C1s–C1r–C1r–C1s [see reviews by Sim (1981a) and Cooper (1985)]. Binding of C1 to activators such as immune complexes occurs through C1q, the recognition subunit, and triggers sequential activation of C1r and C1s, the components of the catalytic subunit, which are both zymogens of serine proteases. Activation involves limited proteolysis and is a two-step process in which C1r plays a key role, the initial step being autolytic activation of proenzyme C1r to its activated form C1r (EC 3.4.21.41), which in the second step activates C1s.

In its proenzyme form, human C1r is a noncovalently linked dimer comprising two apparently identical, single-chain monomers of M_r ~90 000. C1r autolytic activation involves the cleavage of a single Arg–Ile bond (Arlaud & Gagnon, 1985), each activated monomer therefore consisting of two chains A (M_r 56 000–60 000) and B (M_r 30 000–36 000), the latter containing the serine active site [see reviews by Sim, (1981b) and Cooper (1985)]. On prolonged incubation at 37 °C, isolated C1r undergoes further autolytic cleavages, occurring in the N-terminal A chain, which splits into three fragments, α, β, and γ (Assimeh et al., 1978; Okamura & Fujii, 1978; Arlaud et al., 1980). The autolytic process successively removes from each monomer fragments α (M_r 35 000) and β (M_r 7000–11 000), ending in a molecule (C1r II) which is still a dimeric association (M_r 110 000) of two monomers, each comprised of the C-terminal fragment of the A chain, γ, disulfide linked to the B chain (Arlaud et al., 1980). C1r II retains a functional active site but has lost the capacity to bind C1s (Arlaud et al., 1980) and calcium ions (Villiers et al., 1980). These observations, and the use of electron microscopy, have allowed us to propose a model of the domain structure and associated functions of C1r (Villiers et al., 1985), in which each α fragment forms a distal interaction domain, linked through a connecting strand (β) to the core of the protein, formed by the dimeric $(\gamma\text{–B})_2$ catalytic domains.

The aim of this study, based on the joint use of limited proteolysis, amino acid sequence, and chemical cross-linking, was to collect further evidences of the structural reality of the catalytic domains of C1r and to get detailed information on the structural organization of these domains at the molecular level. Such information is of particular importance, considering the critical role played by these domains in the activation mechanism of C1r and C1.

Experimental Procedures

Materials

V8 protease from *Staphylococcus aureus* was obtained from Miles Laboratories, and human plasmin was from Kabi, Stockolm, Sweden. Subtilisin BNP′ (protease type VII), thermolysin (protease type X), porcine elastase (type IV), bovine α-chymotrypsin (type IV), and papain (type IV) were purchased from Sigma. [1,3-^{3}H]iPr_2P–F[1] (6.5 Ci/mmol) was

† This work was partly supported by CNRS and the Foundation pour la Recherche Médicale. A preliminary report of this work was presented at the XIth International Complement Workshop in Miami, FL, November 1985.

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from the Radiochemical Centre, Amersham. EDAC was obtained from Sigma. Spectrapor 6 dialysis tubing (M_r cutoff, 1000) was purchased from Spectrum Medical Industries Inc.

Methods

C1r Autolytic Cleavage. C1r was isolated from human serum as described previously (Arlaud et al., 1979). Autolytic cleavage was performed by incubation of purified C1r (0.46 mg/mL) for 7 h at 37 °C in 145 mM NaCl/15 mM triethanolamine hydrochloride (pH 7.4). Autolytically cleaved C1r was then further incubated for 1 h at 30 °C in the presence of 0.25 mM [1,3-^{3}H]iPr_2P–F (1 Ci/mmol), and excess reagent was removed by exhaustive dialysis of the labeled protein against 145 mM NaCl/15 mM triethanolamine hydrochloride (pH 7.4), using Spectrapor 6 dialysis tubing.

Limited Cleavage of C1r by Extrinsic Proteases. In order to prevent autolytic cleavage, C1r submitted to limited proteolysis by extrinsic proteases was first incubated twice at 30 °C in the presence of 5 mM iPr_2P–F, and excess reagent was removed by dialysis. iPr_2P–C1r (1.6–1.9 mg) was then submitted to extrinsic cleavages, which were performed in 145 mM NaCl/15 mM triethanolamine hydrochloride (pH 7.4) or, in the case of V8 protease, in 0.1 M NH_4HCO_3/2 mM EDTA (pH 7.8). All incubations were carried out at 37 °C, for varying periods and with varying enzyme to iPr_2P–C1r weight ratios, as follows: chymotrypsin, 15 min, 1:50; thermolysin, 45 min, 1:25; subtilisin, 60 min, 1:50; plasmin, 90 min, 1:50; papain, 3 h, 1:50; elastase, 4 h, 1:50; V8 protease, 24 h, 1:25.

Isolation and Analysis of C1r Catalytic Domains. Fragments generated from C1r autolytic cleavage and from limited proteolysis of iPr_2P–C1r by extrinsic proteases were separated by high-pressure gel permeation on a TSK-G 3000 SW column (7.5 mm × 600 mm) (LKB), equilibrated in the same buffer as used for cleavage and run at 1.0 mL/min. In each case, fractions containing the $(\gamma\text{–B})_2$ catalytic domains of C1r were pooled, dialyzed exhaustively against 0.5% (v/v) acetic acid, and freeze-dried. Each pool was analyzed by SDS–polyacrylamide gel electrophoresis under reducing and nonreducing conditions, using 12.5% polyacrylamide gels as described by Laemmli (1970). Protein bands were stained with Coomassie blue, and the following proteins were used as molecular weight markers: phosphorylase a (M_r 92 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 42 000), rabbit IgG heavy and light chains (M_r 52 000 and 23 000), and lysozyme (M_r 14 000).

The major part of each $(\gamma\text{–B})_2$ pool (6.9–12.4 nmol, as estimated from amino acid analysis) was submitted to N-terminal sequence analysis, which was performed on an upgraded Beckman 890C sequencer, as described by Arlaud and Gagnon (1985).

Chemical Cross-Linking of C1r Catalytic Domains. This was performed on the catalytic domains obtained from C1r autolytic cleavage, subsequently labeled with [1,3-^{3}H]iPr_2P–F and purified by high-pressure gel permeation under nondenaturing conditions, as described above. The protein (A_{280} = 0.28) was dialyzed against 150 mM NaCl/20 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 7.0) and then incubated for 1 h at 30 °C in the presence of 10 mM EDAC. After dialysis against 0.5% (v/v) acetic acid and freeze-drying, the cross-linked products were analyzed by SDS–polyacrylamide gel electrophoresis under reducing and nonreducing conditions, using 6% polyacrylamide gels, as described by Fairbanks et al. (1971). Protein bands were stained with Coomassie blue, and radioactivity was measured on 1-mm slices. Proteins used as molecular weight markers were the same as described above.

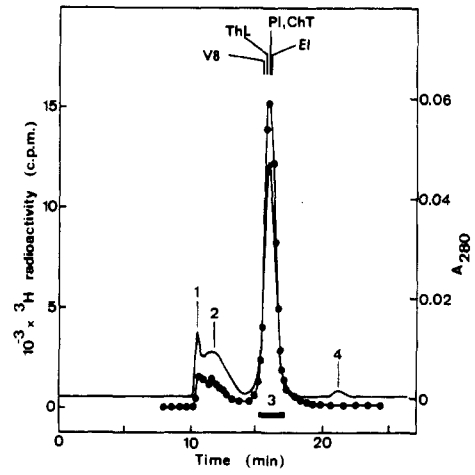

FIGURE 1: Separation of the fragments generated from C1r autolytic cleavage. Autolytically cleaved C1r was labeled with [1,3-^{3}H]iPr_2P–F, and the fragments were separated by high-pressure gel permeation on a TSK G-3000 SW column. The elution positions of the $(\gamma\text{–B})_2$ catalytic domains yielded by extrinsic proteolytic cleavage of iPr_2P–C1r by V8 protease, thermolysin, plasmin, chymotrypsin, and elastase are indicated. Phosphorylase *a* (M_r 92 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 42 000), and lysozyme (M_r 14 000) were used to calibrate the column.

RESULTS

Isolation of C1r Catalytic Domains. After incubation of C1r for 7 h at 37 °C, and labeling with [1,3-^{3}H]iPr_2P–F, the fragments generated from autolytic cleavage were separated by high-pressure gel permeation under nondenaturing conditions, as illustrated in Figure 1. As judged from SDS–polyacrylamide gel electrophoresis, peaks 1 and 2 both contained aggregated material and incompletely cleaved molecules, the major component of peak 2 being fragment α, eluted as aggregates of high molecular weight (M_r ~300 000), whereas peak 4 contained fragment β. Peak 3 contained the [1,3-^{3}H]iPr_2P-labeled $(\gamma\text{–B})_2$ catalytic domains of C1r (Villiers et al., 1985), eluted with an apparent M_r of 92 000, consistent with a dimeric association of two γ–B monomers. Further characterization of C1r catalytic domains by electron microscopy after rotary shadowing, and by sucrose gradient ultracentrifugation, confirmed the dimeric nature of these domains, as previously reported (Villiers et al., 1985; Arlaud et al., 1980).

Activated C1r, blocked with iPr_2P–F, was submitted to extrinsic proteolytic cleavage by plasmin, chymotrypsin, thermolysin, elastase, and V8 protease, and the resulting fragments were separated by high-pressure gel permeation. Each of these cleavages resulted in one major peak detected at 280 nm, eluted with an apparent M_r (88 000–96 000) comparable to that determined for the catalytic domains generated from C1r autolytic cleavage (Figure 1). In contrast, papain and subtilisin further degraded iPr_2P–C1r into smaller fragments.

Analysis of C1r Catalytic Domains. The catalytic domains obtained from C1r autolytic cleavage, and the material contained in the major peaks obtained from extrinsic cleavage of iPr_2P–C1r by plasmin, chymotrypsin, thermolysin, elastase, and V8 protease, were analyzed by SDS–polyacrylamide gel electrophoresis (Figure 2). The material obtained from autolytic cleavage and from extrinsic cleavage by plasmin, elastase, chymotrypsin, and thermolysin gave, in nonreducing

[1] Abbreviations: iPr_2P–F, diisopropyl phosphorofluoridate; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

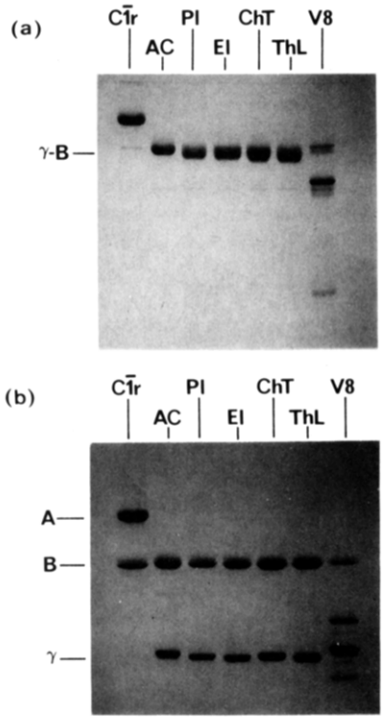

FIGURE 2: Analysis, by SDS–polyacrylamide gel electrophoresis, of the catalytic domains yielded from C$\bar{1}$r autolytic cleavage (AC) and extrinsic cleavage of iPr_2P–C$\bar{1}$r by plasmin (PI), elastase (EI), chymotrypsin (ChT), thermolysin (ThL), and V8 protease (V8): (a) unreduced samples; (b) reduced and alkylated samples.

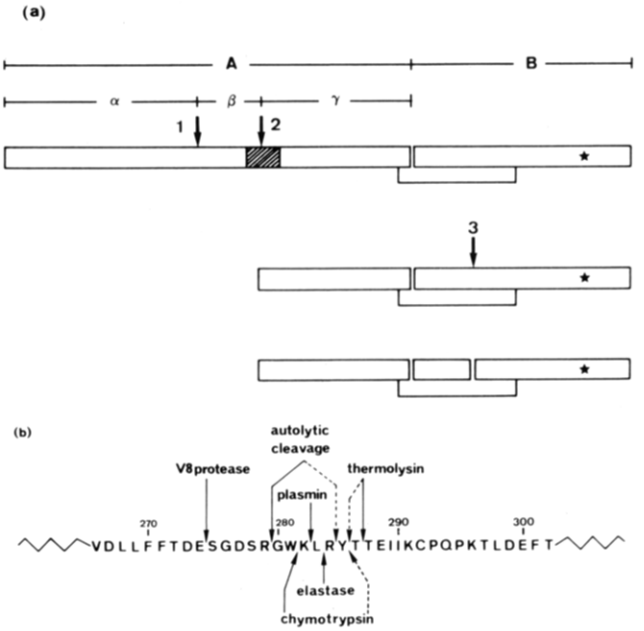

FIGURE 3: Location of the sites of limited proteolysis in human C$\bar{1}$r. (a) Schematic representation of the cleavage pattern. The positions of autolytic cleavages (1, 2) are indicated, and striping covers the portion of the sequence (detailed in panel b) where extrinsic cleavages occur. The location of the additional cleavage site (3) of V8 protease in C$\bar{1}$r B chain is also shown. The relative sizes of C$\bar{1}$r chains and fragments are proportional to the length of their peptide backbone, and the position of the single interchain disulfide bridge is indicated (Arlaud & Gagnon, 1983, 1985; Arlaud and Gagnon, unpublished data). (*) indicates the position of the iPr_2P–F-reactive serine. (b) Precise location of the peptide bonds cleaved during the second step of C$\bar{1}$r autolytic cleavage and during limited proteolysis by extrinsic proteases. The amino acid sequence shown and the numbering are derived from Gagnon and Arlaud (1985) and from Arlaud and Gagnon (unpublished data). Dotted arrows indicate sites of partial cleavage.

conditions, a major band of apparent M_r 53 000–57 000 (Figure 2a) and, after reduction and alkylation (Figure 2b), two bands, corresponding to the intact B chain (M_r 34 000) and a γ fragment of varying apparent molecular weight, estimated as follows: autolytic cleavage, M_r 18 500; plasmin, M_r 18 000; elastase, M_r 17 800; chymotrypsin, M_r 18 200; thermolysin, M_r 17 800. In the case of V8 protease, a residual band of apparent M_r 58 000 was also observed in nonreducing conditions, although most of this material was further degraded into two major bands of apparent M_r 40 000 and 15 000 (Figure 2a). Analysis of the reduced and alkylated sample (Figure 2b) indicated that further cleavage occurred in the B chain, split into two major bands of apparent M_r 23 500 and 15 000, visible on both sides of the γ fragment (apparent M_r 19 200).

In order to locate the cleavage sites at the molecular level, the γ–B fragments generated by each extrinsic cleavage were submitted to N-terminal sequence analysis. Each run showed two or three sequences, one of them being in all cases Ile-Ile-Gly-Gly-Gln-Lys..., corresponding to the N-terminal end of C$\bar{1}$r B chain (Arlaud & Gagnon, 1983). In the cases of plasmin and elastase cleavage, a single additional sequence was observed, identified as Leu-Arg-Tyr-Thr-Thr-Glu... and Arg-Tyr-Thr-Thr-Glu-Ile..., respectively, thus allowing precise location of the cleavage sites of these enzymes at peptide bonds 282 and 283 of C$\bar{1}$r A chain, as indicated in Figure 3. Chymotrypsin yielded two additional sequences, Lys-Leu-Arg-Tyr-Thr-Thr... and Thr-Thr-Glu-Ile-Ile-Lys..., present in relative amounts 4:1, thus indicating that this enzyme cleaved both the Trp–Lys bond at position 281 of C$\bar{1}$r A chain and the Tyr–Thr bond at position 285 (Figure 3). Similarly, it was concluded that the Thr–Thr bond at position 286 and, to a lesser extent, the Tyr–Thr bond a position 285 were both cleaved by thermolysin as, in addition to the N-terminal sequence of C$\bar{1}$r B chain, two sequences were obtained, a major sequence commencing Thr-Glu-Ile-Ile-Lys-(Cys)... and a minor sequence commencing Thr-Thr-Glu-Ile-Ile-Lys.... In the case of V8 protease, again, two additional sequences were found. One sequence, Ser-Gly-Asp-Ser-Arg-Gly..., resulted from cleavage of the Glu–Ser bond position 274 of C$\bar{1}$r A chain (Figure 3), whereas the other sequence, Leu-Met-Lys-Leu-Gly-Asn..., arose from cleavage of the Glu–Leu bond located at position 65 of C$\bar{1}$r B chain (Arlaud & Gagnon, 1983; Figure 3).

The sequence data thus confirmed interpretation of SDS–polyacrylamide gel electrophoresis. It clearly indicated that each extrinsic cleavage of iPr_2P–C$\bar{1}$r yielded disulfide-linked γ–B fragments originating from cleavage of peptide bonds located within residues 274 and 286 of C$\bar{1}$r A chain (Figure 3), in the neighborhood of the bonds cleaved during the second step of C$\bar{1}$r autolytic cleavage (Gagnon & Arlaud, 1985; Villiers et al., 1985). With regard to the cleavage by V8 protease, it is clear that this involved extra cleavage of the C-terminal B chain of C$\bar{1}$r, split into two major fragments (Figure 3) corresponding to the additional bands observed in Figure 2b. All the identified cleavage sites were consistent with the known specificity of the proteases used (Heinrikson, 1977; Houmard & Drapeau, 1972; Robbins & Summaria, 1970; Shotton, 1970; Wilcox, 1970).

Chemical Cross-Linking of C$\bar{1}$r Catalytic Domains. The $(\gamma\text{–B})_2$ catalytic domains obtained from C$\bar{1}$r autolytic cleavage were labeled with [1,3-^{3}H]iPr_2P–F, purified by high-pressure gel permeation in nondenaturing conditions (Figure 1), and submitted to chemical cross-linking with EDAC. The cross-linked products were analyzed by SDS–polyacrylamide gel electrophoresis (Figure 4) and identified on the double basis of molecular weight estimate and [1,3-^{3}H]iPr_2P radioactivity,

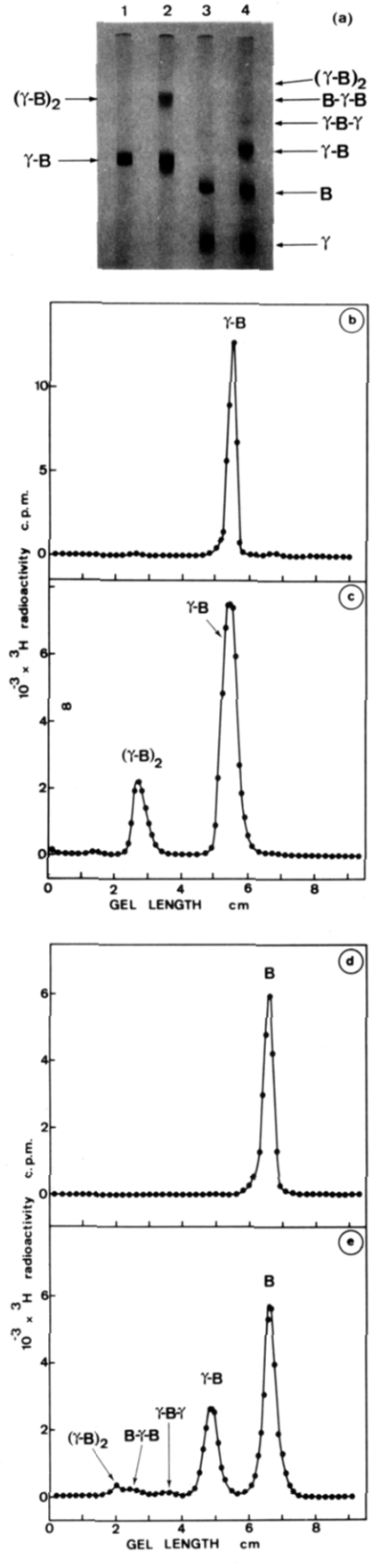

FIGURE 4: Analysis by SDS–polyacrylamide gel electrophoresis of the chemical cross-linking of [1,3-^{3}H]iPr$_2$P-labeled C$\bar{1}$r catalytic domains by EDAC. (a) Gels: 1, untreated sample, unreduced; 2, cross-linked sample, unreduced; 3, untreated sample, reduced and alkylated; 4, cross-linked sample, reduced and alkylated. (b, c, d, and e) radioactivity profiles corresponding to gels 1, 2, 3, and 4.

used as a specific marker of the serine active site located in C$\bar{1}$r B chain. In nonreducing conditions, untreated samples appeared as a single γ–B band of apparent M_r 55000 (Figure 4a, gel 1; Figure 4b), whereas chemical cross-linking led to the formation of stable $(\gamma\text{–B})_2$ dimers (21% of total radioactivity, apparent M_r 110000) visible on the gel beside the monomeric form (Figure 4a, gel 2; Figure 4c).

In reducing conditions, untreated samples gave two bands, corresponding to the γ fragment (apparent M_r 19000) and the iPr$_2$P-labeled B chain (apparent M_r 34000) (Figure 4a, gel 3; Figure 4d). Analysis of cross-linked samples revealed, beside fragment γ and the B chain, the presence of a major cross-linked product, identified as γ–B (apparent M_r 53000), and of minor species of higher M_r, identified as γ–B–γ, B–γ–B, and $(\gamma\text{–B})_2$ (apparent M_r 71000, 90000, and 104000, respectively) (Figure 4a, gel 4; Figure 4e). The relative amounts of radioactivity contained in the B chain, the major cross-linked product γ–B, and the minor cross-linked species were estimated at 60%, 31%, and 9%, respectively. Careful analysis of the gels showed no evidence of γ–γ or B–B cross-linked products, as judged from both Coomassie blue staining and radioactivity pattern.

Discussion

Previous studies (Arlaud et al., 1980) have shown that autolytic C$\bar{1}$r cleavage is a two-step process leading to a truncated molecule (C$\bar{1}$r II). This is formed by the dimeric association of two monomers, each comprised of γ, the C-terminal part of the A chain, disulfide linked to the B chain. Recent sequence studies (Gagnon & Arlaud, 1985; Arlaud and Gagnon, unpublished data) have characterized fragment γ and the precise location within C$\bar{1}$r A chain sequence of the bonds cleaved at the N-terminus of this fragment. On the basis of electron microscopy, a model of the C1r molecule has been proposed (Villiers et al., 1985) in which the dimeric $(\gamma\text{–B})_2$ domains form the core of the protein and fulfill specific catalytic functions associated with the serine protease site located in the B chain (Arlaud & Gagnon, 1983).

The data reported here indicate that limited proteolytic cleavages of iPr$_2$P–C$\bar{1}$r by proteolytic enzymes of various specificities (plasmin, elastase, chymotrypsin, thermolysin) also produce dimeric $(\gamma\text{–B})_2$ associations comprising the intact B chain and γ fragments of various lengths. A comparable process is observed with V8 protease, although additional cleavage occurs within C$\bar{1}$r B chain, maybe due in part to the fact that optimal proteolysis by this enzyme requires an extended incubation period at 37 °C (24 h), which can lead to thermal denaturation and thereby allow further cleavage. In contrast, it is noteworthy that iPr$_2$P–C$\bar{1}$r is extensively digested by papain and subtilisin, two proteases known for their wide specificity (Arnon, 1970; Ottesen & Svendsen, 1970).

In regard to the cleavages generating the γ fragments, it is noteworthy that they all occur at peptide bonds that are located within a very short sequence region, extending from the glutamyl bond at position 274 of C$\bar{1}$r A chain (V8 protease cleavage site) to the threonyl bond at position 286 (thermolysin cleavage site). Therefore, it can be concluded that this sequence stretch is exposed to the solvent and that its secondary structure allows access of a protease site. Such a conclusion is compatible with our present concept of the domain structure of human C1r (Villiers et al., 1985). This sequence represents the N-terminal end of the β region. It is considered as a relatively unordered strand connecting the distal domains of the protein, α, to the central $(\gamma\text{–B})_2$ domains. The precise location of the cleavage sites (Figure 3b) allows a tentative definition, at the sequence level, of the N-terminal limit of the

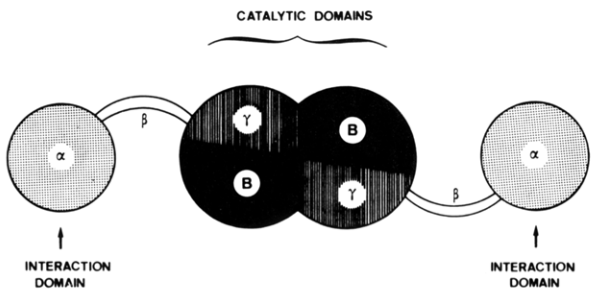

FIGURE 5: Schematic model of dimeric C1r illustrating the head to tail configuration of the central $(\gamma\text{–B})_2$ domains.

γ moiety of the $(\gamma\text{–B})_2$ domains. Such a limit could be assigned to threonine residue at position 287, in agreement with the observation that glutamyl bonds at positions 288 and 300 and lysyl bonds at positions 291 and 296 are resistant to cleavage by V8 protease and plasmin, respectively, and therefore likely buried within the $(\gamma\text{–B})_2$ domains.

The main purpose of this work was to focus on the cleavage sites generating the $(\gamma\text{–B})_2$ domains, but we did not investigate the cleavages occurring, during extrinsic proteolysis of iPr_2P–$C\bar{1}r$, within the region extending from the N-terminus to the β–γ junction. However, comparison of the high-pressure gel permeation profiles (not shown) clearly indicated that, contrary to autolytic cleavage, extrinsic cleavages did not yield fragment α. It is therefore noteworthy that, although this fragment is presently assigned to a single domain with specific interaction functions (Villiers et al., 1985), it is split during extrinsic proteolysis and therefore possibly divided into "subdomains", as suggested for $C\bar{1}s$ (Villiers et al., 1985).

In contrast, the $(\gamma\text{–B})_2$ domains can be considered as "true" globular domains, characterized by a remarkable resistance to extrinsic proteolysis, a property that can arise partly from both the presence of two N-linked oligosaccharides in the B chain (Arlaud & Gagnon, 1983) and the dimeric structure itself, which likely brings about mutual protection of the monomers.

The cross-linking experiments reported here are direct evidence of the dimeric nature of the $(\gamma\text{–B})_2$ catalytic domains and allow an insight into intermonomer interactions at the molecular level. A detailed analysis of the data indicates that all the identified cross-linked molecular species involve cross-linking between γ and B, both inside a γ–B monomer (homologous cross-linking) and between two monomers (heterologous cross-linking). This leads us to propose a schematic model of $C\bar{1}r$, illustrated in Figure 5, where all intermonomer interactions are mediated through the γ moiety of one monomer and the B chain moiety of the other. Such a "head to tail" configuration of the $(\gamma\text{–B})_2$ domains allows the formation of the γ–B, γ–B–γ, B–γ–B, and $(\gamma\text{–B})_2$ cross-linked species reported in this study (see Figure 4) and is also compatible with the observed lack of cross-linking between two B chains or between two γ fragments.

From a functional point of view, the head to tail model proposed for C1r is consistent with present knowledge of the mechanism of C1r autoactivation (Arlaud et al., 1985) and favors a cross-mechanism involving activation of the γ–B domain by its neighbor (Figure 6). Placed within the overall domain organization of the eight-shaped model that we have proposed for the catalytic C1s–C1r–C1r–C1s subunit of C1 (Colomb et al., 1984a,b; Villiers et al., 1985), the head to tail model is also compatible with subsequent activation of C1s by activated $C\bar{1}r$, the active site of each γ–B domain of $C\bar{1}r$ cleaving in turn a susceptible bond in the neighboring C1s catalytic domain (Figure 6). In this model, the double catalytic

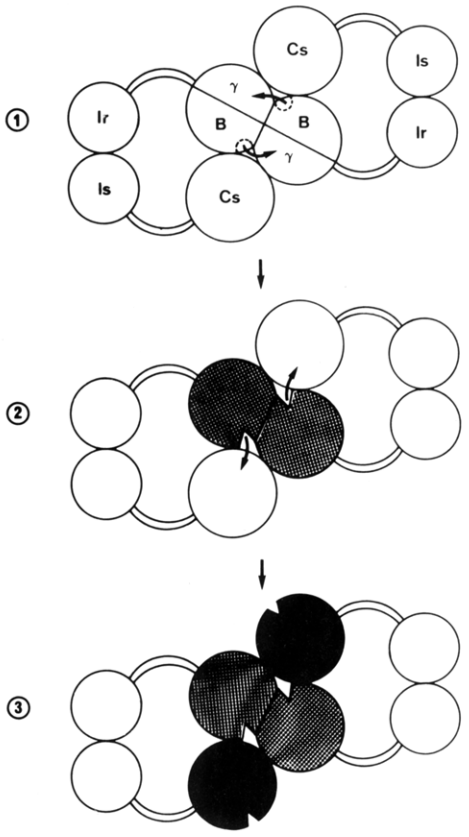

FIGURE 6: Schematic representation of the eight-shaped C1s–C1r–C1r–C1s catalytic subunit of C1, illustrating C1r autoactivation and C1s activation by $C\bar{1}r$. (1) C1r autoactivation; (2) C1s activation; (3) fully activated $C\bar{1}s$–$C\bar{1}r$–$C\bar{1}r$–$C\bar{1}s$. Prosites are symbolized by dotted circles, and active sites by nicks. Proteolytic cleavages are represented by curved arrows. Cs, catalytic domains of C1s; Ir and Is, interaction domains of C1r and C1s.

function of the C1s–C1r–C1r–C1s subunit of C1 is rendered possible through location within the same neighborhood of the following: (i) the serine protease site of one C1r catalytic domain; (ii) the bond cleaved during activation of the other C1r catalytic domain; (iii) the bond cleaved during activation of the adjacent C1s catalytic domain. These conditions represent an essential structural prerequisite.

Further studies involving precise identification at the sequence level of the sites of chemical cross-linking in the catalytic domains of C1r would allow a better knowledge of the residues involved in intermonomer interactions and possibly a better understanding of the activation mechanism of this protein.

ACKNOWLEDGMENTS

We are grateful to M. Lacroix for expert technical assistance, A. C. Willis for amino acid sequence determination, and M. Brenier for skillful secretarial services.

SUPPLEMENTARY MATERIAL AVAILABLE

A table giving characteristics of N-terminal amino acid sequence analyses performed on the γ–B fragments generated by chymotrypsin, elastase, plasmin, thermolysin, and V8 protease (1 page). Ordering information is given on any current masthead page.

Registry No. Activated complement C1r, 80295-69-8.

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Bromoacetophenone as an Affinity Reagent for Human Liver Aldehyde Dehydrogenase†

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ABSTRACT: Human liver aldehyde dehydrogenase isozymes E1 and E2 (EC 1.2.1.3) are both completely and irreversibly inactivated by bromoacetophenone (2-bromo-1-phenylethanone). Steady-state kinetics with both acetophenone and chloroacetophenone indicated interaction with the same enzyme form as the aldehyde substrate. Saturation kinetics with chloroacetophenone and bromoacetophenone indicated interaction at a specific site on the enzyme surface and gave a dissociation constant similar to that from steady-state kinetics, suggesting that the same processes were being observed by both methods and that the active site may be involved. Protection against inactivation was afforded by chloral and NAD together. Stoichiometry of inactivation showed the first 2 equiv per tetramer to abolish the majority of catalytic activity; 4 equiv inactivated both isozymes with complete loss of esterase, NAD-stimulated esterase, and dehydrogenase activities. Peptide mapping of enzyme modified with [*carbonyl*-^{14}C]bromoacetophenone of CNBr digests (E1) and tryptic digests (E1 and E2) showed one peptide to be preferentially labeled. The above results together with the similarity of bromoacetopenone to the substrate benzaldehyde suggest bromoacetophenone may react with a residue in the active site of aldehyde dehydrogenase. Amino acid analysis of the labeled E1 tryptic fragment indicated reaction with a different peptide from that with which iodoacetamide reacts.

The catalytic mechanism of aldehyde dehydrogenase is thought to involve an enzyme nucleophile that forms a covalent intermediate with the aldehyde substrate (Dunn & Buckley, 1985). Currently, it is suspected that the enzyme nucleophile is a cysteine residue. Evidence for this residue being a cysteine, however, is most circumstantial, arising from comparison with glyceraldehyde-3-phosphate dehydrogenase, sensitivity of the enzyme to sulfhydryl reagents, and the identification of a hyperreactive cysteine residue (Hempel & Pietruszko, 1981).

Chemical modification offers a strong tool for identification of catalytically important residues. Three approaches may be used in the chemical modification of protein. One involves the use of a reagent specific for a certain type of amino acid residue to selectively modify a hyperactive residue, which is often found in the active site (Baker, 1967). The second approach involves the design of a reagent that is structurally similar to a substrate or transition-state intermediate (Pauling, 1948) and contains a reactive functional group that is positioned on the reagent enabling formation of a covalent bond with residues positioned in the active site. This approach may or may not label the catalytic residue; however, it will always label residues within the active site (Baker, 1967; Singer, 1967; Vallee & Riordan, 1969; Shaw, 1970). Third is more specialized approaches including suicide inactivators (Abeles & Maycock, 1976) and photoaffinity reagents (Chowdry & Westheimer, 1979).

Previous work with human liver aldehyde dehydrogenase used the general reagent approach with the sulfhydryl reagent iodoacetamide (Hempel & Pietruszko, 1981). The work led

† Financial support of NIAAA Grant AA00186, Research Scientist Development Award AA00046, and the Charles and Johanna Busch Memorial Fund is acknowledged.
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0006-2960/86/0425-5182$01.50/0