Variability in Purified Dysfunctional C1̄-Inhibitor Proteins from Patients with Hereditary Angioneurotic Edema

Functional and Analytical Gel Studies

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Abstract

C1̄-inhibitor (C1̄-INH) proteins from normal persons and members of eight different kindred with dysfunctional C1̄-INH proteins associated with hereditary angioneurotic edema (HANE) were compared with respect to their inhibitory activity against purified preparations of C1s̄, plasma kallikrein, activated forms of Hageman factor, and plasmin. Each dysfunctional C1̄-INH protein showed a unique spectrum of inhibitory activity against these enzymes. Although none of the dysfunctional C1̄-INH proteins significantly impaired amidolysis by plasmin, all but one inhibited activated Hageman factor. One purified dysfunctional C1̄-INH (Ta) inhibited purified C1s̄ to a normal degree. Another C1̄-INH (Za) had almost seven times as much inhibitory activity as normal C1̄-INH against activated Hageman factor, but had decreased activity against C1s̄ and no activity against plasmin. Analyses of mixtures of plasmin and C1̄-INH proteins in SDS gel electrophoresis revealed variability in the patterns of complex formation and cleavage of dysfunctional proteins after exposure to C1s̄ and plasmin. Some bound to plasmin and were cleaved, even though none significantly impaired the amidolytic activity of plasmin. Two were cleaved by C1s̄, whereas neither normal or other dysfunctional C1̄-INH were cleaved. Dysfunctional C1̄-INH proteins from patients with HANE are thus heterogeneous in their inhibitory properties and there must be different structural requirements for the inhibition of the various plasma enzymes that can be regulated by normal C1̄-INH. The data suggest that in addition to common sites of interactions between these proteases and C1̄-INH, there are also points of contact that are specific for each protease. Genetic mutations leading to structural changes at some of these sites may have differing effects on the interaction between individual proteases and abnormal C1̄-INH proteins. These alterations may allow these proteins to serve as probes for structural requirements for inhibitory actions of normal C1̄-INH.

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Introduction

Plasmas from persons with hereditary angioneurotic edema (HANE)[1] are either markedly deficient in C1̄-inhibitor (C1̄-INH) activity, as well as C1̄-INH antigens (type I), or they contain normal or elevated concentrations of dysfunctional C1̄-INH protein (type II). Both types of C1̄-INH deficiency are inherited as autosomal dominant traits in kindred with HANE. Plasma from patients with type II HANE do not adequately inhibit C1̄, or its C1s̄ subunit, and C4 and C2, both of which are substrates of C1̄, were readily inactivated in plasma from patients with type II HANE as well as those with type I (1–3).

The abnormal C1̄-INH proteins in plasma and serum of patients with type II HANE were electrophoretically and functionally heterogeneous (3). At least four structural variants of C1̄-INH have been identified within this group of patients (3). Despite the heterogeneity among this group of patients, both the tendency to bouts of HANE, and the biochemical disorder, were inherited as autosomal dominant traits in affected kindred (3). Because of the heterogeneity of these proteins, dysfunctional C1̄-INH was isolated from plasma of 12 affected individuals from eight different kindred with type II HANE. This report describes the capacity of each of these proteins to inhibit C1s̄, plasma kallikrein, activated Hageman factor (HFa, or Factor XIIa), Hageman factor fragments (HFf), and plasmin; normal C1̄-INH can inhibit each of these proteases in vitro. The inhibition of purified preparations of each of these enzymes by individual dysfunctional C1̄-INH proteins differed from one enzyme to another. In addition, the dysfunctional proteins appeared to have unique patterns of inhibition of each enzyme when compared to one another. This suggests that the sites of contact between inhibitor and enzyme are, in part, different and specific to each protease.

Methods

Materials

Resins used in column chromatography included SP-Sephadex C-50, DEAE-Sephadex A-50, DEAE-Sephadex C-50, CM-Sephadex C-50, DEAE, activated Sepharose 4B, Sephacryl S-200 superfine and Sephadex

1. *Abbreviations used in this paper:* HFa, activated Hageman factor; C1̄-INH, C1̄-inhibitor; HFf, Hageman factor fragments; HANE, hereditary angioneurotic edema; HTP, hydroxylapatite; ALTEE, *N*-acetyl-L-tyrosine ethyl ester; PNA, paranitroaniline; PAGE, polyacrylamide gel electrophoresis.

G-150, all of which were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Hydroxylapatite (HTP) was obtained from Bio-Rad Laboratories, Richmond, CA; AcA 54 Ultragel from LKB Instruments (Bromma, Sweden or Rockville, MD). Polyacrylamide was obtained from Fisher Chemical Co., Norwood, OH. Synthetic chromogenic amides used as substrates in assays for enzymatic activities included S-2302 (Pro-Phe-Arg-paranitroaniline [PNA]), S-2251 (Val-Leu-Lys-paranitroaniline) and S-2160 (*N*-Benz.-Phe-Val-Arg-PNA), which were obtained from Kabi, Stockholm, Sweden, or Stamford, CT. CBZ-L-tyrosine-*O*-paranitroaniline was obtained from Sigma Chemical Co., St. Louis, MO. *Nα*-BZ-Pro-Phe-Arg-PNA hydrochloride was obtained from Pentapharm, Basle, Switzerland. *N*-acetyl-L-tyrosine ethyl ester (ALTEE) was obtained from the Aldrich Chemical Co., Milwaukee, WI; soybean trypsin inhibitor from Worthington Chemical Corp., Freehold, NJ; streptokinase (SK) (high purity) from Lederle Laboratories, Pearl River, NY; benzamidine and L-lysine hydrochloride from Matheson, Coleman and Bell, Norwood, OH; sodium dodecyl sulfate (SDS) from Fisher Chemical Co., Norwood, OH; and BRIJ-35 from Calbiochem-Behring Corp., La Jolla, CA. Lysine-Sepharose was prepared using the instructions of the manufacturer for conjugating the lysine with Sepharose. Soybean trypsin inhibitor was similarly complexed with Sepharose.

Ellagic acid (4,4',5,5',6,6' hexahydroxydiphenic acid 2,6,2',6'-dilactone) used to activate Hageman factor was synthesized as described (4). Stock solutions were prepared in barbital saline buffer, pH 7.4, by homogenization in a mechanical homogenizer and centrifuged at 15,000 *g* for 20 min. These solutions did not contain microscopically visible crystals. The barbital-saline buffer used in coagulation assays contained 2.06 g of sodium barbital, 2.76 g of barbital, and 7.8 g of sodium chloride in 1 liter of distilled water.

Plasmas were obtained from normal individuals and from persons with HANE associated with dysfunctional C1̄-INH protein previously characterized (3, 5) after informed consent was given by the donors. The plasmas were separated by plasmapheresis, and immediately mixed with benzamidine and disodium EDTA (see below).

Methods

Protein isolation. C1̄-INH was isolated from normal plasma by the procedure of Harrison (6, 7), except that in some instances gel filtration on Sephacryl S-200 replaced that on Sephadex G-150. This provided C1̄-INH, which was usually homogeneous in SDS gel electrophoresis, but in some preparations two closely approximated bands were seen (8) with as much as 70% recovery.

Plasma kallikrein. Plasma kallikrein was isolated by the method of Nagase and Barrett (9), modified in that a second affinity step on SBTI-Sepharose was usually added after chromatography on Ultrogel AcA 54, and immune adsorption on a column of Sepharose-antihuman IgG was used when necessary. This kallikrein was homogeneous in SDS electrophoresis gels (Fig. 1), and had specific activity as high as 235–446 U of coagulant activity (1 U being the amount in 1.0 ml of pooled normal human plasma) per mg protein.

Human Hageman factor. Hageman factor was extracted from normal plasma using minor modifications of previously published procedures (10–12). All test tubes and vessels were either coated with silicone (GE-Dri film, SC-87, Pierce Chemical Co., Rockford, IL) or made of polypropylene to prevent surface activation of Hageman factor. Blood was drawn into polypropylene bottles containing 1/50 of final volume of a solution of 0.5 M citrate buffer, pH 5.0, 0.1 M benzamidine hydrochloride, and 100 mg hexadimethrine bromide (Polybrene, Aldrich Chemical Co.) per 10 ml of solution. Plasma was rendered platelet deficient by recentrifugation of cell-free plasma at 12,000 *g* in a Sorvall RC-2B centrifuge for 20 min at 2°C. The Hageman factor was isolated by sequential chromatography upon columns of QAE-Sephadex A-50, DEAE cellulose, SP-Sephadex C-50, and AcA 54 Ultrogel. All equilibrating and eluting buffers used in QAE-Sephadex A-50 and DEAE Sephadex chromatography contained 2 mM benzamidine HCl, 50 mg Polybrene/liter, 0.1 mM disodium EDTA, 0.1 M sodium chloride, and 0.02% sodium azide. Columns and glassware were coated with silicone. Fractions containing 0.25 U of Hageman factor activity per milliliter or more were pooled, examined in SDS polyacrylamide gels and the specific activity of the Hageman factor determined. The specific activity was usually >100 U/mg of protein. An SDS gel of the product is shown in Fig. 1.

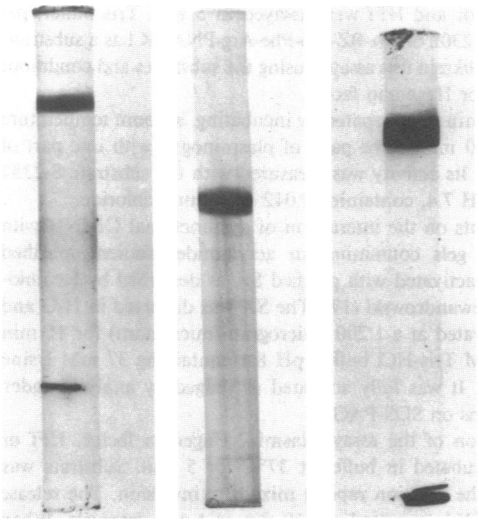

Figure 1. SDS polyacrylamide gel (7.5%) electrophoresis of Hageman factor (*left*) isolated from normal plasma by the method described, having a specific coagulant activity of 95 U/mg protein. Kallikrein (*center*), isolated as described, had a specific coagulant activity of 440 U/mg protein. The plasminogen preparation (*right*) had a sp act of 23 CTA U/mg. The samples applied to gels contained: kallikrein, 320 μg, Hageman factor, 100 μg, plasminogen, 40 μg. These gels were run separately; dye markers are anodal (down).

Plasminogen. Plasminogen was prepared by affinity chromatography according to the method of Deutsch and Mertz (13). This material had a sp act of 17–21 CTA U/mg protein.

The plasminogen used in experiments testing the interactions with dysfunctional C1̄-INH as analyzed on SDS-polyacrylamide gel electrophoresis (PAGE) containing a gradient of acrylamide concentrations (Fig. 4) was the generous gift of Dr. Eileen Remold-O'Donnell of the Center for Blood Research, Boston, MA.

C1s̄. Already activated C1s̄, was prepared according to the method of Bing et al. (14, 15). It gave a homogeneous single band in SDS-PAGE and had a sp act of 1,060 U/mg protein (16).

Enzymatic assays

Assays for enzymatic activity using the synthetic chromogenic substrates were performed in a 1-cm cuvette in a Gilford model 240 spectrophotometer (Gilford Instruments, Inc., Oberlin, OH) with a jacketed cuvette holder maintaining the temperature of the assay at 37°C. Synthetic chromogenic substrates were dissolved in distilled water, as recommended by the manufacturer, unless otherwise noted. The total volume of the assay mixture was 600 μl.

Hageman factor. Hageman factor was activated by incubation of the purified protein in barbital-saline buffer, pH 7.4, containing 1% bovine serum albumin, with an equal volume of 10^{-4} M ellagic acid in a silicone-coated test tube at 37°C. Preliminary tests showed incubation for 2 h before assay was required for optimal activation, and these conditions were used throughout.

HFf were generated spontaneously during storage at −70°C and thawing of solutions of purified Hageman factor in pH 5.2 acetate buffer. This process was visualized by SDS-PAGE, the original single cathodal band, of a molecular weight of ~80,000, disappearing as two more anodal bands were generated. At this point the coagulant activity of Hageman factor had also largely decayed.

Hageman factor and HFf were assayed in 5 mM Tris buffer, pH 7.8, using either S-2302 or Nα-BZ-Pro-Phe-Arg-PNA HCl as a substrate.

Kallikrein. Kallikrein was assayed using the substrates and conditions described above for Hageman factor.

Plasmin. Plasmin was prepared by incubating, at room temperature and pH 7.4 for 10 min, three parts of plasminogen with one part of SK (1,000 U/ml). Its activity was measured with the substrate S-2251 in 0.05 M Tris, pH 7.4, containing 0.012 M sodium chloride.

For experiments on the interaction of dysfunctional C1̄-INH with plasmin in SDS gels containing an acrylamide gradient, purified plasminogen was activated with purified SK as described by Remold-O'Donnell and Lewandrowski (17). The SK was dissolved in H_2O and plasminogen activated at a 1:200 (microgram/microgram) for 15 min at 37°C in 15 mM Tris-HCl buffer, pH 8.1 containing 37 mM lysine and 25% glycerol. It was fully activated as judged by analysis under reducing conditions on SDS-PAGE.

Before initiation of the assay, plasmin, Hageman factor, HFf or kallikrein was incubated in buffer at 37°C for 5 min. Substrate was then added and the solution rapidly mixed by inversion. The release of chromogenic PNA was read at 410 nm at 1-min intervals. When inhibitor proteins were being assessed they were added to enzymes before the 5-min preincubation period. Because the activity of the SK-plasmin deteriorated significantly at 4°C or at 37°C during and between assays, it was necessary to investigate the inhibition of plasmin using a carefully controlled procedure. Each dysfunctional C1̄-INH protein was therefore assessed individually, by simultaneous assay in a set of cuvettes, using a freshly activated SK-plasmin mixture, and an uninhibited (buffer) plasmin standard in each individual assay.

C1s̄ activity was initially measured using the substrate CBZ-L-Tyr-*O*-PNA. This was dissolved in acetone at 1 mM and incubated, at 37°C for 2 min, in the assay cuvette with 0.005 M Tris, pH 8.05, containing 0.09 M NaCl. The reaction was started by the addition of C1s̄. C1s̄ inhibition was measured in assays reported here (Table II and Fig. 2) according to the method of Levy and Lepow (16) using ALTEE as substrate.

The rate of hydrolysis of the chromogenic substrates by each enzyme, expressed as nanomoles of PNA released per minute per microgram enzyme, was calculated from the data obtained during the first 5 min of assay. Table I summarizes the rates of hydrolysis of the synthesis substrates used. The rate of hydrolysis by mixtures containing inhibitor proteins was also calculated, and the inhibitory activity expressed as a decrease in nanomoles of PNA hydrolyzed per minute per microgram inhibitor protein. The percent inhibition of each dysfunctional protein with respect to inhibition by the normal inhibitor was then calculated. In amidolytic assays, two or more concentrations of each C1̄-INH were tested (Table II) and 40 μg or more of each C1̄-INH were used in assays using ALTEE as a substrate for C1s̄ (16).

Table I. Specific Amidolytic or Esterolytic Activities of Purified Enzymes Tested with Synthetic Substrates

Enzyme	Substrate	Rate of hydrolysis
		nmol PNA released/ min per μg enzyme
Kallikrein	Pro-Phe-Arg-PNA (S-2302)	4.509±0.76
HFa (Factor XIIa) (ellagic acid activated)	Pro-Phe-Arg-PNA	0.57±0.069
HFf	Pro-Phe-Arg-PNA	0.296±0
SK-activated plasminogen	Val-Leu-Lys-PNA (S-2251)	0.447±0.025
C1s̄	CBZ-L-tyrosine-O-PNA	0.087±0.023
C1s̄	ALTEE*	1.06±U/μg±0.26

* Esterolytic assay used (16).

Coagulation assays to quantify Hageman factor or kallikrein during fractionation were carried out by methods described (10) in which the correction of delayed clotting of a plasma of a person with a severe hereditary deficiency of the coagulation factor in question was measured. For these assays, column fractions were diluted 1:5 or 1:20 in either distilled water, or, when measuring Hageman factor, in barbital-saline buffer, pH 7.4, containing 1% bovine serum albumin. Fractions containing Hageman factor were added to a mixture of kaolin and Centrolex-O phospholipid in the presence of albumin or other protein.

Assays for C1s̄ hydrolytic activity using the standard assay of Levy and Lepow (16) were performed in 0.067 M phosphate, pH 7.4, and the final acid released titrated in a microformol titration using a Radiometer automatic titrator and pH meter, model PHM 82, with a TTT 80 titrator and an ABU 80 autoburette (Radiometer, Copenhagen, Denmark).

SDS-polyacrylamide gradient gels were made by an adaptation of the method of Laemmli (18). The gradient was 7.5 to 12.5% acrylamide prepared from stocks of 29.4% acrylamide and 0.6% *bis*-acrylamide. Polymerization was initiated with ammonium persulfate and *N,N,N',N'*-tetramethyl-ethylene diamine (Eastman Kodak Co., Rochester, NY) at a final concentration of 0.036 and 0.017%, respectively. After polymerization of the gradient gel (usually within 50 min) of acrylamide and *bis*-acrylamide, a stacking gel of 3.5% acrylamide prepared from the same stock solution with identical concentrations of ammonium persulfate and *N,N,N',N'*-tetramethyl-ethylene diamine (Eastman Kodak Co.) was added. The gels were electrophoresed vertically at room temperature at 3–3.5 V/cm for 18 h or until the tracking dye was within 1–2 cm from the bottom of the gel. In this system C1s̄ (M_r = 87,000) and plasmin (M_r = 77,000) migrated at about the same position corresponding to an ~65,000–70,000 M_r. This system, however, was determined optimal for resolution of free inhibitor from complex and/or free enzyme and degradation products derived by apparent action of the proteases on the inhibitor. In standard 7.5 and 10% SDS-PAGE gels both proteases had molecular weights corresponding to previously reported values (13, 14), and exhibited no evidence of degradation under any of those conditions (data not shown). The enzymes and inhibitors were incubated together at 37°C for 15 min, SDS added and incubation continued for 25 min before electrophoresis.

Results

Functional studies. As shown in Table II, purified dysfunctional C1̄-INH proteins exhibited a wide range of inhibitory activity against the enzymes tested. The different dysfunctional C1̄-INH proteins are indicated by the first two letters of each family name. With the exception of C1̄-INH Ri, all gave a significant degree of inhibition of C1s̄ (19–90%) (Table II) using an esterolytic assay (ALTEE substrate). In an assay measuring C1s̄ activity by esterolysis of CBZ-L-tyrosine-*O*-PNA, the depression of the hydrolytic rate of the C1s̄ by the C1̄-INH proteins was in the range of 10^{-3} to 10^{-4} nmol of substrate hydrolyzed per minute per microgram inhibitor, but the inhibitor proteins depressed ALTEE hydrolysis by C1s̄ from 6.7 to 100.5 nmol/min per μg inhibitor. Although it appears that ALTEE is a more satisfactory substrate for determining the inhibition of C1s̄, it is possible that certain of the dysfunctional proteins block the hydrolysis of ALTEE more effectively than of CBZ-L-tyrosine-*O*-PNA. The levels of inhibition of preparations of C1̄ by infractionated serum samples from the patients studied are shown in column 2, Table II.

Inhibition of the amidolytic properties of purified human plasma kallikrein by dysfunctional C1̄-INH proteins also varied widely compared to the activity of the normal C1̄-INH (Table II). Inhibition values ranged from 1% (Ri) to 64% (Za) of that provided by normal C1̄-INH. There was a wide variation in

Table II. Inhibitory Activities of Dysfunctional C1̄-INH Proteins Against Plasma Enzymes: Percent Normal C1̄-INH Activity

Dysfunctional C1̄-INH	Serum inhibition of C1̄ (16)	Enzyme inhibition by purified C1̄-INH proteins				
		C1s̄	Kallikrein	HFa	HFf	SK-Plasmin
Da	0	75 (72–86)	50 (45–55)	70 (57–97)	194 (118–276)	20.2 (20–47)
Ta	0–15	90 (65–91)	10 (0–21)	93 (78–108)	24 (24, 24)	1.3 (0–5)
We	0	19 (2–35)	33 (30–35)	97 (97, 97)	39 (35–43)	1.8 (0–5)
Ri	0–30	4 (1–10)	1 (0–2.6)	0 (0)	0 (0)	1.2 (0–5)
At	0	50 (30–70)	2.5 (0–5)	39 (30–49)	46 (40–52)	0.35 (0–1.4)
Bo	0	61 (32–100)	16 (14–18)	64 (57–70)	64 (53–79)	0.7 (0–3)
Mo	<20	52 (22–81)	6 (0–11)	119 (119–120)	70 (67–73)	2.2 (0–6)
Za	0–20	19 (0–32)	64 (41–97)	687 (483–893)	(NT)	0 (0)
Normal	100	100	100	100	100	100

Each dysfunctional C1̄-INH protein is designated by the first two letters of each family name. Ranges in multiple assays in parentheses. NT, not tested. The inhibition of each enzyme by dysfunctional C1̄-INH proteins is given as a percentage of inhibition by normal C1̄-INH, which was derived from the depression of the rate of hydrolysis (nanomoles per minute per microgram of inhibitor) of synthetic substrate. Each dysfunctional protein was tested from two to six times against each enzyme. The amount of enzyme used in assays was: C1s̄, 28 μg; kallikrein, 0.13 μg; HFa, 2.3 μg; HFf, 2.1 μg; plasmin, 4.9 μg. C1s̄ was tested with ALTEE as substrate; kallikrein, HFa and HFf with S-2302, and SK-plasmin with S-2251. The amounts of each C1̄-INH protein tested were as follows: C1s̄ assay: Normal C1̄-INH, 20, 40 μg; C1̄-INH Da, Ta, We, Ri, At, Bo, Mo, 40 μg; C1̄-INH Za, 40, 80 μg. Kallikrein assay: Normal C1̄-INH, 6 μg; All dysfunctional C1̄-INH proteins, 6, 12 μg. HFa assay: Normal C1̄-INH, 12 μg; C1̄-INH Da, We, Mo, 6 μg; C1̄-INH Ta, At, Bo, Za, 6, 12 μg; C1̄-INH Ri-18 μg. HFf assay: Normal, 6 μg; C1̄-INH Da, 8.4, 16.8 μg; All other C1̄-INH's, 6, 12 μg. Plasmin assay: Normal C1̄-INH, 6, 7, 12, 14 and 36 μg; C1̄-INH Da, 17, 68 μg; C1̄-INH Ta, We, Ri, 10, 20 μg; At, Bo, Mo, Za, 6, 12 μg. The mean value of percent inhibition provided by each inhibitor protein is given with the range of values obtained in different assays in parentheses beneath the mean value. Column 2 indicates the amount of inhibition of C1̄ provided by sera from which the C1̄-INH proteins were isolated. This information has been published previously (1, 3, 31–33).

the ratios of anti-C1s̄ to anti-kallikrein activity among dysfunctional inhibitors. For example, C1̄-INH Ta exhibited a high degree of inhibition of C1s̄ esterolytic activity (90% normal), but little antikallikrein activity (10% normal). A similar ratio was found with C1̄-INH At, which inhibited C1s̄ esterolytic activity ~50% as well as normal C1̄-INH, but had <3% of the antikallikrein activity of the normal C1̄-INH protein. In contrast, C1̄-INH Za had only 19% of the normal amount of activity against C1s̄, but considerably more antikallikrein activity (64%).

When 40 μg of C1̄-INH Ta was added to Ta plasma, no inhibition of C1s̄ could be measured, but the protein had a specific activity against C1s̄ of 12 U/mg of inhibitor before being added to the plasma from which it was derived. When 40 μg of a stored preparation of normal C1̄-INH was added to Ta plasma, the specific inhibition of C1s̄ was 52 U/mg of this protein, as it was in mixtures containing buffer in place of Ta plasma. Therefore, the anti-C1s̄ activity of the C1̄-INH Ta was markedly reduced by the Ta plasma.

When the amidolytic activity of ellagic acid HFa, Factor XIIa was measured, six of eight dysfunctional C1̄-INH proteins had >60% of the inhibitory activity found in the normal C1̄-INH. Only one (Ri) had essentially no inhibitory activity. The inhibition of HFf by dysfunctional proteins did not parallel the inhibition of HFa, and most had reduced activity against HFf. C1̄-INH Da, however, had enhanced activity against HFf. C1̄-INH Mo had essentially normal activity against both activated forms of Hageman factor, but its action against C1s̄ and kallikrein was only 52 and 6%, respectively, of that of the normal inhibitor. C1̄-INH Za had exceptionally high anti-HFa activity, but little anti-C1s̄ activity.

All of the dysfunctional C1̄-INH proteins were deficient in their capacity to block the amidolytic activity of SK-plasmin (Table II), but C1̄-INH Da had more activity than any of the others. In examining the inhibition of the amidolytic activity of SK-plasmin, each C1̄-INH protein was examined individually with its own standard SK-plasmin mixture run simultaneously. This was necessary because the activity of the SK-plasmin deteriorated significantly at 4°C or at 37°C during and between assays.

The levels of inhibition by each dysfunctional C1̄-INH protein against each enzyme tested are summarized diagrammatically in Fig. 2. These data are expressed as a depression in hydrolytic rate of each enzyme in nanomoles, or units in

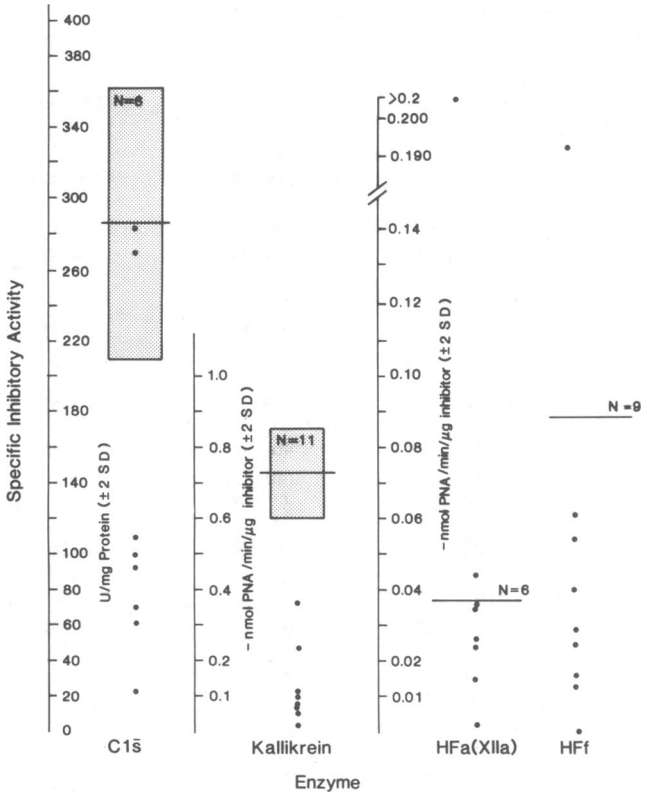

Figure 2. A diagrammatic representation of the inhibition of C1s̄ (*left*), kallikrein, ellagic acid-activated HFa, and HFf by normal C1̄-INH and dysfunctional C1̄-INH proteins. The transverse line in each set indicates the mean value of inhibition by purified normal C1̄-INH and the shaded boxes indicate ±2 SD. *N*, the number of assays done with purified normal C1̄-INH in each set of experiments. Individual points (·) represent the mean value of inhibition of enzymatic activity by each dysfunctional protein in two to six experiments. The ratios of amounts micrograms of inhibitor protein to enzyme used were: C1s̄ assay: 0.8–2.8:1; kallikrein: 46–92:1; HFf, 2.9–8:1; HFa: 8–16:1; SK-plasmin: 1.2–13.8:1. (see legend, Table II). The substrate used for C1s̄ was ALTEE; for kallikrein and HFa, S-2302 was used.

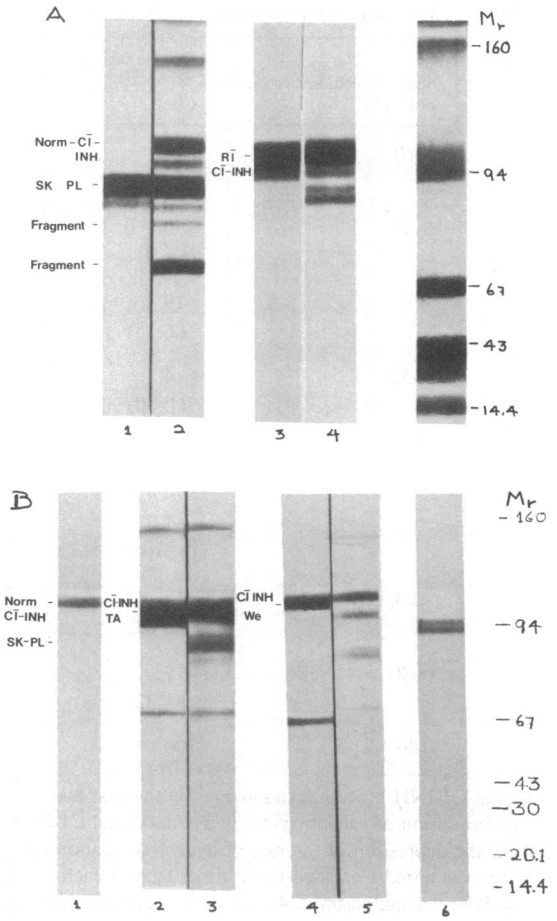

Figure 3. Samples of dysfunctional and normal C1̄-INH proteins were incubated with 12 μg of SK-plasmin and pH 7.4, 0.15 M Tris-HCl buffer at 37°C for 5 min. Then 50 μl of 0.01 M S-2251 was added and the chromogen released was measured during a continuous titration at 37°C for 5 min. Each sample was then transferred to 100 μl of 10% SDS and the mixtures placed in a boiling water bath for 3 min. These were stored at −30°C until all samples had been incubated and boiled. The amounts of inhibitor proteins in the incubation mixtures were: normal, 36 μg, Ta, 50 μg, Ri, 50 μg, and We, 100 μg. The contents of each mixture were then applied to SDS gels and electrophoresis carried out according to the procedure of Laemmli under nonreduced conditions (18). The samples shown in panel *A* were run simultaneously in disc gels, and those in panel *B* run simultaneously in a different set of disc gels. Panel *A:* 1, SK-plasmin; 2, SK-plasmin and normal C1̄-INH, treated as above; 3, C1̄-INH Ri; 4, mixture of C1̄-INH Ri and SK-plasmin; 5, molecular weight (M_r) markers $\times 10^3$. Panel *B:* 1, normal C1̄-INH; 2, C1̄-INH Ta; 3, mixture of C1̄-INH Ta and SK-plasmin; 4, C1̄-INH We; 5, C1̄-INH We and SK-plasmin; 6, SK-plasmin used with C1̄-INH We. Numbers to the right indicate $M_r \times 10^3$ derived from mobility of a mixture of proteins of known M_r (Pharmacia Fine Chemicals) run concurrently (see panel *A*).

the case of C1s̄, per minute per microgram of inhibitor protein, rather than as a percentage of the activity of the normal protein. In each set of Hageman factor assays, the standard deviation of repeated assays using normal C1̄-INH was 0, probably because the same preparation of Hageman factor at the same thawing was used in these experiments.

SDS gel analyses of C1s̄ and plasmin with C1̄-INH proteins. Since plasmin can cleave normal C1̄-INH (8), several of the dysfunctional C1̄-INH proteins were examined in SDS disc gel electrophoresis to see if they were more extensively cleaved by plasmin than the normal inhibitor, or if they were cleaved differently than normal C1̄-INH. Mixtures used in experiments measuring antiplasmin activity were boiled in SDS and applied to the gels. Normal C1̄-INH inhibited plasmin and was partially cleaved by the amount of plasmin used (Fig. 3, panel *A*). A complex that has an approximate molecular weight of 145,000 formed between normal C1̄-INH and plasmin, and cleavage fragments having apparent molecular weights of 81,000 and 69,000 were released (Fig. 3, panel *A*, gels 1 and 2). C1̄-INH Ri, which had essentially no antiplasmin activity, did not form visible complexes with the plasmin and there was no apparent cleavage of the inhibitor (Fig. 3, panel *A*, gels 3 and 4). The preparation of C1̄-INH Ta contained two readily visible contaminants, the anodal of which was albumin (Fig. 3, panel *B*, gels 2 and 3). C1̄-INH We was also contaminated with albumin (Fig. 3, panel *B*, gel 4). C1̄-INH Ta did not form complexes with plasmin that were detectable in these SDS gels, and was

not apparently cleaved by the enzyme (Fig. 3, panel *B*, gel 3). C1̄-INH We, on the other hand, was cleaved by the plasmin and small amounts of complexes having approximate molecular weights of ~150,000 were visible in these mixtures, even though the inhibitor did not block the action of plasmin (Fig. 3, panel *B*, gels 4 and 5). Cleavage fragments were released from C1̄-INH We of apparent molecular weights of 102,000, 89,000, and 61,000 after incubation with plasmin (Fig. 3, panel *B*, gel 5). Thus, fragments cleaved from C1̄-INH We appeared to have different molecular weights than those released from normal C1̄-INH by plasmin.

These interactions were then examined in thin-layer SDS gels in which the concentration of polyacrylamide was graded (Fig. 4). In these experiments, mixtures of C1s̄ or plasmin and inhibitors were not boiled in SDS before being applied to the gels, and varied ratios of plasmin to inhibitor proteins were tested.

The interaction of normal C1̄-INH with C1s̄ and plasmin is shown in Fig. 4, panel *A*. At molar ratios of 23:1, 3:1, and 1:1, C1s̄ formed a complex with C1̄-INH, with no evidence of proteolytic degradation of the inhibitor. In contrast, plasmin in molar ratios of inhibitor to enzyme of 3:1 or 1:1 formed both a complex with C1̄-INH and also caused limited proteolysis of the inhibitor to a lower molecular form, in agreement with the observations of Harpel and Cooper (8). C1̄-INH Ta formed a high molecular weight complex with C1s̄ when mixed at ratios of about 3:1 (Fig. 4 *B*, lane 3) and was cleaved by the C1s̄ (lane 3). A mixture containing C1̄-INH Ta and plasmin at molar ratios of 23:1, inhibitor to enzyme, showed no evidence of complex formation, but by changing the inhibitor to enzyme ratio to 3:1, C1̄-INH Ta could be shown to form a high molecular weight complex and was simultaneously converted to a lower molecular weight form, which we call I′ according to the terminology of Weiss and Engel (19) (Fig. 4 *B*, lanes 5 and 6). When C1̄-INH Ta and plasmin were mixed in approximately equimolar amounts (0.74:1), there appeared to be an increase in the formation of I′, but the predominant species was a higher molecular weight complex (Fig. 4 *B*, lane 6). C1̄-INH We formed a complex with C1s̄ and with plasmin while being converted to a lower molecular weight form by each enzyme. Although C1̄-INH We as isolated was somewhat more heterogeneous in these gels than the other dysfunctional inhibitors, it was still possible to demonstrate that this particular inhibitor was proteolytically degraded by both enzymes, in

INTERACTION OF C1̄ INH AND DYSFUNCTIONAL C1̄ INH WITH C1s̄ AND PLASMIN

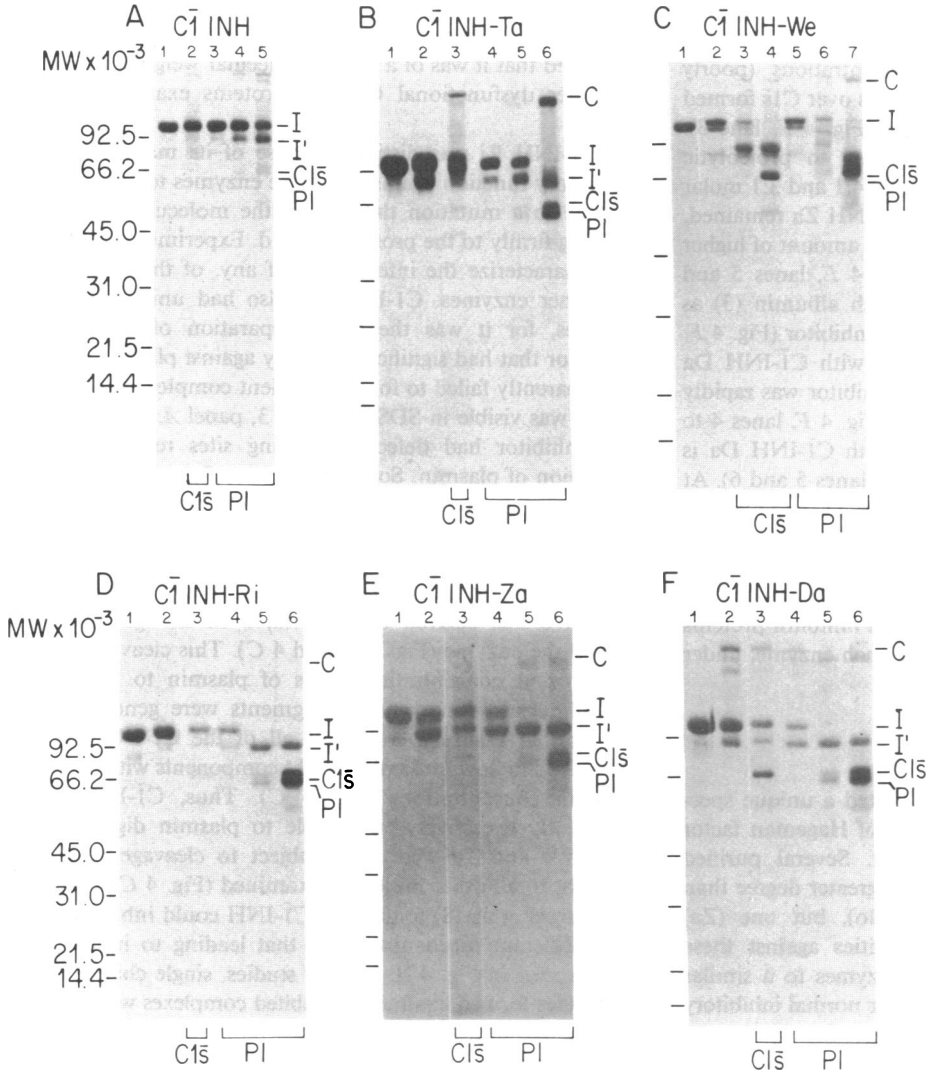

Figure 4. Interactions of C1s̄ and SK-activated plasmin (SK-P1) with C1̄-INH proteins: Thin-layer gels containing polyacrylamide in graded concentrations from 7.5 to 12% (*top* to *bottom*) and 1% SDS. The cathode is at the top of each set. The samples in the lanes are as follows: Panel *A:* lane 1, 7.6 μg normal C1̄-INH; lane 2, 7.6 μg normal C1̄-INH + 2.3 μg C1s̄; lane 3, 7.6 μg normal C1̄-INH + 0.24 μg SK-P1; lane 4, 7.6 μg normal C1̄-INH + 1.9 μg SK-P1; lane 5, 7.6 μg normal C1̄-INH + 7.5 μg SK-P1. Panel *B:* lane 3 contains 15.1 μg C1̄-INH Ta + 4.6 μg C1s̄. Other mixtures are as in panels *D–F*. Panel *C* (C1̄-INH We): As panel *A* except that lane 4 contains 6.2 μg C1s̄; lanes 5, 6, and 7 contain SK-PL mixtures as in other panels. Panels *D–F:* lane 1, normal C1̄-INH, 15.1 μg; lane 2, dysfunctional C1̄-INH, 15.1 μg; lane 3, dysfunctional C1̄-INH, 7.6 μg + 2.3 μg C1s̄; lane 4, dysfunctional C1̄-INH, 7.6 μg + 0.24 μg SK-P1; lane 5, dysfunctional C1̄-INH, 7.6 μg + 1.9 μg SK-P1; lane 6, dysfunctional C1̄-INH, 7.6 μg + 7.5 μg SK-P1.

contrast to the behavior of normal or other dysfunctional inhibitors except C1-INH Ta. With C1s at a 3:1 molar ratio of inhibitor to enzyme, a higher molecular weight complex formed and the C1-INH We was converted to a lower molecular weight form. (Fig. 4 *C*, lane 3). When C1s and C1-INH We were present in equimolar amounts, no intact inhibitor remained, either in a complex or a proteolyzed form (Fig. 4 *C*, lane 4). Similarly with plasmin at a molar ratio of 23:1, inhibitor to enzyme, there appeared to be conversion to a lower molecular weight form (Fig. 4 *C*, lane 5). At a molar ratio of 3:1 inhibitor to enzyme, the proteolysis of C1-INH We by plasmin was very evident, as multiple bands were found probably ranging in molecular weight from 100,000 to ~60,000 (Fig. 4 *C*, lane 6). At 0.74:1 inhibitor to enzyme, all of the inhibitor was in the lower molecular weight form or was present as a higher molecular weight complex (Fig. 4 *C*, lane 7). As previously noted (see Methods), it was difficult to obtain an accurate estimate of the molecular weight of the fragments because in the gradient gels the proteins used did not migrate according to their true molecular weight. C1-INH Ri formed a trace of a higher molecular weight complex when incubated with C1s at a molar ratio of 3:1 inhibitor to enzyme (Fig. 4 *D*, lane 3). C1-INH Ri appeared to be much more susceptible to limited proteolysis by plasmin, being completely converted to the I′ form when incubated with plasmin at inhibitor to enzyme ratios of 3:1 and 0.74:1 (Fig. 4 *D*, lanes 5 and 6). There appeared to be a small amount of a higher molecular weight complex at higher enzyme concentrations (poorly visible). C1-INH Za at threefold molar excess over C1s formed a small amount of complex with the C1s (Fig. 4 *E*, lane 3). Addition of small amounts of plasmin led to proteolytic conversion of I to I′ (Fig. 4 *E*, lane 4). At 0.74:1 and 3:1 molar ratios of inhibitor to enzyme, no intact C1-INH Za remained, being completely converted to I′ with a small amount of higher molecular weight complex remaining (Fig. 4 *E*, lanes 5 and 6). C1-INH Da, is partially complexed with albumin (3) as readily seen in the lane containing purified inhibitor (Fig. 4 *F*, lane 2). C1s formed little if any complex with C1-INH Da (Fig. 4 *F*, lane 3), but this dysfunctional inhibitor was rapidly degraded at all concentrations of plasmin (Fig. 4 *F*, lanes 4 to 6). Apparently the complex of albumin with C1-INH Da is susceptible to plasmin proteolysis (Fig. 4 *F*, lanes 5 and 6). At approximately equimolar amounts of C1-INH Da and plasmin there was a suggestion of formation of a high molecular weight complex. (Fig. 4 *F*, lanes 5 and 6).

Table III summarizes the observations on formation of complexes between C1s and plasmin and the inhibitor proteins and designates those inhibitors cleaved by each enzyme, under conditions of these experiments.

Table III. Summary of Complex Formation and Cleavage of Inhibitor Proteins with C1s and Plasmin

	Enzyme			
	C1s		Plasmin	
Inhibitor	Complex formation	Cleavage	Complex formation	Cleavage
Normal	+	0	+	+
Ta	+	+	+	+
We	+	+	+	+
Ri	±	0	0	+
Da	+	0	±	+
Za	±	0	+	+

+, Complex or cleavage visualized in graded SDS gels described in Methods. 0, No visible complex between inhibitor and enzyme or cleavage of inhibitor. ±, Trace of complex or cleavage visualized.

Discussion

Each dysfunctional C1-INH protein exhibited a unique spectrum of activity against C1s, active forms of Hageman factor (HFa and HFf), kallikrein, and plasmin. Several purified dysfunctional inhibitors inhibited C1s to a greater degree than they inhibited kallikrein (Ta, Da, At, Mo), but one (Za) demonstrated the opposite ratio of activities against these enzymes, and one (We) inhibited both enzymes to a similar degree. All but C1-INH Ri had significant, or normal inhibitory activity against activated forms of Hageman factor, but the amount of activity against HFa and HFf could not be correlated with that directed against kallikrein or C1s. These data indicate that there are different structural requirements for the inhibition of each enzyme by C1-INH. Normal and dysfunctional C1-INH proteins are single chain molecules, with the exception of C1-INH Da, part of which exists in plasma complexed through disulfide linkages with albumin (3, 7). C1-INH Ta was unique among dysfunctional proteins in that earlier work indicated that it was of a higher molecular weight than normal or other dysfunctional C1-INH proteins examined to date (7, 20).

C1-INH Ri was distinct because of its markedly limited inhibitory function against all of the enzymes tested. This may be due to a mutation that makes the molecule incapable of binding firmly to the proteases tested. Experiments in progress will characterize the interactions, if any, of this protein with the other enzymes. C1-INH Da also had unique functional features, for it was the only preparation of dysfunctional inhibitor that had significant activity against plasmin. C1-INH Ri apparently failed to form a covalent complex with plasmin, which was visible in SDS gels (Fig. 3, panel *A;* Fig. 4 *D*), as if this inhibitor had defective binding sites required for the inhibition of plasmin. Some visible complex was formed with C1s under different conditions of electrophoresis (Fig. 4 *D*). C1-INH Ta also failed to form a visible complex with plasmin in the SDS disc gels (Fig. 3, panel *B*) but complexes were visible under different experimental conditions (Fig. 4 *B*). C1-INH We was cleaved by plasmin, even though it did not inhibit the enzyme (Figs. 3 *B* and 4 *C*). This cleavage actually occurred at concentration ratios of plasmin to inhibitor at which no additional anodal fragments were generated from the normal C1-INH. Moreover all of the C1-INH We was degraded into low molecular weight components with increasing plasmin concentrations (Fig. 4 *C*). Thus, C1-INH We is apparently excessively susceptible to plasmin digestion. C1-INH We and Ta were both subject to cleavage by C1s in contrast to all other inhibitors examined (Fig. 4 *C*, lane 4).

Harpel et al. (8) found that C1-INH could inhibit plasmin by a different mechanism than that leading to inhibition of C1s as seen in Fig. 4 *A*. In their studies, single chain C1-INH molecules formed equimolar inhibited complexes with plasmin, as ocurred with C1s. In addition, plasmin could produce a lower molecular weight C1-INH molecule (I′, Fig. 4 *A*) that

could also form an inhibited complex with the plasmin. Cleavage of other antiproteases is known to occur during the formation of inhibited complexes with certain enzymes (21–23).

$C1\bar{s}$ was not thought to cleave normal $C\bar{1}$-INH before forming an inhibited stoichiometric complex and neither Harpel or Sim et al. (8, 24) detected cleavage of $C\bar{1}$-INH by $C1\bar{s}$. Weiss and Engel (19) recently reported that normal $C\bar{1}$-INH was cleaved by $C1\bar{s}$ and this was enhanced by heparin. We, however, failed to demonstrate cleavage of normal $C\bar{1}$-INH by $C1\bar{s}$ in the presence or absence of heparin. Cleavage of $C\bar{1}$-INH by $C1\bar{s}$ is, therefore probably not directly related to the formation of an inhibited complex (25). Only $C\bar{1}$-INH We and Ta were cleaved by $C1\bar{s}$ in the present experiments (Fig. 4, *B* and *C*).

When conditions of electrophoresis were varied and graded concentrations of polyacrylamide were used in SDS gel studies (Fig. 4), further information regarding the variability of the interactions of the dysfunctional proteins tested and plasmin and $C1\bar{s}$ were obtained, some of which was not evident in the SDS gels shown in Fig. 3 that were derived from kinetic experiments. In these studies, high molecular weight complexes were found in mixtures of $C\bar{1}$-INH Ta, We, Za, and Da when these proteins were incubated with $C1\bar{s}$ or with increasing amounts of plasmin. A high molecular weight complex was only faintly visible, visible between $C1\bar{s}$ and $C\bar{1}$-INH Za (Fig. 4). Moreover, an I′ fragment of $C\bar{1}$-INH was generated in each of these mixtures except $C\bar{1}$-INH We, as was seen with the normal $C\bar{1}$-INH as reported by Harpel and Cooper (8). These gels were done with mixtures of enzymes and inhibitor were incubated in SDS and applied to graded polyacrylamide concentrations in gels without boiling, which may account for some of the additional detail not seen in Fig. 3. It is clear that in several instances when sufficient plasmin was used, complexes can be formed with all dysfunctional $C\bar{1}$-INH proteins tested in these gels, except $C\bar{1}$-INH Ri, as seen in Fig. 3 (panel *A*). These studies confirm the heterogeneity of these proteins and support the likelihood that $C\bar{1}$-INH We is unduly susceptible to cleavage by plasmin, for this protein was broken into four fragments in mixtures containing 1.9 μg of plasmin in contrast to the effect of plasmin on the other dysfunctional and normal proteins. $C\bar{1}$-INH Ri did not form visible complexes with three different concentrations of plasmin, but the I, or normal inhibitor band, was readily converted to I′ despite lack of demonstrated inhibitory effect. Similar cleavage occurred in the case of $C\bar{1}$-INH Za and Da. It is noteworthy that the high molecular weight complex of $C\bar{1}$-INH Da with albumin did not protect it from plasmin cleavage (Fig. 4, panel F, lanes 5 and 6).

$C\bar{1}$-INH Za was distinct in that it had an extraordinarily high activity against HFa, while its activities against $C1\bar{s}$, plasmin and kallikrein were reduced. This enhanced activity against HFa is reminiscent of the altered function found in "antithrombin Pittsburgh" (26, 27). Antithrombin Pittsburgh is an α_1 proteinase inhibitor variant that acquired potent antithrombin activity because of a single amino acid substitution of an arginine for a methionine (27). This substitution was apparently sufficient to generate the potent antithrombin activity and induce a fatal hemorrhagic diathesis in the propositus (27). It is unlikely that the variable properties of dysfunctional $C\bar{1}$-INH proteins reported here could be explained on the basis of a single amino acid substitution.

Earlier reports suggested dysfunctional $C\bar{1}$-INH proteins might inhibit kallikrein (28), and clearly demonstrated heterogeneous electrophoretic and functional properties of several dysfunctional $C\bar{1}$-INH proteins from patients with HANE (3). Some dysfunctional proteins interacted with $C1\bar{s}$ when the $C1\bar{s}$ was added to unfractionated plasma while others failed to bind to $C1\bar{s}$ (3). Heterogeneity of structure of dysfunctional proteins has also been demonstrated in studies of isolated preparations of $C\bar{1}$-INH (7, 8).

The information obtained from studies of purified reagents can not be applied directly to events which occur in unfractionated plasma. In our earlier description of the interactions of $C\bar{1}$-INH in plasmas from patient We and three others, not described here, there was no apparent interaction between $C\bar{1}$-INH and kallikrein, Hageman factor, or high molecular weight kininogen (29). On the other hand, $C\bar{1}$-INH proteins in plasma from patients We, Da and Ri interacted with streptokinase-activated plasmin (29), in contrast to the present observations. The behavior of purified $C\bar{1}$-INH proteins from these individuals differed from their behavior in plasma, in part due to differences in the conditions of assay. Furthermore, all of the dysfunctional proteins were isolated from plasmas of individuals with excessive consumption of the natural substrates of $C\bar{1}$ (C4 and C2), because of defective regulation of $C\bar{1}$. Therefore, the ability of purified $C\bar{1}$-INH Da and Ta to inhibit $C1\bar{s}$ is probably due to differences between their interactions with the physiologic enzymes which can form in plasma (macromolecular $C\bar{1}$ or $C1\bar{r}$-$C1\bar{s}$ complexes) and its purified $C1\bar{s}$ subcomponent.

$C\bar{1}$-INH Ta may also be susceptible to digestion by one or more serum proteases to which normal $C\bar{1}$-INH is resistant, for the anti-$C1\bar{s}$ activity of $C\bar{1}$-INH Ta was lost when it was added to Ta serum, whereas that of the normal $C\bar{1}$-INH was not. The in vivo behavior of three of the dysfunctional proteins described herein has been reported (5). $C\bar{1}$-INH Ta and At behaved similarly in being cleared more slowly than the normal inhibitor from the vascular space of normal individuals, patients with HANE (5), and other patients with excessive C1 activation (30, 31). This suggested that catabolism was independent of inhibitory functions of these proteins. $C\bar{1}$-INH We was cleared more rapidly than $C\bar{1}$-INH Ta and At, as if $C\bar{1}$-INH We may be excessively susceptible to proteolytic digestion by enzymes such as plasmin, which may play a role in its catabolism.

These studies confirm earlier reports of genetically determined heterogeneity of dysfunctional $C\bar{1}$-INH proteins found in HANE plasmas, and additionally demonstrate a complexity of interactions with plasma proteases which was not previously appreciated. Work in progress will characterize the interactions of dysfunctional $C\bar{1}$-INH proteins and other plasma proteases and assess the relevance of these interactions to the pathogenesis of hereditary angioneurotic edema.

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