Interaction of Chymotrypsinogens with α_1-Protease Inhibitor†

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ABSTRACT: In a previous report [Largman, C., Brodrick, J. W., Geokas, M. C., Sischo, W. M., & Johnson, J. H. (1979) *J. Biol. Chem. 254*, 8516–8523] it was demonstrated that human proelastase 2 and α_1-protease inhibitor react slowly to form a complex that is stable to denaturation with sodium dodecyl sulfate and β-mercaptoethanol and that the zymogen can be recovered from the isolated complex following dissociation by hydroxylamine. The present report demonstrates that bovine chymotrypsinogen A reacts with human α_1-protease inhibitor in a very similar manner. The rate of complex formation was measured by two methods. In the first, the reaction was followed by determining the loss of the inhibitory activity of α_1-protease inhibitor as a function of time. A second-order rate constant for complex formation (pH 7.6, 37 °C) of 12.9 ± 2.4 M^{-1} s^{-1} was obtained. In the second procedure, the reaction of fluorescein isothiocyanate labeled chymotrypsinogen A with α_1-protease inhibitor was measured by fluorescence polarization. A second-order rate constant (pH 7.6, 37 °C) of 13.9 ± 2.1 M^{-1} s^{-1} was obtained. The rate of complex formation is $\sim 10^{-5}$ of that measured for the reaction of bovine chymotrypsin with α_1-protease inhibitor. Dissociation of the complex was not observed after dilution or the addition of excess bovine α-chymotrypsin. As judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis experiments, human chymotrypsinogens I and II react with α_1-protease inhibitor at rates that are approximately equivalent to that determined for bovine chymotrypsinogen A. In contrast, bovine trypsinogen reacts very slowly with α_1-protease inhibitor, at a rate that is at most 10^{-2} of that of bovine chymotrypsinogen A. These results suggest that zymogens react with α_1-protease inhibitor by virtue of partially formed active sites and that the potential active-site specificity of the zymogen in part determines the rate of complex formation.

There has been intense interest in recent years in the elucidation of the mechanism of action of α_1-PI[1] because of the established relationship between genetic deficiency of this serum protease inhibitor and the premature development of pulmonary emphysema (Laurell & Eriksson, 1963). This protein is capable of inhibiting serine proteases of widely different specificities. In addition, it is generally believed that full catalytic activity is a prerequisite for interaction of endopeptidases with α_1-PI, since the reports of Cohen (1973) and of Bloom & Hunter (1978) suggested that neither zymogens nor proteases treated with diisopropyl fluorophosphate could react with the inhibitor.

We have previously reported that proelastase 2 is present in normal human plasma as a complex with α_1-PI (Largman et al., 1980). We have also demonstrated that proelastase 2 can react directly with α_1-PI in vitro and that the zymogen can be recovered from the complex (Largman et al., 1979). The rate of reaction in vitro is very slow, however, suggesting that the zymogen is functioning like an active protease with a much less efficient catalytic apparatus. This hypothesis is consistent with the work of Neurath and co-workers (Morgan et al., 1972; Robinson et al., 1973; Gertler et al., 1974) which demonstrated that bovine chymotrypsinogen and trypsinogen have partially formed active sites and can thus react with certain substrates and inhibitors at rates that are 10^{-4}–10^{-5} of that of corresponding rates observed with the active proteases.

In the present report, these findings have been extended by using bovine chymotrypsinogen A (CTGN) in order to quantitate the reaction of zymogens with α_1-PI. We have demonstrated that CTGN reacts with α_1-PI in essentially the same manner as human proelastase 2. It has also been shown that human chymotrypsinogens I and II react with α_1-PI, while bovine trypsinogen reacts much more slowly with the inhibitor. Two independent methods have been devised to determine the second-order rate constant for the reaction of CTGN with α_1-PI. In the first, the rate of complex formation was determined by monitoring the loss of the inhibitor activity of α_1-PI as a function of time. The extent of complex formation with α_1-PI was also determined by monitoring the change in the fluorescence polarization of FITC-labeled CTGN upon binding of the zymogen to α_1-PI. Fluorescence polarization is an extremely sensitive indicator of molecular volume, and hence molecular size, and thus is a powerful method for studying intermolecular interactions which involve significant changes in apparent molecular size through digestion, dissociation, or, in the study described herein, binding of macromolecules.

Experimental Procedures

Materials. CTGN (3× crystallized) was obtained from Worthington Biochemical Corp., while bovine trypsinogen was supplied by Sigma Chemical Co. Human chymotrypsinogens I and II were purified as described previously (Geokas et al., 1979). Human α_1-PI was purified according to a slight modification (Largman et al., 1979) of the procedure of

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[1] Abbreviations used: α_1-PI, α_1-protease inhibitor (also known as α_1-antitrypsin); CTGN, bovine chymotrypsinogen A; FITC, fluorescein isothiocyanate; F-CTGN, bovine chymotrypsinogen labeled with FITC; $NaDodSO_4$, sodium dodecyl sulfate; GdnBzMum, 4-methylumbelliferyl *p*-guanidinobenzoate hydrochloride; MUSAD, 4-methylumbelliferyl *p*-[*N*-(dimethylsulfonic)acetamido]benzoate bromide; DEAE, diethylaminoethyl; DIFP, diisopropyl fluorophosphate.

0006-2960/80/0419-4865$01.00/0

Pannell et al. (1974). GdnBzMum and MUSAD were the generous gifts of Dr. M. Laskowski, Jr., Purdue University, Lafayette, IN. FITC isomer I was purchased from Sigma Chemical Co.

Methods. The kinetics of complex formation between α_1-PI and CTGN were followed by determining the loss of α_1-PI activity as a function of time. CTGN (26–52 μM) was incubated with α_1-PI (32 μM) at 37 °C in 50 mM Tris-HCl (pH 7.6) containing 0.14 M NaCl. Aliquots were removed at various times into a solution containing a 20% excess of chymotrypsin for titration of α_1-PI inhibitory activity. After a 5-min incubation at room temperature, chymotrypsin activity was determined with succinyl-Ala_2-Pro-Leu-*p*-nitroanilide (Del Mar et al., 1980), which is a very sensitive substrate for chymotrypsin as well as for human elastase 2. Two controls were incubated and assayed in the same manner. The first, which did not contain α_1-PI (CT_0^a), was used to establish the uninhibited chymotrypsin activity and demonstrated that CTGN was not activated under the incubation conditions. The second control, which did not contain CTGN (CT_0^b) was employed to determine the chymotrypsin inhibitory activity of the total α_1-PI. The concentration of α_1-PI as a function of time $[\alpha_1\text{-PI}]_t$ was then calculated from the chymotrypsin activity at each time point CT_t by eq 1. The data were plotted

$$[\alpha_1\text{-PI}]_t = \frac{CT_t - CT_0^a}{CT_0^b - Ct_0^a}[\alpha_1\text{-PI}]_0 \quad (1)$$

according to standard for second-order kinetics in order to estimate the rate constant according to eq 2. The reaction

$$\ln \frac{[CTGN_0] - [\alpha_1\text{-PI}\cdot CTGN]}{[\alpha_1\text{-PI}]_0 - [\alpha_1\text{-PI}\cdot CTGN]} = ([\alpha_1\text{-PI}]_0 - [CTGN]_0)kt + \ln([\alpha_1\text{-PI}]_0/[CTGN]_0) \quad (2)$$

followed second-order kinetics under the conditions used; however, no attempt was made to verify the order of the reaction over a wide concentration range.

Formation of complexes between α_1-PI and various zymogens was monitored by $NaDodSO_4$ gel electrophoresis. All incubations were performed at 37 °C in 50 mM Tris-HCl (pH 7.6) containing 0.14 NaCl. Aliquots were removed at the indicated incubation times into electrophoresis sample buffer containing 1% $NaDodSO_4$ and 1% β-mercaptoethanol as denaturants and immediately placed in a boiling water bath for 2 min. Discontinuous $NaDodSO_4$–polyacrylamide slab gel electrophoresis was performed in an apparatus (Model 220) from Bio-Rad Laboratories, Inc., by using the Laemmli (1970) buffer system in a 12.5% acrylamide gel.

The preparations of human chymotrypsinogens I and II contained peptide impurities that could not be separated except by use of strong denaturing conditions. An indirect method was used to determine the concentrations of potential active sites in these zymogens following activation with bovine trypsin and titration with the fluorogenic burst substrate MUSAD (Laskowski et al., 1974). The fluorescence intensity of the 4-methylumbelliferone was determined by measuring the fluorescence produced by hydrolysis of the trypsin-specific fluorogenic burst substrate GdnBzMum (Jameson et al., 1973) by a reference bovine trypsin solution that had previously been titrated with *p*-nitrophenyl *p'*-guanidinobenzoate by the method of Chase & Shaw (1970). The fluorescence intensity generated from MUSAD hydrolysis by the human chymotrypsins was taken to be equal to that produced by titration of the reference trypsin solution with GdnBzMum.

CTGN was labeled with a fivefold molar excess of FITC in 0.1 M Tris-HCl and 9 mM $CaCl_2$ (pH 8.5) for 5 h in the dark at room temperature and was then separated from unreacted reagent by gel filtration on Sephadex G-25 followed by extensive dialysis against 0.1 mM HCl. The final product was analyzed for protein content (Lowry et al., 1951) with CTGN as standard and for the content of fluorescent label by absorbance at 490 nm with a molar extinction coefficient of 61 000. The product was determined to have 0.87 mol of fluorescein/mol of CTGN. It was stored at −20 °C and used within 3 weeks.

Fluorescence polarization measurements were carried out with the MAC-2 polarization spectrophorometer (Japan Immunoresearch Co., Ltd., Takasaki, Japan—sold in the U.S.A. by Meloy Laboratories, Springfield, VA). This instrument is water-jacketed for constant temperature and uses a rotating emission polarizer which allows the detection of both parallel and perpendicular fluorescence components as a cosine function of rotation time by using a single photomultiplier tube. The fluorescence polarization value (P by value) is defined as $(I_\parallel - I_\perp)/(I_\parallel + I_\perp)$ where I designates the fluorescence intensity in the parallel or perpendicular mode. In this instrument, the P value is calculated by a microcomputer which prints the average value of 200 readings every 85 s. In addition, the machine utilizes three-cavity filters (Ditric Optics, Hudson, MA) that pass near-monochromatic light with little depolarization. The filters used are 490 nm for excitation and 520 nm for emission to make the instrument particularly applicable for studying molecular interactions of fluorescein-labeled proteins.

The kinetics of complex formation between α_1-PI- and FITC-labeled CTGN were followed by adding the zymogen (0.14 μM final concentration) to a cuvette containing a 20–200-fold molar excess of α_1-PI in 50 mM Tris-HCl and 0.14 M NaCl (pH 7.6) at 37 °C and monitoring the fluorescence polarization as a function of time. The P value of the fully complexes species was determined by direct measurement of the P value of the complex after isolation by gel filtration on a Sephadex G-75 column equilibrated with the same buffer.

At any point in time, the measured polarization is the weighted average of the polarization contributions of free and bound F-CTGN (F-CTGN–α_1-PI complex) so that

$$P_m = \frac{P_f[\text{F-CTGN}]_f Q_f + P_b[\text{F-CTGN}]_b Q_b}{[\text{F-CTGN}]_f Q_f + [\text{F-CTGN}]_b Q_b} \quad (3)$$

where P_m is the measured polarization, P_f and P_b are the polarization values of free and bound F-CTGN, $[\text{F-CTGN}]_f$ and $[\text{F-CTGN}]_b$ are the respective concentrations of free and found [F-CTGN], and Q_f and Q_b are the respective fluorescence intensities per mole of free and bound F-CTGN. The relative fluorescence intensities of free F-CTGN and F-CTGN–α_1-PI complex were determined by incubating 70 nM F-CTGN solutions with a 65–870-fold molar excess of α_1-PI for 20 h at 37 °C to allow complex formation. At this point, the relative fluorescence intensity of each solution was determined by using a Perkin-Elmer 610-S spectrofluorometer water-jacketed at 37 °C. The excitation and emission monochromators were set at 490 and 520 nm, respectively, with a 5-nm band-pass. The fluorescence values obtained were compared to a control solution containing 70 nM F-CTGN containing no α_1-PI and incubated under the same conditions.

Algebraic manipulation of eq 3 yields

$$f = \frac{\frac{Q_b}{Q_f}(P_b - P_m)}{\frac{Q_b}{Q_f}(P_b - P_m) + P_m - P_f} \times 100 \quad (4)$$

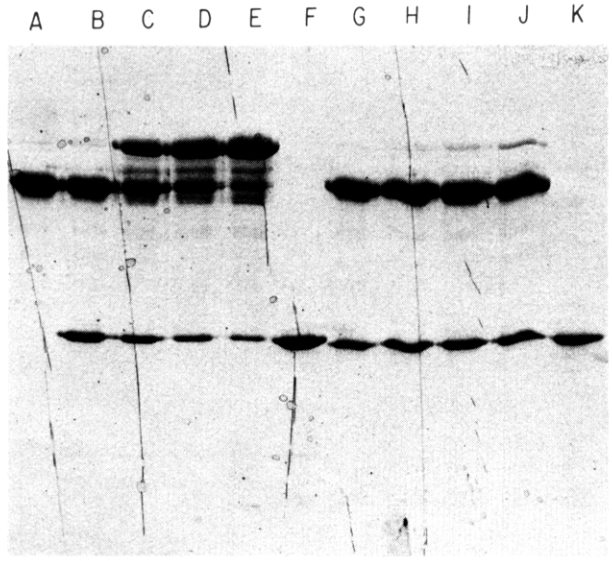

FIGURE 1: Reaction of CTGN and bovine trypsinogen with human α_1-PI. Reaction mixtures containing 32 μM α_1-PI and either 31 μM chymotrypsinogen or 29 μM trypsinogen were incubated and subjected to $NaDodSO_4$ gel electrophoresis as described under Experimental Procedures. (Lane A) α_1-PI control; (lanes B–E) α_1-PI and chymotrypsinogen at 15 s, 15 min, 45 min, and 2 h of incubation at 37 °C; (lane F) chymotrypsinogen control; (lanes G–J) α_1-PI and trypsinogen at 15 s, 15 min, 45 min, and 2 h of incubation at 37 °C; (lane K) trypsinogen control.

where

$$f = \frac{[\text{F-CTGN}]_f}{[\text{F-CTGN}]_f + [\text{F-CTGN}]_b} \times 100$$

the percentage of F-CTGN in the free form. The second-order rate constant was then obtained from a semilog plot of $[\text{F-CTGN}]_{free}$ vs. time of incubation. From the half-time obtained from such a plot, the rate constant is calculated from eq 5.

$$k = 0.693/(t_{1/2}[\alpha_1\text{-PI}]) \quad (5)$$

Dilution experiments were carried out in which FITC-labeled CTGN was incubated in 50 mM Tris-HCl (pH 7.6) containing 0.14 M NaCl with varying amounts of α_1-PI for 2.0 h at 37 °C to allow for complex formation and then diluted 10- or 100-fold with the same buffer. The P values were measured prior to dilution and 2–3 h after dilution to ascertain whether the complex had dissociated.

Results

Figure 1 demonstrates that CTGN forms a 1:1 molar complex with α_1-PI (M_r = 77 000) that is stable to denaturation by heating in $NaDodSO_4$ and β-mercaptoethanol. The relatively high concentrations of the reactants, as well as the extended incubation time required for a high yield of complex (Figure 1, lines B–E), indicate that rate of complex formation is much slower than for the active enzyme [$k = 2 \times 10^6$ M^{-1} s^{-1}; Bieth et al. (1974)]. Incubation of bovine trypsinogen with human α_1-PI under essentially the same conditions results in only a small amount of material with a molecular weight equivalent to that of a 1:1 molar complex (Figure 1, lines G–J). α_1-PI and bovine trypsinogen were incubated under the same conditions at a nine-fold higher product [α_1-PI][trypsinogen] to determine whether a significant amount of complex formation could be observed. Amounts of protein equivalent to those given in the legend to Figure 1 were removed after 0, 1, and 4 h of incubation and subjected to $NaDodSO_4$ gel electrophoresis. The amount of complex formation observed after 4 h was greater than that shown in Figure 1, line J, but significantly less complex was formed than the amount observed after incubation of α_1-PI and CTGN for only 15 min (Figure 1, lane C). This finding implies that the rate of reaction of trypsinogen with α_1-PI is on the order of 10^{-2}–10^{-3} lower than the rate of reaction of CTGN with the inhibitor.

The data presented above, while strongly suggesting that CTGN reacts directly with α_1-PI, do not rule out the possibility that complex formation results from slow activation of CTGN and subsequent reaction of chymotrypsin with α_1-PI. The direct reaction of the zymogen with α_1-PI was demonstrated by the recovery of CTGN but no chymotrypsin after incubation of the isolated complex with hydroxylamine. The experiments that led to this conclusion were essentially the same as those we have previously reported for the characterization of an α_1-PI–human proelastase 2 complex (Largman et al., 1979) and can be summarized as follows. After incubation of CTGN and α_1-PI under conditions similar to those shown in Figure 1, the complex was isolated by DEAE-cellulose chromatography. The α_1-PI–CTGN complex has an ionic character that is intermediate that of α_1-PI, which elutes late in the salt gradient employed, and CTGN, which is not adsorbed by the column. Incubation of the isolated complex with 0.5 M hydroxylamine (pH 10, 1 h at 37 °C) releases a protein with the molecular weight of CTGN as determined by $NaDodSO_4$ gel electrophoresis, accompanied by the appearance of activatable chymotrypsin activity. Gel filtration of the isolated complex on Sephadex G-100 after hydroxylamine treatment yields a protein peak with the M_r of CTGN that contains CTGN (chymotrypsin activity after activation with bovine trypsin) but no inherent chymotrypsin activity. The α_1-PI released from the complex is reduced in molecular weight by ~7000 and no longer inhibits trypsin or chymotrypsin.

In the case of the formation of a trypsinogen–α_1-PI complex, only indirect evidence has been obtained that indicates that autoactivation is not a factor. Prolonged incubation of bovine trypsinogen with α_1-PI in the presence of bovine pancreatic trypsin inhibitor (0.1 mg/mL) or 10^{-4} M Phe-Ala-Arg-CH_2Cl (Kettner et al., 1978) results in the formation of essentially the same amount of complex as incubation of the two proteins in the absence of inhibitors. However, we cannot rule out slow autoactivation of trypsinogen accompanied by reaction with α_1-PI prior to inhibition by pancreatic trypsin inhibitor or the chloromethyl ketone.

Reaction of Human Chymotrypsinogens with α_1-PI. As shown in Figure 2, human chymotrypsinogens I and II also react with α_1-PI to form 1:1 molar complexes after prolonged incubation at high concentrations. However, a comparison of the amounts of complex band at 15 min of incubation (lane C vs. lane H) indicates that chymotrypsinogen II reacts more rapidly with α_1-PI. This result is consistent with our previous observation (Geokas et al., 1979) that incubation of ^{125}I-labeled chymotrypsinogen II with normal human plasma converted the zymogen to a bound form with a molecular weight equivalent to that of an α_1-PI complex more rapidly than did labeled chymotrypsinogen I incubated with plasma under the same conditions.

Kinetics of the Reaction of Bovine CTGN with α_1-PI. After incubation of equimolar amounts of CTGN and α_1-PI for sufficient time to allow partial complex formation, the remaining unreacted α_1-PI could be easily titrated by the addition of a 1.5-fold excess of bovine α-chymotrypsin, as demonstrated by a reduction in the chymotrypsin activity compared to a control. It was observed that the chymotrypsin activity

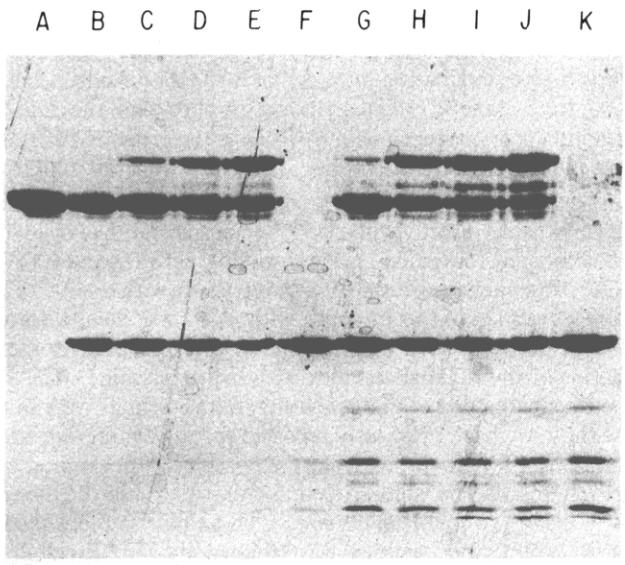

FIGURE 2: Reaction of human chymotrypsinogens I and II with human α_1-PI. Reaction mixtures containing 32 μM α_1-PI and 30 μM chymotrypsinogen I or chymotrypsinogen II were incubated and subjected to $NaDodSO_4$ gel electrophoresis as described under Experimental Procedures. (Lane A) α_1-PI control; (lanes B–E) α_1-PI and chymotrypsinogen I at 15 s, 15 min, 45 min, and 2 h of incubation at 37 °C; (lane F) chymotrypsinogen I control; (lanes G–J) incubation of α_1-PI and chymotrypsinogen II at 37 °C for 15 s, 15 min, 45 min, and 2 h, respectively; (lane K) chymotrypsinogen II control.

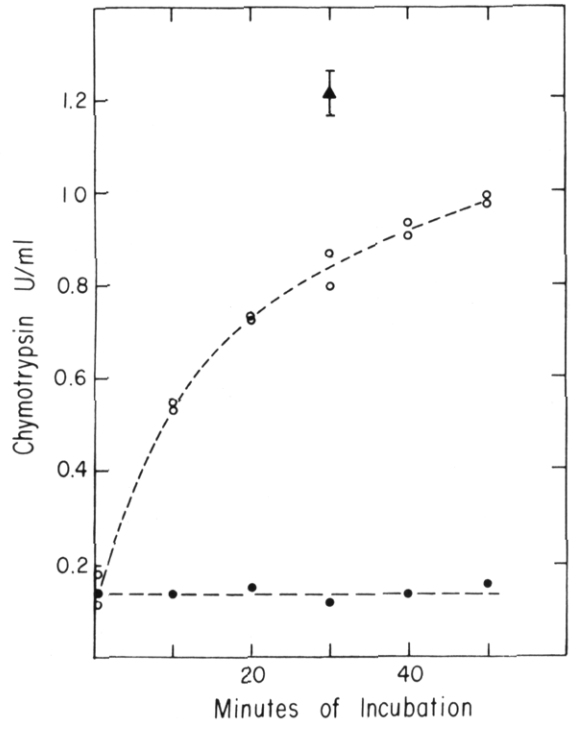

FIGURE 3: Reaction of CTGN with α_1-PI measured by loss of inhibitory activity of α_1-PI. CTGN (52 μM) was incubated with α_1-PI (32 μM) at 37 °C as described under Methods. At the times indicated, aliquots were removed and added to tubes containing a 1.2-fold molar excess of bovine chymotrypsin. The experimental points (○) show the remaining chymotrypsin activity after 5 min of incubation at 25 °C. (●) Control incubation with no CTGN. (▲) Mean (with standard deviation) of four determinations of chymotrypsin activity in control without α_1-PI at 0, 30, 60, and 120 min of incubation.

remained unchanged after further incubation of this mixture for up to 1 h at 37 °C, indicating that the α_1-PI–CTGN complex does not dissociate to a measurable degree in this time period in the presence of excess chymotrypsin. This observation suggested that the rate of reaction of α_1-PI with CTGN could be followed by titrating the α_1-PI remaining after appropriate incubation times with excess chymotrypsin. The results of such an experiment are shown in Figure 3. Control incubations without α_1-PI or without CTGN were included. It can be seen that the chymotrypsin inhibitory activity in aliquots of the incubation mixture is reduced as a function of time, reflecting a decrease in free α_1-PI and thus an increasing amount of complex. A plot of these data by the standard second-order treatment as described under Experimental Procedures is shown in Figure 4. It can be seen that a linear plot was obtained when the data were plotted according to eq 2, indicating that second-order kinetics are indeed being observed. In four separate determinations with constant α_1-PI and a twofold variation in CTGN concentration, an average value of 12.9 ± 2.4 M^{-1} s^{-1} was obtained for the second-order rate constant.

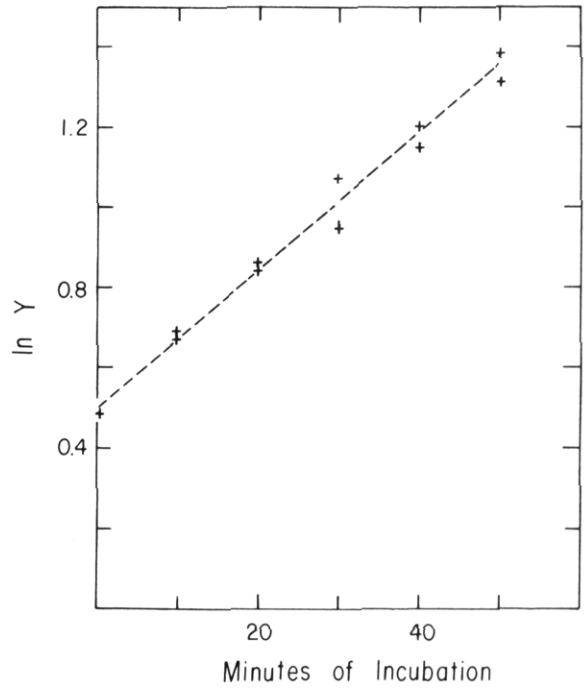

FIGURE 4: Second-order plot of reaction of CTGN with α_1-PI. The concentrations of α_1-PI at various incubation times were calculated from the data in Figure 3 according to eq 1. Equation 2 was employed for the second-order kinetic treatment. Ln Y represents the term on the left side of eq 2.

The kinetics of CTGN–α_1-PI complex formation were also studied by fluorescence polarization. When F-CTGN was incubated with excess α_1-PI under pseudo-first-order conditions, a time-dependent increase in P value was observed, as shown in Figure 5. The asymptote indicates a P value 76% above the control, which agrees quite closely with a 78% increase in P value for the isolated complex obtained by gel filtration as described under Methods. The latter value was used in all calculations. The relative fluorescence per mole of the F-CTGN–α_1-PI complex was found to be 65% of that of F-CTGN. By use of these values, all measured polarization data were converted to the percent of free F-CTGN as described by eq 4 under Methods. Figure 6 is a representative plot of one such experiment and shows the decrease in the percent of free F-CTGN with time. The second-order rate constant derived from nine such experiments was 13.9 ± 2.1 M^{-1} s^{-1}. Dilution of the complex by 10- or 100-fold failed to show any dimunition of the P values, indicating that the complex has no measurable tendency to dissociate.

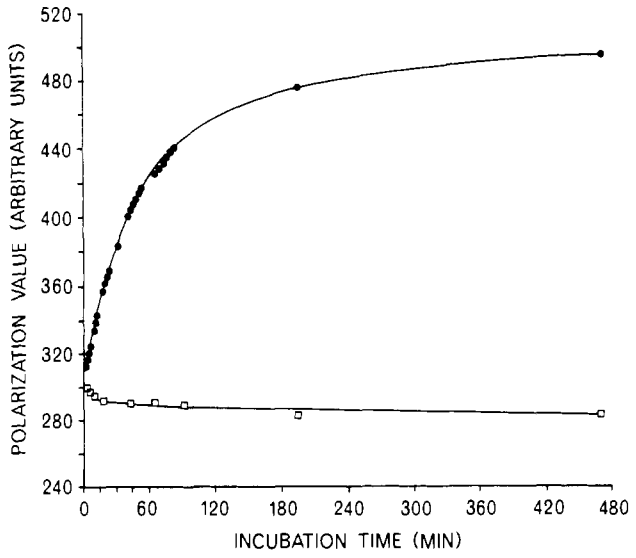

FIGURE 5: Detection of α_1-PT–CTGN complex formation by fluorescence polarization. F-CTGN (0.14 μM) was incubated with 28 μM α_1-PI as described under Methods. The polarization values shown are in arbitrary units that depend upon instrument calibration. The F-CTGN solution was kept on ice prior to addition to the cuvette; this accounts for the initial drop in P value seen in the control, since polarization is inversely related to temperature. (●) F-CTGN and α_1-PI; (□) F-CTGN alone.

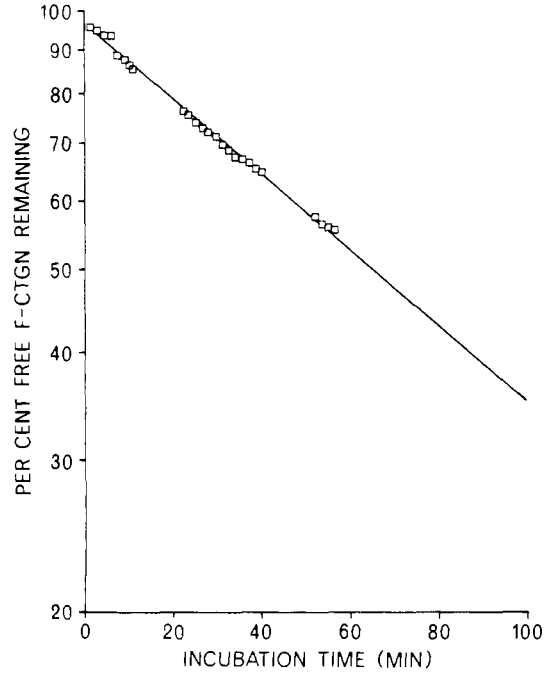

FIGURE 6: Kinetics of F-CTGN–α_1-PI complex formation measured by fluorescence polarization. F-CTGN (0.14 μM) was incubated with 10.5 μM α_1-PI. The percent of free CTGN was calculated as described under Methods. This particular experiment yielded a rate constant of 14.9 M^{-1} s^{-1}.

Discussion

The results obtained in this work provide a quantitative confirmation that certain zymogens of pancreatic serine proteases can form irreversible complexes with α_1-PI. We have demonstrated that the isolated complexes of α_1-PI with CTGN and with human proelastase 2 (Largman et al., 1979) can both be cleaved by incubation with hydroxylamine to yield apparently unaltered zymogen and an inactive fragment of α_1-PI whose M_r is the same as that which is obtained when complexes of α_1-PI with bovine trypsin or chymotrypsin are incubated under similar conditions. These observations suggest that the nature of the CTGN–α_1-PI complex is similar to or identical with that formed between α_1-PI and the active protease. The second-order rate constant for the reaction of CTGN with α_1-PI, obtained by two independent methods, is $\sim 10^{-5}$ of that reported for the interaction of bovine chymotrypsin with α_1-PI under somewhat different conditions (Bieth et al., 1974). Neurath and co-workers have shown that bovine trypsinogen (Morgan et al., 1972) and chymotrypsinogen (Robinson et al., 1973) react specifically with DIFP at the respective active-site serine residues of these proteins, at rates that are 10^{-4}–10^{-5} of those of the respective enzymes but are much faster than the rates of reaction of model compounds with DIFP. The relative rates of reaction of CTGN and chymotrypsin with α_1-PI are thus consistent with the concept that zymogens of serine proteases possess inherent catalytic activity, albeit at a much reduced efficiency (6–8).

Our results show qualitatively that trypsinogen reacts much more slowly than CTGN with α_1-PI. This finding is consistent with the reported second-order rate constants (Bieth et al., 1974) for the association of bovine chymotrypsin and trypsin with α_1-PI of 2.6×10^6 and 10^5 M^{-1} s^{-1}, respectively. Johnson & Travis (1978) have demonstrated a methionine residue at the point of cleavage of α_1-PI when a trypsin–α_1-PI complex is uncubated with benzamidine. Further work has demonstrated that this residue is at the reactive site of α_1-PI with respect to interaction of the inhibitor with several serine proteases (Johnson & Travis, 1978). Thus the relative rates of reaction of the zymogens, which parallel those of the respective active enzymes, can be explained at least in part by the presumed preferential interaction of the chymotrypsin(ogen) binding pocket with the methionine side chain.

Fehlhammer et al. (1977) have recently reported a high-resolution X-ray crystallographic structure for bovine trypsinogen. Their data demonstrate statistical or thermal disorder in three polypeptide chain segments which in part form the specificity pocket in active trypsin. Vincent & Lazdunski (1976) have reported that bovine trypsinogen forms a rapidly reversible complex with bovine pancreatic trypsin inhibitor (PTI) (Kuntiz) ($k_d < 4 \times 10^{-2}$ s^{-1}). X-ray crystallographic studies (Bode et al., 1978) demonstrated that trypsinogen assumes a trypsin-like conformation in crystals of a trypsinogen–pancreatic trypsin inhibitor complex, in which the partially disordered structure is no longer evident and the binding pocket is similar to that of trypsin. We propose that a similar phenomenon accounts for the ability of CTGN to react with α_1-PI. The relative rates of reaction of CTGN and chymotrypsin with α_1-PI would thus reflect the percentage of the zymogen in the active conformation, as well as quantitative differences in the catalytic efficiency of the zymogen active center with respect to that of the active protease.

Further comparison of our data with results obtained by others on the interaction of trypsin with pancreatic trypsin inhibitor suggests one other important difference between protease zymogen–α_1-PI interactions and those involving the pancreatic inhibitor. X-ray crystallographic studies of the trypsin-PTI complex have demonstrated multiple close contacts between the respective protein molecules. The high degree of stability of the complex ($K_d \simeq 10^{-14}$ M) has been in part attributed to the combined energy of these interactions. The role of interactions between the active-site serine of trypsin and the reactive lysine in PTI has been considered to be relatively minor, however, since anhydrotrypsin, in which the active-site serine is converted to dehydroalanine, interacts

nearly as strongly with PTI as does trypsin. Therefore, the greatly reduced stability of the trypsinogen–PTI complex can be mainly attributed to the conformational differences between the zymogen and trypsin that were mentioned above. In the present case, however, the irreversible nature of the complex that is formed between CTGN and α_1-PI implies that the role of the active-site serine is dominant and suggests greatly reduced stability of anhydrochymotrypsin–α_1-PI complexes when compared to those involving the active enzyme. Studies on the interaction of certain modified chymotrypsins with α_1-PI are currently in progress.

Two independent methods have been employed in this work to quantitate the rate constant for the reaction of chymotrypsinogen with α_1-PI. The enzymatic method, in which the loss of α_1-PI activity is followed, suffers to some extent because of a lack of sensitivity and the indirect nature of the measurement. The fluorescence polarization method, however, has several appealing features as a technique for the study of interactions of macromolecules. The principle of the method is as follows. Plane polarized light excites the sample so that properly oriented fluorochromes are preferentially excited. To the extent that the excited molecules remain stationary within their fluorescent lifetimes, the emitted light will also be polarized. Random rotation of the excited molecules within this time frame causes depolarization of the emitted light. Since rotational rates are inversely related to molecular volume, small fluorescent molecules have lower fluorescence polarization values (P values) than do larger molecules (Weber, 1973), and increases in P values can therefore be used to monitor macromolecular associations.

Since fluorescence polarization senses only the rotational characteristics of a macromolecule, it is by nature not concentration dependent over a very wide range and is limited only by viscosity changes at high concentration and instrument sensitivity at low concentration. However, the method easily distinguishes changes in the average molecular weight of the fluorescent species. When, as in the case of the data reported here, it can be determined that the fluorescent molecule (F-CTGN) can exist in two states with different molecular weights (free and bound to α_1-PI), both kinetic and equilibrium studies can be performed on the interaction, and the results can be analyzed in a straightforward manner (Dandliker et al., 1976).

The fluorochrome used here, FITC, has the additional advantage that it reacts preferentially with α-amino groups under the conditions employed (Maeda et al., 1969). Since it appears to react easily with many proteins, many potential applications of the method can be foreseen. Fluorescence polarization has found relatively little use in biochemistry in the past due to cumbersome instrumentation which necessitated extensive calibration and calculation of P values from measured parallel and perpendicular light intensities. The instrument used in our studies has eliminated these problems and allows for the routine use of fluorescence polarization in the study of macromolecular interactions. It is hoped that the hitherto unrecognized power of this technique will be utilized more extensively in the future.

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