Fluorescence Polarization Studies on the Interaction of Active Site Modified Chymotrypsins with α_1-Protease Inhibitor†

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ABSTRACT: Fluorescence polarization has been used to study the interaction of human α_1-protease inhibitor (α_1PI; also called α_1-antitrypsin) with two active site modified chymotrypsins (CT), dehydroalaninyl-195-α-CT (AnhCT) and *N*-methylhistidinyl-57-α-CT (MeCT). For the reaction of the fluorescein-labeled AnhCT (FAnhCT) with α_1PI (Pi type MM, the predominant allelic form), a K_{assoc} of 1.8×10^7 M^{-1} was obtained by Scatchard analysis, which also indicated 1.3 binding sites. An alternate analysis using a direct dissociation plot, which assumes 1:1 binding, gave a K_{assoc} of 2.2×10^7 M^{-1}. Fluorescein-labeled MeCT (FMeCT) binds somewhat more weakly to α_1PI ($K_{assoc} = 1.2 \times 10^6$ M^{-1}; 0.87 binding site). Similar results were obtained by using the proflavin displacement method to determine the binding constant for MeCT with α_1PI ($K_{assoc} = 1.0 \times 10^6$ M^{-1}). With α_1PI (ZZ type) in which the serum level is reduced and there is a strong tendency to develop chronic obstructive pulmonary disease, the K_{assoc} found by the fluorescence polarization method was similar to that for α_1PI (MM type) for both CT derivatives. α_1PI (MM type), modified by oxidation with *N*-chlorosuccinimide, shows a reduced binding affinity for FAnhCT ($K_{assoc} = 6.5 \times 10^5$ M^{-1}) and no measurable binding with FMeCT ($K_{assoc} < 1 \times 10^4$ M^{-1}). Previous studies have demonstrated that bovine CT forms very stable complexes with α_1PI. In contrast, complexes formed with both active site modified CT derivatives undergo rapid dissociation as shown by the drop in the polarization value on dilution or on the addition of excess unlabeled chymotrypsin derivative. This weakened association suggests that, for reaction with α_1PI, the enzyme active site serine is important in stabilizing the enzyme–inhibitor complex.

α_1PI[1] is the major protease inhibitor in human serum. It is a glycoprotein of 52000 daltons (Glaser et al., 1975). It is present at a concentration of ~1.3 mg/mL and inhibits a wide spectrum of serine proteolytic enzymes. Individuals who inherit a modified form of this protein (ZZ type) have low serum levels of α_1PI and a marked predisposition to develop chronic obstructive pulmonary disease (Laurell & Eriksson, 1963). Substantial evidence now exists suggesting that this is the result of elastolysis in the lung due to the low level of inhibitor (Janoff et al., 1977). For this reason there has been a considerable interest in the biochemistry of α_1PI.

Previous reports have demonstrated that human proelastase 2 and bovine CTGN can react slowly, apparently through partially formed active sites, to form covalent complexes with α_1PI that are stable to denaturation by sodium dodecyl sulfate and β-mercaptoethanol (Largman et al., 1979; Brodrick et al., 1980). The respective complexes can be dissociated by hydroxylamine to yield unaltered zymogen and an inactive form of α_1PI of reduced molecular weight.

In studies from other groups, it has been shown that active site modified derivatives of trypsin and CT that have little or no catalytic activity nevertheless retain the ability to bind to a variety of protease inhibitors, including basic pancreatic trypsin inhibitor, soybean trypsin inhibitor, lima bean inhibitor, and avian ovomucoid inhibitors (Ako et al., 1974; Ryan & Feeney, 1975). The association equilibrium constant for binding of bovine anhydrotrypsin to bovine basic pancreatic trypsin inhibitor is nearly as large as that for the native enzyme (Vincent et al., 1974). Similarly, MeCT binds somewhat more strongly to turkey ovomucoid than does CT (Ryan & Feeney, 1975). In contrast, bovine trypsinogen binds very weakly to bovine basic pancreatic trypsin inhibitor (Vincent & Lazdunski, 1976).

Fluorescence polarization experiments are a measure of the rotational freedom of the fluorescent group and, consequently, are sensitive to changes in molecular volume. Thus, fluorescence polarization is a powerful tool for studying intermolecular interactions which involve significant changes in molecular size. Previously, using fluorescein as an extrinsic label, we have utilized this technique to study the rate constant for the formation of complexes between CTGN and α_1PI (Brodrick et al., 1980). In the present investigation, fluorescence polarization is applied to obtain equilibrium constants for the association of two active site modified CT's with α_1PI from both normal individuals (MM type) and persons with the genetic variant form of this protein (ZZ type). In addition, the effects of oxidation of methionine at the inhibitory site of α_1PI on binding are investigated.

Experimental Procedures

Materials

Lima bean inhibitor and CT (3 times crystallized) were purchased from Worthington Biochemical Corp. Phenylmethanesulfonyl fluoride was obtained from Calbiochem and was used after recrystallization from benzene–heptane. FITC was a product of Sigma Chemical Co. Sephadex resins were purchased from Pharmacia Fine Chemicals.

Methods

Protein Preparations. α_1PI from normal (MM type) and deficient (ZZ type) individuals was purified to homogeneity

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[1] Abbreviations used: α_1PI, α_1-protease inhibitor (also known as α_1-antitrypsin); CT, bovine α-chymotrypsin; AnhCT, dehydroalaninyl-195-α-CT; MeCT, *N*-methylhistidinyl-57-α-CT; FITC, fluorescein isothiocyanate isomer I; FAnhCT, AnhCT labeled with FITC; FMeCT, MeCT labeled with FITC; CTGN, bovine chymotrypsinogen A.

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as previously reported (Glaser et al., 1975), except that plasma was used as a starting material instead of Cohn fraction IV-I and Cibacron blue dye coupled to agarose was used for the removal of albumin. The trypsin inhibitory capacity was determined by using benzoyl-DL-arginine-*p*-nitroanilide as the substrate (Erlanger et al., 1961), and active site titration of trypsin was carried out with *p*-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1967). The specific activity of the MM type preparation was 1.03 mol of active trypsin inhibited/mol of α_1PI and 0.82 mol/mol for the ZZ type protein. The extinction coefficient used for all α_1PI preparations is $E^{0.1\%}_{280nm}$ = 0.50, as previously determined (Glaser et al., 1975).

Methionine-oxidized α_1PI (MM type) was prepared according to the procedure of Shechter et al. (1975). To a solution of α_1PI (8 mg/mL) in 0.1 M Tris-HCl (pH 8.8) was added a 20-fold excess (mole/mole α_1PI) of *N*-chlorosuccinimide. After reaction for 20 min at 25 °C, the reaction mixture was subjected to gel filtration on Sephadex G-25 in 0.05 M Tris-HCl (pH 7.6) containing 0.14 M NaCl. The product was quick frozen in aliquots until use. An aliquot subjected to gel filtration on Sephadex G-150 indicated a single symmetrical peak, demonstrating that the product was free from aggregate. The oxidized inhibitor reacted with bovine trypsin at a rate similar to that reported by Beatty et al. (1980), indicating that an intact but less efficient reactive site remained.

Dehydroalaninyl-195-α-CT (AnhCT) was prepared from CT by the method of Ako et al. (1974) modified to eliminate side products as described by Matta et al. (1977). This procedure includes affinity chromatography with lima bean trypsin inhibitor immobilized on agarose. A recent report (Schultz et al., 1979) indicated that lyophilized preparations of this protein differ from unlyophilized material in parameters for binding substrate analogues. The preparation used in this study was not lyophilized. *N*-Methylhistidinyl-57-α-CT (MeCT) was prepared from bovine α-chymotrypsin as described by Ryan & Feeney (1975). MeCT was purified free of inactive side products by affinity chromatography with turkey egg white protease inhibitor immobilized on agarose. The extinction coefficient of CT is $E^{0.1\%}_{280nm}$ = 2.0 after correcting for light scattering. This value was also used for the other CT derivatives described. CT activity was determined for the native enzyme or its derivatized forms by using succinyl-Ala_2-Pro-Leu-*p*-nitroanilide (Del Mar et al., 1980).

The modified CT's were labeled with a 1:1 molar ratio of FITC/CT in 0.1 M Tris-HCl and 9 mM $CaCl_2$ (pH 8.5) for 3 h in the dark at room temperature and then separated from unreacted reagent by gel filtration on Sephadex G-25 in 0.05 M Tris-HCl (pH 7.6) containing 0.14 M NaCl. The final product was analyzed for protein content (Lowry et al., 1951) with CT as the standard and for the content of fluorescent label by absorbance at 490 nm with a molar extinction coefficient of 61 000. The FAnhCT product, in several preparations, was found to have 0.55–1.03 mol of fluorescein/mol of AnhCT, while for MeCT, this ratio was 0.92–1.28. Each preparation was divided into aliquots, quick frozen, and stored at −20 °C until use.

Polarization Experiments. Fluorescence polarization measurements were carried out with the MAC-2 polarization spectrophotometer (Japan Immunoresearch Co., Ltd., Takasaki, Japan; sold in the United States by Meloy Laboratories, Springfield, VA), as previously reported (Brodrick et al., 1980). Cuvettes containing FAnhCT (12–800 nM) or FMeCT (200–1200 nM) in 0.14 M NaCl and 50 mM Tris at pH 7.6 were placed in the polarizer and equilibrated to 37 °C. The polarization of the free FITC-labeled material, P_f, was measured prior to addition of α_1PI. Several additions of prewarmed α_1PI were then made to each cuvette (final concentration 10–2000 nM), and the polarization, P_m, was measured after each addition. The measured polarization values were normalized to P_f to avoid cuvette to cuvette variation.

Since loss of fluorescence was originally noted when FAnhCT solutions were mixed in a cuvette with Pasteur pipets or with disposable pipet tips, an experimental protocol was devised in order to minimize adsorption of FAnhCT to glass and plastic surfaces. Whenever an aliquot of frozen FAnhCT was thawed, a disposable plastic pipet tip was conditioned to FAnhCT by drawing the FAnhCT solution into the pipet tip 10 times. The same conditioned pipet tip was then used for all subsequent additions of FAnhCT to buffer in cuvettes and α_1PI was added to cuvettes which already contained FAnhCT. In some experiments, capillary pipets whose ends were sealed by flaming were used to mix solutions within the cuvette, and the same capillary was used for mixing all serial additions to a given cuvette. In later experiments, which gave comparable results, a micro magnetic stirring bar was placed directly within the cuvette, and mixing was achieved by the motion of an external magnet.

The relative molar fluorescence intensities of bound and free FAnhCT were determined on a Perkin-Elmer 650-105 fluorescence spectrophotometer by measuring the fluorescence intensities of a 1-mL solution of 24 nM FAnhCT in Tris buffer (490-nm excitation; 520-nm emission; 5-nm band-passes) and then adding 100- and 400-μL additions of buffer alone or of 5.2 μM α_1PI (MM or ZZ type) in the same buffer. An analogous procedure was used for FMeCT.

To be certain that an equilibrium approach was correct for measuring the interaction of α_1PI with FAnhCT or FMeCT, we conducted reversibility experiments in which 1 μM α_1PI was incubated with 1 μM FAnhCT or FMeCT to allow complex formation. After P_m values were measured, the solutions were diluted 10-fold and the polarization was measured immediately (within 10 s of dilution). These P_m values were compared to the polarization of control solutions containing 0.1 μM α_1PI (MM or ZZ type) and 0.1 μM FMeCT or FAnhCT.

K_{assoc} for Binding of FAnhCT and FMeCT to α_1PI (MM and ZZ Type). The derivation given below closely follows that given by Dandliker et al. (1964) for use of fluorescence polarization in the determination of equilibrium binding constants. If the labeled molecule is assumed to be in two distinct states (free and bound), then the measured polarization P_m is defined as the weighted average of the polarization due to the bound and free species.

$$P_m = \frac{P_b Q_b F_b + P_f Q_f F_f}{Q_b F_b + Q_f F_f} \quad (1)$$

Q = molar fluorescence, P = polarization, F = molar concentration of fluorescent material, and subscripts f and b refer to the free and bound species. If $Q_b/Q_f = 1$ (see Results), then

$$P_m = \frac{P_b F_b + P_f F_f}{F_b + F_f} \quad (2)$$

Let $M = F_b + F_f$ = total concentration of labeled material. Then

$$F_b = \frac{M(P_m - P_f)}{P_b - P_f} \quad (3)$$

$$\frac{F_b}{F_f} = \frac{P_m - P_f}{P_b - P_m} \quad (4)$$

Table I: K_{assoc} and Number of Binding Sites, n, for the Interaction of CT Derivatives with α_1PI Determined by Fluorescence Polarization [a]

	AnhCT		MeCT	
	K_{assoc} (M^{-1})	n	K_{assoc} (M^{-1})	n
α_1PI (MM type)				
Scatchard analysis	$(1.8 \pm 0.7) \times 10^7$	1.3 ± 0.2	$(1.2 \pm 0.03) \times 10^6$	0.87 ± 0.2
dissociation plot	$(2.2 \pm 0.8) \times 10^7$		$(0.96 \pm 0.2) \times 10^6$	
proflavin binding			$(1.0 \pm 0.26) \times 10^6$	
α_1PI (ZZ type)				
Scatchard analysis	$(0.93 \pm 0.21) \times 10^7$	0.88 ± 0.1	$(1.8 \pm 0.3) \times 10^6$	0.72 ± 0.03
dissociation plot	$(1.0 \pm 0.2) \times 10^7$		$(1.1 \pm 0.3) \times 10^6$	
α_1PI (MM type), oxidized				
Scatchard analysis	$(6.5 \pm 4.2) \times 10^5$	0.46 ± 0.2	$<1 \times 10^4$	not determined
dissociation plot	$(2.8 \pm 0.1) \times 10^5$		not determined	

[a] Values represent the mean ± the standard deviation of at least three determinations.

Equations 3 and 4 in conjunction with the linear form of the mass action law introduced by Scatchard (1949) can be used to determine K_{assoc} and $F_{b,max}$ ($F_{b,max}$, the maximum value of F_b, is taken to be equal to the molar concentration of binding sites in α_1PI).

$$F_b/F_f = K_{assoc}F_{b,max} - K_{assoc}F_b \quad (5)$$

An alternative plot can be derived from the definition of the dissociation constant for the interaction of two species involved in 1:1 binding where

$$K_{dissoc} = \frac{F_f[\alpha_1PI]_f}{F_b} \quad (6)$$

$$1 + \frac{K_{dissoc}}{[\alpha_1PI]_f} = \frac{F_f + F_b}{F_b} \quad (7)$$

Substituting from eq 3 and 4

$$\frac{F_f + F_b}{F_b} = \frac{P_b - P_f}{P_m - P_f} = 1 + \frac{K_{dissoc}}{[\alpha_1PI]_f} \quad (8)$$

Competition with Unlabeled MeCT and AnhCT. For determination of the effect of the fluorescein label on K_{assoc}, α_1PI (MM type) was added to solutions with different ratios of labeled/unlabeled AnhCT (or MeCT), the polarization was measured, and the data were analyzed by the method of Dandliker et al. (1978) to determine the association constant for the unlabeled material.

Proflavin Binding. Binding of MeCT to α_1PI was also measured by the proflavin displacement method originally described by Brandt et al. (1967) by using a Cary Model 16 spectrophotometer with a wavelength scanning attachment and water-jacket cuvette holders (held at 37 °C). This procedure is based on determining the decrease in ΔA at 465 nm due to the competitive displacement of proflavin from the active site by α_1PI. The equations of Brandt et al. (1967) were modified for use at concentrations of α_1PI not in excess over those of the other components.

Results

Relative Molar Fluorescence Intensities for Bound and Free FAnhCT. When 1-mL solutions of 24 nM FAnhCT were diluted with buffer or a 108-fold molar excess of α_1PI (MM or ZZ type), there was essentially no difference in the resulting fluorescence intensities. Since most of the FAnhCT is bound to α_1PI under these conditions, this indicates that the relative molar fluorescence intensities of bound and free FAnhCT are identical within experimental error. FMeCT was also found to have the same molar fluorescence intensity in the bound and free state. Since $Q_b/Q_f = 1$, then eq 2 can be applied for the analysis of polarization measurements.

Determination of K_{assoc} Using Fluorescence Polarization. Both the K_{assoc} and the total number of binding sites are obtained from a Scatchard plot. To evaluate F_b and F_b/F_f from P_m, using eq 4 and 5, one needs to know P_f and P_b. P_f is easily determined experimentally since it is the polarization of the labeled material prior to addition of any α_1PI. P_b is obtained by an extrapolation to a state where all labeled material is bound. From eq 5, as $F_b \rightarrow 0$, then $F_b/F_f \rightarrow K_{assoc}F_{b,max}$ and let $P_m \rightarrow P'$. With these substitutions in eq 4, by rearrangement one obtains

$$P' = P_b - \frac{P' - P_f}{K_{assoc}F_{b,max}}$$

Since $F_{b,max}$ is the total concentration of α_1PI, P_b is the intercept as $[(P' - P_f)[\alpha_1PI]^{-1}] \rightarrow 0$. The results of a representative binding experiment are shown in Figures 1–4, where the interaction of FMeCT with α_1PI (MM type) was studied. The normalized P_m values are plotted vs. [FMeCT] for a series of α_1PI (MM type) concentrations in Figure 1. The extrapolated polarizations at zero [FMeCT] are denoted P'. These P' values are then plotted vs. $(P' - P_f)/[\alpha_1PI]$, and the intercept as $1/[\alpha_1PI] \rightarrow 0$ is P_b (Figure 2). A Scatchard plot of these data by using eq 5 (Figure 3) yields a $K_{assoc} = 1.2 \times 10^6$ M^{-1} with 0.80 binding site. A similar value of $K_{assoc} = 0.86 \times 10^6$ M^{-1} is obtained when the data are plotted according to eq 8 (Figure 4).

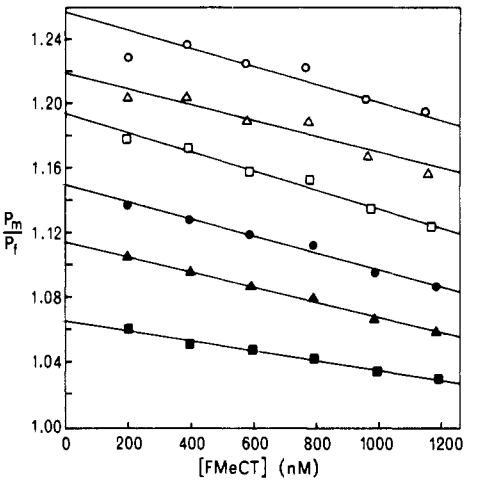

FIGURE 1: Normalized polarization as a function of [FMeCT] for different concentrations of α_1PI (MM type): 1600 nM (○), 1200 nM (△), 900 nM (□), 600 nM (●), 400 nM (▲), and 200 nM (■). Intercepts were calculated and lines projected by linear regression. P' is the limit of P_m at [FMeCT] → 0 for each α_1PI concentration.

Table I gives the values for the K_{assoc} and number of binding sites for the interaction of modified CT's (AnhCT; MeCT) with α_1PI (MM type, ZZ type, and oxidized MM type) as determined by fluorescence polarization. The table also includes the K_{assoc} determined by the proflavin displacement

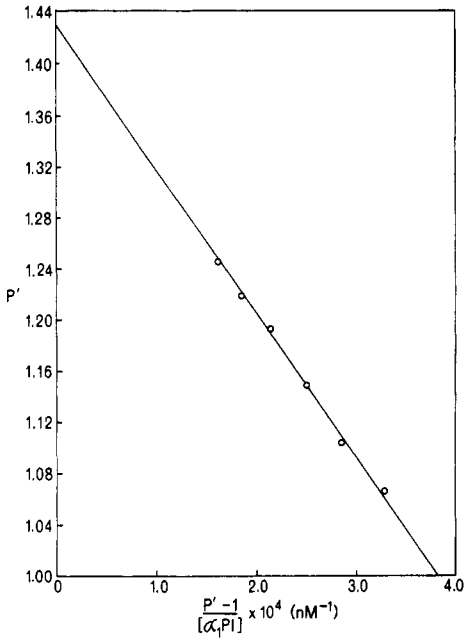

FIGURE 2: Measurement of P_b for FMeCT and α_1PI (MM type). The intercept was calculated and the line projected by linear regression to give P_b = 1.430.

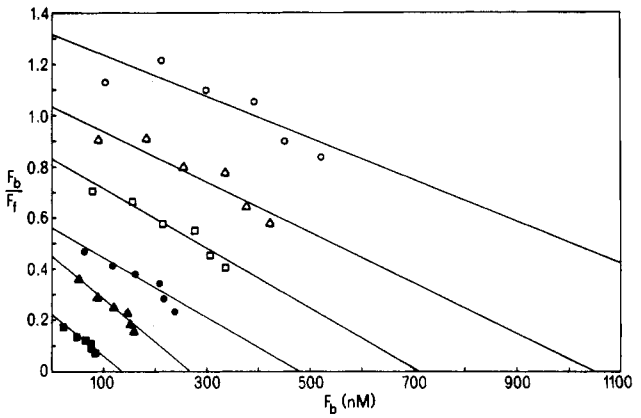

FIGURE 3: Scatchard plot of binding data for FMeCT and α_1PT (MM type). Slopes and intercepts were calculated and the lines projected by linear regression to give K_{assoc} = 1.2 × 10^6 M^{-1} and 0.80 binding site. The symbols are the same as those used in Figure 1.

method for MeCT and α_1PI (MM type). The binding constant obtained for MeCT by these two independent procedures was very similar. The dye displacement technique could not be employed with AnhCT because of the higher affinity of this protein for α_1PI.

Reversibility. When α_1PI (MM or ZZ type) and FMeCT or FAnhCT solutions were allowed to form complexes and then diluted 10-fold, the polarization decreased to the polarization of solutions containing 10-fold less α_1PI (MM and ZZ type) and FAnhCT or FMeCT, indicating that the interaction between α_1PI and FMeCT and FAnhCT is reversible. The final polarization value was reached within approximately 10 s of dilution, which represents the minimum time required to get a measurement (i.e., dilution, mixing, and replacement of the cuvette in the instrument).

Competition with Unlabeled MeCT and AnhCT. For determination of the effect of the fluorescein label on K_{assoc}, α_1PI (MM type) was added to a series of AnhCT or MeCT solutions containing both labeled and unlabeled material. Each solution had the same total concentration of modified CT; however, the ratio of labeled/unlabeled material was varied between 1:1 and 1:9. The polarization was measured before

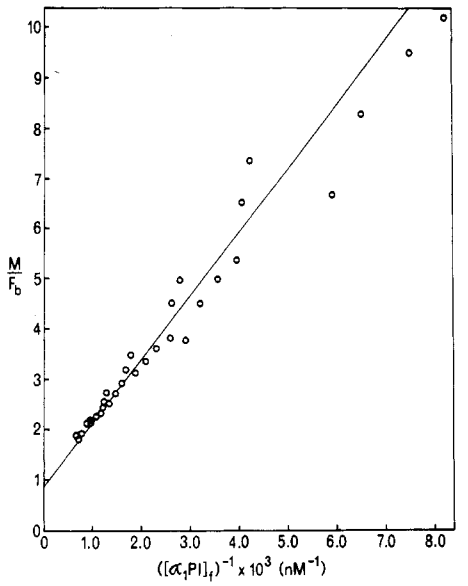

FIGURE 4: Dissociation plot of binding data for FMeCT and α_1PI (MM type). The slope was calculated and the line projected by linear regression to give K_{assoc} = 0.86 × 10^6 M^{-1}.

and after addition of α_1PI. The data were analyzed by the method of Dandliker et al. (1978) with the assumption that the unlabeled protein (AnhCT or MeCT) is a competitive inhibitor for the interaction of the corresponding labeled protein with α_1PI. The association constant for the unlabeled protein, K_N, is obtained from

$$K_N = \frac{K_{assoc}[F_{b,max} - F_b - [F_b/(F_f K_{assoc})]]}{(F_b/F_f)[W - [F_{b,max} - F_b - [F_b/(F_f K_{assoc})]]]} \quad (9)$$

where W is the total concentration of unlabeled protein. Scatchard plots of data obtained in the absence of unlabeled CT derivatives were used to determine the constants K_{assoc} and $F_{b,max}$. The results of these competition experiments indicate a small but measurable decrease in binding affinity as a consequence of fluorescein labeling. With AnhCT, the K_{assoc} for the unlabeled material was 3.4 × 10^7 M^{-1} (vs. 1.8 × 10^7 M^{-1} for FAnhCT) while the value for MeCT was found to be 1.7 × 10^6 M^{-1} (vs. 1.2 × 10^6 M^{-1} for FMeCT).

Discussion

The interaction of proteases with protein protease inhibitors has been under active investigation for many years. The detailed structure of the complexes has remained controversial, although recent evidence tends to rule out certain previously proposed structures. Beginning with the report of Finkenstadt & Laskowski (1965) suggesting that the interaction of trypsin with soybean trypsin inhibitor involved an acyl-enzyme inhibitor linkage, considerable experimental data were obtained that supported the notion that an acyl linkage can serve a stabilizing role in complex formation (Laskowki & Sealock, 1971). Initial X-ray crystallographic data suggested that the carbonyl carbon in the reactive site residue of the inhibitor is in a tetrahedral conformation in the complex, leading to the proposal that the tetrahedral intermediate in peptide bond hydrolysis is stabilized in the enzyme-inhibitor complex (Ruhlman et al., 1973; Blow et al., 1972; Sweet et al., 1974). More recent, refined X-ray data on the trypsin–pancreatic trypsin inhibitor complex suggest, however, that the reactive site carbonyl carbon adopts a conformation intermediate between trigonal and tetrahedral and that the oxygen of the trypsin active site serine is 2.6 Å from the reactive site carbonyl carbon in the inhibitor, which is too great a distance for co-

valent bond formation (Huber et al., 1974; Huber & Bode, 1978). Recent data, based on NMR studies of complexes of trypsin with soybean trypsin inhibitor and pancreatic trypsin inhibitor, in which the reactive site carbonyl carbons were selectively enriched with ^{13}C by semisynthetic methods, do not support the hypothesis that this carbon is in a tetrahedral conformation (Richarz et al., 1980; Baillargeon et al., 1980).

There is substantial thermodynamic and kinetic evidence demonstrating that proteases which have been chemically modified such that they cannot be acylated nevertheless bind nonplasma protease inhibitors with high affinity. These results generally point to the conclusion that the active site serine does not contribute greatly to the stability of the complex. Thus Ako et al. (1974), using an equilibrium competitive binding technique, demonstrated that potato inhibitor I and pancreatic trypsin inhibitor each bind with equal avidity to AnhCT and CT. In addition, it was shown that lima bean inhibitor forms a complex with AnhCT that is actually more stable by 3 kcal than the corresponding complex with the native enzyme. These authors also studied the interaction of trypsin and anhydrotrypsin with soybean trypsin inhibitor, lima bean inhibitor, and chicken ovomucoid. The ratio of equilibrium constants indicated less stability with anhydrotrypsin in each case, but the inactive derivative was able to compete with trypsin for the inhibitor to some extent. Vincent et al. (1974) demonstrated, however, that anhydrotrypsin forms an extremely stable complex with bovine pancreatic trypsin inhibitor, the K_{dissoc} being nearly as small as that for the native enzyme. Similar results were obtained with MeCT by Ryan & Feeney (1975). They showed that the equilibrium constant K_{assoc} for the interaction of MeCT with turkey ovomucoid was only 13-fold less than the value for the native protein. The half-lives of the respective complexes were estimated to be 8 and 13 min, respectively. The above results provide substantial evidence against the notion that an acyl-enzyme intermediate contributes substantially to the strength of interaction of trypsin and CT with the protease inhibitors described above. Thus the stability of the well-characterized protease-inhibitor complexes may be primarily due to a summation of multiple noncovalent contacts, as shown by the X-ray crystal structure of the trypsin–pancreatic trypsin inhibitor complex (Ruhlmann et al., 1973).

The present report provides evidence that two active site modified derivatives of bovine CT, MeCT and AnhCT, form moderately stable complexes with α_1PI. However, experiments in which preformed complex was rapidly diluted demonstrated in each case that complexes of α_1PI with both MeCT and AnhCT dissociate to a new equilibrium concentration at a rate that is too fast to be observed with the instrumentation used in this study. The instability of the complexes of the two CT derivatives with α_1PI is in marked contrast to results obtained previously on the reaction of chymotrypsin and chymotrypsinogen with the inhibitor. Both proteins form extremely stable complexes with α_1PI that are resistant to denaturation by sodium dodecyl sulfate and β-mercaptoethanol and cannot otherwise be dissociated at pH values near neutrality without the addition of benzamidine (Johnson & Travis, 1976) or hydroxylamine (Largman et al., 1979). Dissociation results in cleavage of α_1PI into two polypeptide fragments, one of which contains the reactive site Met of α_1PI at its C terminus (Johnson & Travis, 1978), suggesting that the active site serine may form an acyl-enzyme or tetrahedral structure in these complexes.

We were unable to determine rate constants for either formation or dissociation of complexes of α_1PI with either CT derivative employed in this study. However, our data can be employed to argue that the forward reaction rate of each derivative with the inhibitor is comparable to that of CT. Since the complex of AnhCT with α_1PI dissociates to a new equilibrium point within 10 s after dilution, the half-life for dissociation of the AnhCT complex must be less than a few seconds. The relationship

$$t_{1/2} = \ln 2/k_{dissoc}$$

suggests that the rate constant k_{dissoc} is in the range of 0.1–1 s^{-1}. The definition

$$K_{assoc} = k_{assoc}/k_{dissoc}$$

therefore indicates that k_{assoc} is on the order of 10^6–10^7 M^{-1} s^{-1} for the interaction of AnhCT with α_1PT which, with the qualification of difference in temperature of observation, is comparable to the rate constant reported by Beatty et al. (1980) for the reaction of CT with α_1PI ($k_{assoc} = 5.9 \times 10^6$ M^{-1} s^{-1}; 25 °C). A similar argument can be made with respect to the rate of association of MeCT with α_1PT.

Kinetic investigations of the reaction of bovine trypsin with soybean trypsin inhibitor (Luthy et al., 1973) and of CT with bovine pancreatic trypsin inhibitor (Quast et al., 1974) have demonstrated that the enzyme–inhibitor reaction is best described as a two-step process with initial formation of a reversible complex that is then converted to a more stable species:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI^* \xrightarrow{k_2} EI$$

Our results are consistent with this type of mechanism for the reaction of α_1PI with CT, CTGN, and the active site modified CT derivatives. For the reaction of CT with α_1PI, k_2 is sufficiently large with respect to the other constants that no kinetic evidence for an intermediate complex has yet been obtained (Beatty et al., 1980). In the case of reaction of CTGN with α_1PI, the rate is sufficiently slow that an intermediate should be identifiable by using conventional kinetic methods. In our previous studies (Brodrick et al., 1980), formation of an intermediate reversible complex would have been indicated by a rapid increase in the fluorescence polarization upon mixing the reactants. However, no such change was detected. This finding supports our previous hypothesis that the slow rate of reaction of chymotrypsinogen with α_1PI probably reflects the small fraction of zymogen molecules in a reactive conformation. On the other hand, the characteristics of the interaction of MeCT and AnhCT with α_1PI are consistent with rapid formation of the reversible, intermediate complex but an inability of the intermediate complex to convert to a stable species. Both derivatives are very similar in structure to CT, with only minor alterations in functional groups at the active site that nevertheless markedly affect the integrity of the active site. The differences in the characteristics of interaction of these derivatives with α_1PI, when compared to those of CT and CTGN, clearly indicate that an intact active site is a prerequisite for formation of a stable complex between serine proteases and α_1PI. This finding suggests that there is a significant difference in the mechanism of interaction of α_1PI with serine proteases compared to the interaction of these enzymes with other well-characterized protein protease inhibitors.

Our results indicate that AnhCT binds somewhat more strongly to α_1PI, by about 1 order of magnitude, than does MeCT. This may be attributed to adverse steric effects imposed on the active site of CT by the introduction of the methyl group into His_{57}, which may directly hinder close contact and/or proper orientation of the His side chain with a complementary site on the inhibitor. Our results also demonstrate

that the integrity of the inhibitory site of α_1PI contributes greatly to the strength of the reversible binding observed with AnhCT or MeCT. Oxidation with *N*-chlorosuccinimide, which has been shown to alter a Met residue in the inhibitory site of α_1PI (Johnson & Travis, 1978, 1979), greatly decreases the rate of reaction of CT with α_1PI (Beatty et al., 1980). Our data show that oxidation of α_1PI by this procedure strongly reduces the strength of binding of the inhibitor to either CT derivative.

Both the common allelic form of α_1PI (Pi type MM) and the ZZ variant, which has a single amino acid substitution [Lys for Glu (Jeppson, 1976)], were employed in these studies. In previous studies using circular dichroism and fluorescence spectroscopy with various quenching agents (Glaser & Karic, 1978), we have demonstrated a conformational difference between the two forms of the inhibitor. However, little difference was observed in their binding to either CT derivative, indicating that the conformational change induced in the ZZ form probably does not involve the inhibitory site of α_1PI.

In these studies, we have relied primarily on the technique of fluorescence polarization to follow a macromolecular interaction in which the complex has a significantly higher molecular weight than the labeled probe molecule. The introduction of a fluorescent label is required to study molecular interactions using this technique unless one of the interacting species has natural fluorescence. Since the biological properties of the unlabeled molecule are actually those of interest, the label must be shown not to adversely interfere with these properties. The competition experiments reported here show that the introduction of an FITC label on AnhCT or MeCT results in only a minor decrease in the ability of either derivative to bind to α_1PI. Additional evidence that the FITC moiety does not greatly interfere in the association of α_1PI with the CT derivatives employed in this study is provided by the finding that the fluorescent intensity of each derivative is the same in the bound and the unbound state.

Our results demonstrate the great potential inherent in the use of fluorescence polarization in the study of macromolecular interactions. The procedure is technically easy to apply and has the unique feature that polarization data are largely independent of concentration and depend directly only on the average molceular weight of the fluorescent probe as derived from the average rotational relaxation time.

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