Dynamics of Ligand Binding to α-Chymotrypsin and to *N*-Methyl-α-chymotrypsin†

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ABSTRACT: K_s values for binding of selected substrates, competitive inhibitors, and a noncompetitive inhibitor were found to be similar for α-chymotrypsin and *N*-methyl-α-chymotrypsin. The rates and steps of binding of a competitive inhibitor and a noncompetitive inhibitor were also found to be similar for α-chymotrypsin and *N*-methyl-α-chymotrypsin. Therefore, *N*-methyl-α-chymotrypsin is an appropriate model for α-chymotrypsin in studying the dynamics of the binding of substrates by temperature-jump techniques in aqueous solvents. 2-Toluidinylnaphthalene-6-sulfonate, a noncompetitive inhibitor, bound to α-chymotrypsin in a single step with rate constants k_1 and k_{-1} of 3.9×10^7 M^{-1} s^{-1} and 1.9×10^3 s^{-1}, respectively, at pH 5.0 (0.2 M acetate, ionic strength of 0.2). Similar values were obtained for *N*-methyl-α-chymotrypsin and chymotrypsinogen A at pH 5.0 and for α-chymotrypsin at pH 7.8 [0.1 M tris(hydroxymethyl)aminomethane–0.03 M $CaCl_2$]. Indole, a competitive inhibitor, bound to α-chymotrypsin in a single step at pH 5.0 and 7.8, with k_1 and k_{-1} of 1.8×10^7 M^{-1} s^{-1} and 7.8×10^3 s^{-1}, respectively, at pH 5.0 while proflavin, another competitive inhibitor, bound to α-chymotrypsin with two observable steps where k_1, k_{-1}, k_2, and k_{-2} were 1.0×10^7 M^{-1} s^{-1}, 7×10^2 s^{-1}, 1.0×10^3 s^{-1}, and 7×10^2 s^{-1}, respectively, at pH 5.0. The specific substrate *N*-acetyl-L-3,5-dinitrotyrosine ethyl ester bound to *N*-methyl-α-chymotrypsin at pH 5.0 in three observable steps where k_1, k_{-1}, k_2, k_{-2}, k_3, and k_{-3} were 3.7×10^7 M^{-1} s^{-1}, 6.2×10^4 s^{-1}, 1.2×10^3 s^{-1}, 3.5×10^2 s^{-1}, 3×10^2 s^{-1}, and 4×10^2 s^{-1}, respectively. Preliminary data indicated that the third step of this reaction is probably absent when Met_{192} of *N*-methyl-α-chymotrypsin is oxidized to methionine sulfoxide. These results confirm the validity of data obtained from reactions at subzero temperatures in 65% dimethyl sulfoxide in indicating multiple steps in the binding of substrates to α-chymotrypsin. The methodology described should make it possible to measure quantitatively the contribution of the binding process to enzyme catalysis (the Circe effect).

The accepted mechanism of α-chymotrypsin-catalyzed hydrolyses of proteins and synthetic substrates is described by eq 1 where E is chymotrypsin, S is the substrate, E·S is the

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES' + P_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P_2 \quad (1)$$

noncovalent Michaelis complex formed between chymotrypsin and substrate, ES' is the covalent acyl-enzyme intermediate, P_1 is the leaving group of the substrate (alcohol or amine), and P_2 is the acid portion of the substrate. The chemical steps involved in chymotrypsin-catalyzed hydrolyses are well described (Bender & Killheffer, 1973; Kraut, 1977) and generally accepted. However, relatively little is known of the dynamic details of binding of substrate with chymotrypsin (k_1 and k_{-1} steps above).

Mechanisms common both to ordinary chemical catalysts and to enzymes, such as nucleophilic and electrophilic or general acid and general base catalysis, only partially explain the tremendous rate accelerations of reactions observed with enzymes. Therefore, a major role of the enzyme–substrate complex in this rate acceleration is generally assumed (Wolfenden, 1972; Lienhard, 1973; Jencks, 1976; Kraut, 1977; DeTar, 1981). It then becomes essential to understand the dynamic nature of the step(s) involved in the combination of enzyme with substrate if the complete mechanism of any enzyme-catalyzed reaction is to be understood.

Static descriptions of chymotrypsin–ligand complexes include X-ray crystallographic results on at least 40 compounds (Steitz et al., 1969; Henderson, 1970; Segal et al., 1971; Blow, 1976; Tulinsky et al., 1973; Fersht, 1977; Kraut, 1977) and equilibrium binding and Michaelis constants for probably more than a 1000 compounds (Hein & Niemann, 1961; Wallace et al., 1963; Ingles & Knowles, 1968; Cohen et al., 1969; Segal, 1972; Bender & Killheffer, 1973; Coll & Whitaker, 1979; Ohno & Karasaki, 1979) and with more than 30 different chemically modified chymotrypsins and chymotrypsinogens (Nakagawa & Bender, 1970; Hess, 1971; Horbett & Teller, 1973; Bender & Killheffer, 1973; Ako et al., 1974; Treadway & Schultz, 1976; Chen et al., 1979; Landis & Berliner, 1980a,b). Therefore, a reasonably detailed description of the Michaelis complex has evolved.

In contrast to the wealth of information on the static nature of the Michaelis complex, few investigations have dealt with the dynamics of the complexation reaction. Fersht, in 1977, reported data on the direct measurement in aqueous solution of the kinetics of binding of only one inhibitor (Havsteen, 1967), one "virtual" substrate (Smallcombe et al., 1972), and one substrate (Hess et al., 1970). The maximum calculated value for one additional substrate was reported (Renard & Fersht, 1973). At subzero temperatures, several (noncovalent) binding steps were resolved that suggested that conformational changes occur in enzyme and/or substrate upon complexation (Fink, 1976a,b, 1977).

While there is considerable evidence supporting conformational changes in chymotrypsin on adding ligands (Labouesse et al., 1962; Hess et al., 1970; Yapel & Lumry, 1964; Himoe et al., 1967; Henderson, 1970; McClure & Edelman, 1967; Edelman & McClure, 1968), relatively few of these investigations establish the direct involvement of the conformational change in the reaction pathway and even fewer directly link the conformational change to the noncovalent association of enzyme and substrate or inhibitor. The majority of reports of altered CD[1] (Hess et al., 1970), ORD and UV

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spectra (Labouesse et al., 1962) are concerned with either the now well-characterized alkaline pH transition (Fersht & Requena, 1971a; Kim & Lumry, 1971; Stoesz & Lumry, 1978) or the influence of covalent intermediate formation (Labouesse et al., 1962; Wootton & Hess, 1962; Havsteen & Hess, 1963; Moon et al., 1965).

Because of the critical importance to the catalytic mechanism of the combination of enzyme with substrate, because of the deficiency of direct data on the dynamics of this combination, and because of the uncertainty in extrapolating the results obtained in dimethyl sulfoxide at subzero temperatures to aqueous systems at ambient temperatues (Fink, 1976a,b, 1977), we have investigated in detail the kinetics of combination of ligands with α-chymotrypsin and *N*-methyl-α-chymotrypsin by temperature jump. We believe these results have direct application to the mechanism of α-chymotrypsin.

Materials and Methods

Materials

α-Chymotrypsin (3 times crystallized) was from Worthington Biochemical Corp. (lots CDI-34S-895 and CDI-36S-771) and Sigma Chemical Co. (lots 58C-8135 and 36C-8095). The Sigma enzyme was purified on a Sephadex G-25 column to remove peptide impurities prior to use in kinetic experiments. The Worthington enzyme was shown to be free of peptide impurities by gel filtration and by the temperature-jump method of Yapel et al. (1966).

Chymotrypsinogen A (Sigma Chemical Co., 6 times crystallized, lot 75C-8380), initially with 0.37 unit/mg chymotrypsin activity, was used as a control in kinetic experiments after incubation with phenylmethanesulfonyl fluoride (PMSF; 1 mM) at pH 7.8 (0.1 M Tris–0.03 M $CaCl_2$) for 30 min at room temperature or until no chymotrypsin activity was detectable.

Benzoyl-L-tyrosine ethyl ester (BTEE; Nutritional Biochemical Corp., lot 8796) was used without further purification. Proflavin sulfate (Mann Research Laboratories, lot T2828) was recrystallized twice from boiling water in the dark and stored in the dark over a desiccant. Phenylmethanesulfonyl fluoride (lot 64C-0355), 2-toluidinylnaphthalene-6-sulfonate (TNS), and *N*-acetyl-L-3,5-dinitrotyrosine ethyl ester (lot A7750) were from Sigma Chemical Co. The TNS was recrystallized from water and protected from light. Methyl *p*-nitrobenzenesulfonate (lot 691), 5-nitro-3*H*-1,2-benzoxathiole 2,2-dioxide (2-hydroxy-5-nitrotoluenesulfonic acid sultone; lot 671-AI), and phenolsulfonphthalein (Phenol Red; lot 541) were from Eastman Organic Chemicals. The 2-hydroxy-5-nitrotoluenesulfonic acid sultone was recrystallized from ethanol and was stored desiccated in the dark. Phenol Red was recrystallized before use (Orndorff & Sherwood, 1923). Indole (99%, lot D11467) was from Aldrich. The indole was recrystallized from water. *N*-Acetyl-L-tryptophan *p*-nitrophenyl ester (lot H-4285) was from Vega Fox and was stored desiccated at −10 °C. *N*-Formyl-, *N*-carbomethoxy-, *N*-chloro-, *N*-dichloro-, and *N*-trifluoro-L-tyrosine ethyl esters and L-tyrosine ethyl ester were gifts from R. J. Coll and synthesized as described (Coll & Whitaker, 1979). All other reagents were of reagent grade.

Methods

All dialyses were performed in large-diameter (4.8-cm) cellophane tubing in order to minimize protein losses (McPhie, 1971).

Turkey Ovomucoid. Turkey ovomucoid was purified by a modification of the procedures of Rhodes et al. (1958, 1960). Egg white (175 mL), dialyzed against 0.1 N acetate buffer, pH 4.5, was mixed with 46 g of DEAE-cellulose previously equilibrated with the same buffer. The DEAE-cellulose–protein complex was washed with 1.5 L of the pH 4.5 buffer to elute most of the egg white proteins. The ovomucoid was eluted by adjusting the pH to 3.6. The eluant was added to CM-cellulose previously equilibrated at pH 3.6 (0.1 M sodium acetate buffer), the CM-cellulose–protein complex washed with 1 L of the pH 3.6 buffer, and the ovomucoid eluted by adjusting the pH to 4.35. The eluant was dialyzed against deionized water and lyophilized to give 900 mg of ovomucoid.

Turkey Ovomucoid–Sepharose 4B. Turkey ovomucoid was covalently coupled to Sepharose 4B via cyanogen bromide activation according to the procedure of Ryan & Feeney (1975), except the coupling step with ovomucoid was performed in pH 7.5 phosphate buffer rather than at pH 9. Approximately 70% of the added turkey ovomucoid was bound, as estimated by the 280-nm absorbance of the washings, with 23% active ovomucoid, as estimated by overloading a small column with α-chymotrypsin. Coupling at pH 9 gave more protein bound but with less activity.

N-Methyl-α-chymotrypsin. One- or two-gram batches of *N*-methyl-α-chymotrypsin were prepared by the method of Ryan & Feeney (1975) with suitable modifications needed for the large batches, reduction of the residual chymotrypsin activity to below 0.01%, and for separation of any peptide impurities. The reaction with methyl *p*-nitrobenzenesulfonate was allowed to proceed until approximately 50% of the chymotrypsin activity was lost, as determined by titration of aliquots with 2-hydroxy-5-nitrotoluenesulfonic acid sultone (Kézdy & Kaiser, 1970). Phenylmethanesulfonyl fluoride was then added and the reaction continued until no chymotrypsin activity was detectable with benzoyl-L-tyrosine ethyl ester (Walsh & Wilcox, 1970) or with *N*-acetyl-L-tryptophan *p*-nitrophenyl ester (Henderson, 1971; preferred method). The mixture of *N*-methyl-α-chymotrypsin and [(phenylmethyl)-sulfonyl]-α-chymotrypsin was separated from modifying reagents and equilibrated with HCl, at pH 4.0, either by dialysis or on a column of Sephadex G-25. The product was lyophilized and stored desiccated at −10 °C.

The mixture of *N*-methyl-α-chymotrypsin and [(phenylmethyl)]sulfonyl-α-chymotrypsin was again treated with phenylmethanesulfonyl fluoride just prior to affinity chromatography to modify any reactivated (desulfonated) [(phenylmethyl)sulfonyl]-α-chymotrypsin. Separation by affinity chromatography was carried out according to Ryan & Feeney (1975) except that the starting buffer was at pH 5.0 (0.025 M acetate–0.2 M KCl) and the eluting solution was deionized water adjusted to pH 2.85 with HCl. The purified proteins were then dialyzed against HCl at pH 4.0 and lyophilized.

When the purified *N*-methyl-α-chymotrypsin still had detectable residual chymotrypsin activity (by *N*-acetyl-L-tryptophan *p*-nitrophenyl ester), just prior to a kinetic determination, the material was again incubated with phenylmethanesulfonyl fluoride (pH 7.8, 0.1 M Tris–0.03 M $CaCl_2$ at room temperature) until no detectable activity was present (<0.02% chymotrypsin) and then rapidly passed through a preequilibrated Sephadex G-10 column to purify the protein and change to the desired buffer. The presence of peptides was checked also just prior to use by the Phenol Red method (Yapel et al., 1966), and they were removed on Sephadex G-25 when necessary.

[1] Abbreviations: CD, circular dichroism; ORD, optical rotatory dispersion; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; T jump, temperature jump; NMR, nuclear magnetic resonance.

Operational Normality of N-Methyl-α-chymotrypsin and α-Chymotrypsin. The active site concentration of chymotrypsin was determined by titration with 2-hydroxy-5-nitrotoluenesulfonic acid sultone (Kézdy & Kaiser, 1970). The active site concentration of *N*-methyl-α-chymotrypsin was determined routinely by titration with *N*-acetyl-L-tryptophan *p*-nitrophenyl ester (Henderson, 1971). To ensure that the burst of *p*-nitrophenolate represented 100% of the active sites (Kézdy & Kaiser, 1970), we also determined *N*-methyl-α-chymotrypsin normality by proflavin displacement with a previously standardized (against standardized α-chymotrypsin) solution of turkey ovomucoid. Since both methods gave equivalent results, and since *N*-acetyl-L-tryptophan *p*-nitrophenyl ester displaces 100% of proflavin from *N*-methyl-α-chymotrypsin, the *p*-nitrophenolate burst must give an accurate measure of the active concentration of *N*-methyl-α-chymotrypsin.

Binding of Proflavin to α-Chymotrypsin and N-Methyl-α-chymotrypsin. The dissociation constant, K_s^{PF}, for proflavin binding to either α-chymotrypsin or *N*-methyl-α-chymotrypsin was determined by the method of Bernhard et al. (1966) in a thermostated Cary 118C spectrophotometer at 10 or 25 °C and pH 5.0 (0.2 M acetate, ionic strength of 0.2). The varying proflavin concentrations were kept in large excess of protein concentration, but well below the proflavin dimer dissociation constant of 2.5 mM (Turner et al., 1972). Proflavin was determined to obey the Beer–Lambert law up to 100 μM, the highest concentration of proflavin used in this work.

Determination of K_s for Binding of Substrates to N-Methyl-α-chymotrypsin. Substrate binding by *N*-methyl-α-chymotrypsin was determined by proflavin displacement (Brandt et al., 1967) at pH 5.0 (0.2 M acetate, ionic strength of 0.2) in a thermostated Cary 118C spectrophotometer. In most cases the enzyme and proflavin were held constant such that [*N*-methyl-α-chymotrypsin] ≪ [proflavin] ≪ [S]. Control experiments were done to correct for binding of proflavin by substrates. *N*-Formyl-L-tyrosine ethyl ester and L-tyrosine ethyl ester were determined to have $K_s^{PF\cdot S}$ of 42 and 130 mM, respectively. The other substrates showed negligible proflavin binding. In an alternative procedure with *N*-carbomethoxy-L-tyrosine ethyl ester, the proflavin concentration was varied with constant substrate and enzyme concentrations so that [*N*-methyl-α-chymotrypsin] ≪ [proflavin] ≪ [S]. Each set of data was analyzed according to Wilkinson's bilinear regression equation (Wilkinson, 1961).

Determination of K_m and k_{cat} for α-Chymotrypsin-Catalyzed Hydrolysis of N-Acetyl-L-3,5-dinitrotyrosine Ethyl Ester. The reactions, at 26.5 °C and pH 5.0 in acetate buffer of ionic strength of 0.2, contained 90 μM α-chymotrypsin and 0.693–6.93 mM *N*-acetyl-L-3,5-dinitrotyrosine ethyl ester. Aliquots were removed at various times near the beginning of the reaction, and the ethanol concentration produced was measured with a Boehringer-Mannheim kit (lot no. 1261229) containing alcohol dehydrogenase, aldehyde dehydrogenase, and NAD^+, under the conditions described by the supplier. Quantitation of the assay was checked by using standard ethanol solutions as well as after complete hydrolysis of *N*-acetyl-L-3,5-dinitrotyrosine ethyl ester. The initial velocities, v_0, were plotted vs. substrate concentration by the Lineweaver–Burk method to obtain K_m and k_{cat}.

T-Jump Experiments. The T-jump experiments were carried out on a type TJ 50A-001 T-jump instrument from Studiengesellschaft mblt at pH 5.0 (0.2 M acetate, ionic strength of 0.2) or pH 7.8 (0.1 M Tris–0.03 M $CaCl_2$ and 0.1 M KCl). The cell held 7 mL of solution but heated only about 1 mL. A 40-kV discharge gave approximately an 8 °C rise in less than 2 μs as checked with a 100 μM Phenol Red solution in 0.2 M Tris buffer, pH 7.8, initially at 10 °C (Czerlinski, 1966). The rise time of each of the signal amplification circuits was also checked with the Phenol Red solution. The time constant of the circuit was kept at least 10 times less than the $t_{1/2}$ of the transient being observed. All jump experiments were performed at an initial temperature of 4 °C and a final temperature of 12 °C.

A tungsten light source was used in most experiments. A xenon lamp was used for fluorescence measurements, with an RCA type 1P28 photomultiplier tube at a 90° angle from the input light and 30 cm from the T-jump cell to minimize pickup of radiation emitted by the high voltage discharge. A circuit similar to that of French & Hammes (1969) was constructed for amplification of the photomultiplier signal. Since photocathode saturation was not a problem in the fluorescence experiments, the last three dynodes were not tied at the same potential as in their circuit. An RC filter was also added to their circuit to maximize the signal to noise ratio.

The signal was recorded on a storage oscilloscope (Tektronix) and photographed with high contrast copy film. A photographic enlarger was used to trace results from the negatives onto graph paper.

Jumps were repeated four to seven times at each of the reagent concentrations used. The solution was then diluted, and the jumps were repeated to maximize use of reagents. Control experiments in which fresh reagents were jumped at some of the concentrations used gave the same results. Alternatively, a single solution was jumped a large number of times over a period of 1 or 2 h without significant change in results. In experiments with 2-toluidinylnaphthalene-6-sulfonate, the solution was protected from the light source in between jumps.

Because of the several species in the solution, the equilibrium reagent concentrations were calculated by a reiterative program written for a Texas Instrument programmable 58 calculator. In these programs, the α-chymotrypsin inactive conformer (present at pH 5.0 and pH 7.8) and dimer (present at pH 5.0) were assumed not to bind the ligands investigated, and proflavin dimer was assumed not to bind α-chymotrypsin. Additional species of *N*-methyl-α-chymotrypsin and chymotrypsinogen A were not considered. *N*-Methyl-α-chymotrypsin does not dimerize (Neet et al., 1974). The equations and techniques discussed by Czerlinski (1966) or by Bernasconi (1976) were used in analyzing the relaxation data. In the plots presented here all data points are reported as the average plus or minus the standard deviation.

Results and Discussion

The objective of this research has been to investigate the dynamics of substrate binding to α-chymotrypsin, a necessary and important event occurring prior to chemical conversion of substrate to products. Because of the rapidity of enzyme–substrate complexation, fast measurement techniques such as temperature jump are essential, which require, for practical reasons, prior equilibration of the system. Therefore, with substrates we have used *N*-methyl-α-chymotrypsin in place of α-chymotrypsin thereby focusing exclusively on noncovalent binding and avoiding the nonequilibrium of α-chymotrypsin hydrolyses. It then became essential to demonstrate that under the experimental conditions used the binding of ligands to *N*-methyl-α-chymotrypsin and α-chymotrypsin is equivalent.

Binding of Ligands to α-Chymotrypsin and to N-Methyl-α-chymotrypsin. Dissociation constants, K_s, for a number of

Table I: Comparison of K_s for α-Chymotrypsin and *N*-Methyl-α-chymotrypsin with Several Substrates and Inhibitors

compound	$K_s^{N\text{-MCT}}$ [a] (mM)	K_s^{CT} [b] (mM)	reference
substrate			
N-acetyl-L-tyrosine ethyl ester	18 ± 6	10 ± 1	Henderson (1971)[c]
N-carbomethoxy-L-tyrosine ethyl ester	3.1 ± 1.7	6.8 ± 1.8	this work;[d,e] Coll & Whitaker (1979)[f]
N-formyl-L-tyrosine ethyl ester	11 ± 3	8 ± 1	this work;[g] Coll & Whitaker (1979)
N-(chloroacetyl)-L-tyrosine ethyl ester	9.7 ± 2.6	13.3 ± 2.7	this work;[h] Coll & Whitaker (1979)
N-mesyl-L-tyrosine ethyl ester	17 ± 4	1.98 ± 0.07[i]	this work;[j] Coll & Whitaker (1979)
N-(dichloroacetyl)-L-tyrosine ethyl ester	7 ± 4		this work[k]
N-acetyl-L-tyrosine *p*-methoxyanilide	11 ± 2	16 ± 2	Lucas & Caplow (1972)[l]
N-acetyl-L-tyrosine *p*-chloroanilide	13 ± 3	2.6 ± 0.2	Lucas & Caplow (1972)
N-acetyl-L-3,5-dinitrotyrosine ethyl ester	1.8 ± 0.4		this work[m]
L-tyrosine ethyl ester	30 ± 10	36.5 ± 2.8[i]	this work;[n] Coll & Whitaker (1979)
competitive inhibitors			
proflavin	0.0744 ± 0.0076	0.0891 ± 0.013	this work[o]
N-methyl-*N*-acetyl-L-tyrosine methyl ester	1.1	1.8	Byers & Koshland (1978)[p]
hydrocinnamoylnitrile	2.5 ± 0.4	12.5	Byers & Koshland (1978)
N-acetyl-D-tryptophan	7.3 ± 0.6	4.2 ± 0.7	Schultz et al. (1977)[q]
N-acetyl-L-tryptophan	16.5 ± 2.4	6.9 ± 0.7	Schultz et al. (1977)
N-acetyl-L-phenylalanine	93 ± 9	93	Byers & Koshland (1978)
noncompetitive inhibitor			
2-*p*-toluidinylnaphthalene-6-sulfonate (TNS)			
pH 5.0	0.044 ± 0.015	0.049 ± 0.016	this work[r]
pH 5.0		0.280 ± 0.050	this work[s]
pH 7.8		0.064 ± 0.034	this work[r]
pH 7.8		0.120 ± 0.015	this work[s]
transition state analogues			
N-benzoyl-L-phenylalaninal	0.0035 ± 0.0006[u]	0.026 ± 0.001[v]	Kennedy & Schultz (1979)[t]
2-phenylethaneboronic acid	18 ± 2[u]	0.045[v]	Byers & Koshland (1978)[t]
protein inhibitors			
lima bean inhibitor	(9.5 ± 2.9) × 10^{-4}[u]	0.2 × 10^{-4}[v]	Byers & Koshland (1978)[t]
potato inhibitor	(9.3 ± 3.7) × 10^{-4}[u]	9.3 × 10^{-4}[v]	Byers & Koshland (1978)[t]
turkey ovomucoid	1.1 × 10^{-4}	0.02 × 10^{-4}	Ryan & Feeney (1975)[t,w]

[a] K_s for *N*-methyl-α-chymotrypsin. [b] K_s for α-chymotrypsin. [c] Determined spectrophotometrically by proflavin displacement at 25 °C and pH 7.9. [d] In this work, unless otherwise stated, the values are at pH 5.0 and 25 °C as determined spectrophotometrically by proflavin displacement from *N*-methyl-α-chymotrypsin. [e] *N*-Methyl-α-chymotrypsin, 5.2 μM; substrate, 2.48 mM; proflavin, varied from 24 to 85 μM. There was no evidence of substrate-proflavin complex. [f] In Coll & Whitaker (1979), K_s was determined at pH 5.0, 26.5 °C, and ionic strength of 0.2 M with a stopped-flow spectrophotometer to monitor displacement of proflavin from α-chymotrypsin. [g] *N*-Methyl-α-chymotrypsin, 5.23 μM; proflavin, 70.1 μM; substrate, varied from 9 to 33 mM. Corrected for complex between substrate and proflavin with K_d = 42 mM. [h] *N*-Methyl-α-chymotrypsin, 8.0 μM; proflavin, 60 μM; substrate, varied from 6 to 17 mM. No evidence for substrate-proflavin complex. [i] K_m value that is not necessarily equal to K_s. [j] *N*-Methyl-α-chymotrypsin, 5.1 μM; proflavin, 60 μM; substrate, varied from 1.3 to 4.7 mM. No evidence for substrate-proflavin complex. [k] *N*-Methyl-α-chymotrypsin, 9.4 μM; proflavin, 67.6 μM; substrate, varied from 0 to 0.48 mM. [l] In Lucas & Caplow (1972), K_s was determined at pH 6.2 and 25 °C by proflavin displacement. [m] *N*-Methyl-α-chymotrypsin, 6.32 μM; proflavin, 74 μM; substrate, varied from 0 to 500 μM. [n] *N*-Methyl-α-chymotrypsin, 6.93 μM; proflavin, 76.8 μM; substrate, varied from 20 to 60 mM. Substrate and proflavin formed complex with K_d = 130 mM. [o] *N*-Methyl-α-chymotrypsin or α-chymotrypsin, 5.0 μM; proflavin, 25–100 μM; K_s = 65.4 ± 10.0 μM for *N*-methyl-α-chymotrypsin and proflavin at 10 °C. [p] In Byers & Koshland (1978), K_s was determined at pH 7.8 and 25 °C by proflavin displacement. [q] In Schultz et al. (1977), K_s was determined at pH 7.8 and 25 °C by proflavin displacement. [r] Calculated from the ratio of k_{-1}/k_1 determined by temperature jump as described in Table II. [s] Determined by fluorescence titration at 12 °C according to the procedure of McClure & Edelman (1967). [t] At 25 °C, pH 7.8. [u] Determined by steady-state inhibition. [v] Determined by proflavin displacement. [w] Determined by competitive enzyme assay (Ryan & Feeney, 1975).

substrates and competitive and noncompetitive inhibitors of α-chymotrypsin have been determined for *N*-methyl-α-chymotrypsin by using proflavin displacement. These results are summarized in Table I. The data have been corrected for the observed binding of proflavin to two of the substrates (Brandt et al., 1967); proflavin binding to the other substrates was negligible.

The data of Table I, including some data from other workers, indicate that the K_s values for all the substrates are almost identical for α-chymotrypsin and *N*-methyl-α-chymotrypsin. The two notable exceptions are for *N*-mesyl-L-tyrosine ethyl ester and *N*-acetyl-L-tyrosine *p*-chloroanilide where the K_m values for α-chymotrypsin are compared with the K_s values for *N*-methyl-α-chymotrypsin. The differences probably reflect buildup of covalent enzyme–substrate intermediates with α-chymotrypsin so that $K_m < K_s$ (Fersht & Requena, 1971a; Coll & Whitaker, 1979).

Data with some other compounds (Table I) indicate a difference in binding between α-chymotrypsin and *N*-methyl-α-chymotrypsin. The transition state analogue 2-phenylethaneboronic acid binds 400 times tighter to α-chymotrypsin than to *N*-methyl-α-chymotrypsin, probably because of covalent adduct formation (Rawn & Lienhard, 1974; Schultz & Cheevra, 1975) possible only for α-chymotrypsin. On the other hand, the transition state analogue *N*-benzoyl-L-phenylalaninal binds significantly tighter to *N*-methyl-α-chymotrypsin than to α-chymotrypsin possibly because of slower dissociation of the inhibitor from *N*-methyl-α-chymotrypsin (Chen et al., 1979; Kennedy & Schultz, 1979). Protein inhibitors seem to have a greater affinity for α-chymotrypsin, which may reflect additional complex stabilization from ionic interaction of His-57 with a group on the inhibitor (Blow et al., 1972) or from acylation with α-chymotrypsin only.

Some experimental data have been interpreted to indicate that there may be small differences in the active site conformations of α-chymotrypsin and *N*-methyl-α-chymotrypsin. X-ray data indicated that crystals of *N*-methyl-α-chymotrypsin may have a slightly altered conformation in the active site region where the conformation resembled more closely that of acylchymotrypsins than that of free α-chymotrypsin (Wright et al., 1972). However, the results of Neet et al. (1974) indicate that the direct effect of His-57 methylation was on

the interacting dimer interface and that the observed conformational change was only incidental to this effect.

CD and ORD spectra of *N*-methyl-α-chymotrypsin and α-chymotrypsin were similar but not identical, as reported by Antonov & Vorotyntseva (1972). However, the spectra were taken at pH 4.8 and 50 μM protein, conditions favorable for α-chymotrypsin dimerization. Byers & Koshland (1978) found no difference between the absorption spectrum or fluorescent properties of the two proteins. The slightly decreased stability of *N*-methyl-α-chymotrypsin observed in denaturation experiments (Byers & Koshland, 1978; Antonov & Vorotyntseva, 1972) is probably a result of disruption of the bonds of the charge relay system (Byers & Koshland, 1978).

Schultz et al. (1977; 1979) reported that while the overall free energy of binding of some substrates and inhibitors is the same for α-chymotrypsin and *N*-methyl-α-chymotrypsin, the enthalpy and entropy of binding are different. They suggested that binding to α-chymotrypsin requires at least one more step than binding to *N*-methyl-α-chymotrypsin, resulting in the observed differences in entropy and enthalpy.

Most experimental data, including that reported in this paper, indicate that binding of most ligands to α-chymotrypsin and *N*-methyl-α-chymotrypsin appears to be very similar if not identical. If any major alterations in the binding site conformation results from methylation of His-57, decreased binding of all ligands to *N*-methyl-α-chymotrypsin might be expected. When observed, the differences in binding probably result from additional (covalent) reaction steps or direct ionic interaction with His-57 rather than alterations in binding site conformation. Therefore, kinetic data on the binding of substrates to *N*-methyl-α-chymotrypsin probably are representative of the same reactions with α-chymotrypsin.

Dynamics of Ligand Binding to α-Chymotrypsin, N-Methyl-α-chymotrypsin, and Chymotrypsinogen A. Three basic approaches were used in the kinetic monitoring of changes at the active site on binding of substrates and substrate-like compounds to α-chymotrypsin or chymotrypsin derivatives. The first approach was the use of UV-invisible substrates, such as *N*-acetyl-L-phenylalanine ethyl ester, which permitted monitoring of changes in the absorbance or fluorescence of groups on the enzyme on binding of substrate. The second approach was the use of the reporter compounds proflavin (competitive inhibitor) and 2-*p*-toluidinylnaphthalene-6-sulfonate (noncompetitive inhibitor) to report changes in absorbance or in fluorescence on binding of other compounds at the active site. The third approach involved use of the substrate *N*-acetyl-L-dinitrotyrosine ethyl ester where the chromophoric group of the substrate undergoes spectral changes due to changes in the environment at the active site.

We achieved only limited success in use of the intrinsic absorbance and fluorescence of the enzyme to monitor binding of ligands, such as *N*-acetyl-L-phenylalanine ethyl ester, because of the small changes observed in absorbance and quenching of fluorescence by the reactants. Therefore, no results are reported here.

Rates of Proflavin Binding to α-Chymotrypsin and N-Methyl-α-chymotrypsin. Proflavin is a competitive inhibitor of α-chymotrypsin and on this basis has been used successfully to measure the equilibrium binding and acylation rate constants for substrates and substrate-like compounds with α-chymotrypsin and with *N*-methyl-α-chymotrypsin (this work, Table I; Bernhard et al., 1966; Brandt et al., 1967; Coll & Whitaker, 1979) and the rate of binding of the competitive inhibitor benzamidine to trypsin (Guillain & Thusius, 1970). Therefore, we hoped to use proflavin as a reporter of changes

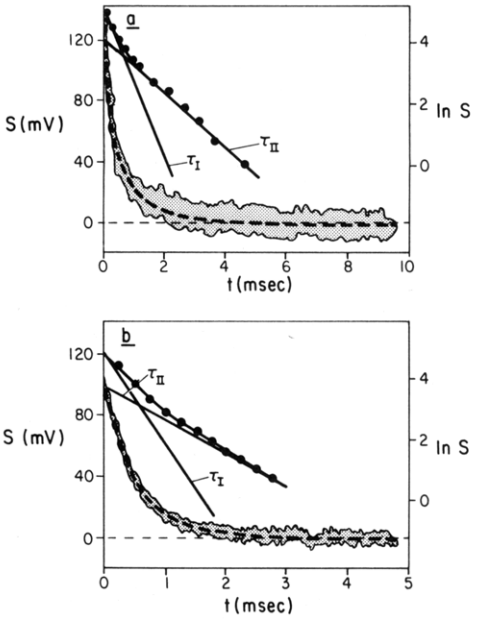


FIGURE 1: Oscilloscope traces for binding of proflavin to chymotrypsin and *N*-methyl-α-chymotrypsin. A solution buffered at pH 5.0 (0.2 M acetate–Cl^-, μ = 0.2 M) containing proflavin and protein was monitored spectrophotometrically at 470 nm after jumping from 4 to 12 °C. Reaction traces for both α-chymotrypsin (a) and *N*-methyl-α-chymotrypsin (b) yielded nonlinear logarithm plots (●) that were resolved graphically into two linear portions, each representing a single exponential. The dashed lines inside the reaction traces were calculated from the sum of the exponentials and therefore indicate the fit to the data. (a) α-Chymotrypsin + proflavin. The sum of the exponentials is given by the equation $S = 86\exp(-4000t) + 50\exp(-880t)$, indicating relaxation rates of 4000 s^{-1} and 880 s^{-1} for τ_I^{-1} and τ_{II}^{-1}, respectively, at 208 μM $\overline{PF}$ + $\overline{CT}$. (b) *N*-Methyl-α-chymotrypsin + proflavin. The sum of the exponentials is given by the equation $S = 81\exp(-3000t) + 39\exp(-1100t)$, indicating relaxation rates of 3000 s^{-1} and 1100 s^{-1} for τ_I^{-1} and τ_{II}^{-1}, respectively, at 140 μM $\overline{PF}$ + $\overline{N\text{-MCT}}$.

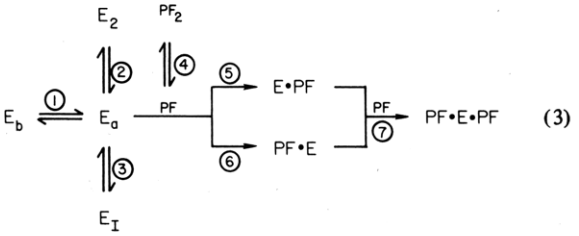
occurring at the active site of α-chymotrypsin and *N*-methyl-α-chymotrypsin on binding of substrates and substrate-like compounds as well as the binding of proflavin alone.

Changes in the absorbance of proflavin with α-chymotrypsin and with *N*-methyl-α-chymotrypsin at pH 5 as a function of time after perturbation of the equilibrium are shown in Figure 1. The plot is biphasic, indicating two observable transients. The data are plotted in Figure 2 according to eq 2 where the

$$E + PF \underset{}{\overset{\tau_I}{\rightleftharpoons}} E{\cdot}PF \overset{\tau_{II}}{\rightleftharpoons} (E{\cdot}PF)' \qquad (2)$$

initial complex, E·PF, formed then undergoes a conformational change to complex (E·PF)′. The rate constants k_1, k_{-1}, k_2, and k_{-2} are given in Table II. They are essentially identical for α-chymotrypsin and *N*-methyl-α-chymotrypsin. K_s^{PF} for α-chymotrypsin calculated from these rate constants is 50 ± 40 μM (at 12 °C) while K_s^{PF} obtained from titration data at 10 °C is 65 ± 10 μM (Table III).

Because of the presence of other species of α-chymotrypsin and proflavin at pH 5, other mechanisms can be proposed that could conceivably lead to a second observed transient (eq 3).



(3)

Table II: Rate Constants for Noncovalent Interaction of Ligands with *N*-Methyl-α-chymotrypsin, α-Chymotrypsin, and Chymotrypsinogen A[a]

compound	enzyme	pH	$k_1 \times 10^{-7}$ (M^{-1} s^{-1})	$k_{-1} \times 10^{-3}$ (s^{-1})	$k_2 \times 10^{-3}$ (s^{-1})	k_{-2} (s^{-1})	k_3 (s^{-1})	k_{-3} (s^{-1})
noncompetitive inhibitors								
TNS[b,c]	chymotrypsinogen A	5.0	1.8 ± 0.2	2.7 ± 1.7				
TNS[d]	*N*-methyl-α-chymotrypsin	5.0	7.7 ± 0.7	3.4 ± 1.1				
TNS[e]	α-chymotrypsin	5.0	3.9 ± 0.3	1.9 ± 0.6				
TNS[f]	α-chymotrypsin	7.8	5.9 ± 0.8	3.7 ± 1.9				
competitive inhibitors								
indole[g]	α-chymotrypsin	5.0	1.8 ± 0.4	7.8 ± 3.6				
indole[h]	α-chymotrypsin	7.8	1.9 ± 0.2	5.8 ± 1.4				
proflavin[i]	α-chymotrypsin (and *N*-methyl-α-chymotrypsin)	5.0	1.0 ± 0.5	0.70 ± 0.30	1.0 ± 0.5	700 ± 400		
proflavin[j]	α-chymotrypsin	5.0	1.2	3.2				
proflavin[k]	α-chymotrypsin	8.4	6.3 ± 0.3	1.2 ± 0.6	0.5 ± 0.4	7000 ± 400		
proflavin[k]	α-chymotrypsin	9.2	11.0 ± 0.2	2.2 ± 0.3	7.3 ± 0.7	2000 ± 300		
F_3Ac-D-Trp[l]	α-chymotrypsin	5.0	1.5	3.9				
F_3Ac-D-Trp[l]	α-chymotrypsin	7.0	0.59	3.5				
substrates								
DNTEE[m]	*N*-methyl-α-chymotrypsin	5.0	3.7 ± 0.5	62 ± 8	1.2 ± 0.1	350 ± 110	300 ± 200	400 ± 200
DNTEE	MS-*N*-MCT[n]	5.0	1.5 ± 0.4	110 ± 10	1.1 ± 0.4	200 ± 100		
N-Ac-Phe-*p*-NA[o]	α-chymotrypsin	7.5			2.0		900	
N-Ac-Try-*p*-NPE[p]	α-chymotrypsin		6	60				
(furylacryloyl)-Try-amide[q]	α-chymotrypsin	7.4	0.62					

[a] In this work, the values were determined by temperature jump (from 4 to 12 °C) at pH 5.0 and ionic strength of 0.2 with absorption spectrometry unless otherwise noted. [b] 2-*p*-Toluidinylnaphthalene-6-sulfonate, by fluorescence spectroscopy; excitation λ was 350 nm; changes in fluorescence were monitored through a cutoff filter ($A_{350} = 2, A_{370} = 0.1$). [c] TNS and chymotrypsinogen concentrations ranged from 30 to 300 μM and 0.1 to 1.0 mM, respectively. [d] TNS and *N*-methyl-α-chymotrypsin concentrations ranged from 6 to 110 μM and 17 to 300 μM, respectively. [e] TNS and α-chymotrypsin concentrations ranged from 60 to 340 μM and 0.1 to 1.0 mM, respectively. [f] TNS and α-chymotrypsin concentrations were 143 μM and 13–500 μM, respectively. [g] Determined with TNS (200 μM) as the "reporter group". Indole and α-chymotrypsin concentrations ranged from 0.04 to 2.0 mM and 13 to 650 μM, respectively. [h] Determined with TNS (200 μM) as the reporter group. Indole and α-chymotrypsin concentrations ranged from 0.14 to 2.0 mM and 33 to 460 μM, respectively. [i] Proflavin and α-chymotrypsin concentrations ranged from 0.04 to 2.2 mM and 40 to 400 μM, respectively; proflavin and *N*-methyl-α-chymotrypsin concentrations both ranged from 40 to 150 μM. The relaxation times for *N*-methyl-α-chymotrypsin and α-chymotrypsin were indistinguishable. [j] In Quast et al. (1974); pH 5 by temperature jump. [k] In Havsteen (1967); determined at 12 °C by temperature jump. [l] (Trifluoroacetyl)-D-tryptophan. In Smallcombe et al. (1972); determined by NMR. [m] *N*-Acetyl-L-3,5-dinitrotyrosine ethyl ester. The DNTEE and *N*-methyl-α-chymotrypsin concentrations ranged from 0.3 to 3.6 mM and 75 to 700 μM, respectively. [n] (Methionine sulfoxide)$_{192}$-*N*-methyl-α-chymotrypsin; concentrations of DNTEE and MS-*N*-MCT were 0.11–4.3 mM and 17–660 μM. [o] *N*-Acetyl-L-phenylalanine *p*-nitroanilide. In Fink (1976a); done at −80 to −40 °C and pH 5.3 in 65% dimethyl sulfoxide; results extrapolated to 25 °C and 0% dimethyl sulfoxide; error of extrapolation of ±50%. [p] *N*-Acetyl-L-tryptophan *p*-nitrophenyl ester. In Renard & Fersht (1973). Calculated from steady-state kinetic experiments as maximum values by assuming rate-limiting association at high pH at 25 °C. [q] (Furylacryloyl)-L-tryptophanamide. In Hess et al. (1970); determined at 15 °C by temperature jump.

Table III: Comparison of K_s Determined by Temperature-Jump Perturbation and by Spectrophotometric Titration of Enzyme with Compound[a]

enzyme	compound	pH	K_s (temp jump)[b] (mM)	K_s (titration) (mM)
α-chymotrypsin	TNS	5.0	0.049 ± 0.016	0.280 ± 0.015
		7.8	0.064 ± 0.034	0.120 ± 0.015
α-chymotrypsin (also *N*-methyl-α-chymotrypsin)	proflavin	5.0	0.050 ± 0.040	0.065 ± 0.010
α-chymotrypsin	indole[c]	5.0	0.430 ± 0.220	0.5[d]
		7.8	0.310 ± 0.080	0.25[d]
N-methyl-α-chymotrypsin	DNTEE	5.0	0.7 ± 0.4	1.8 ± 0.4

[a] Reaction conditions given in Tables I and II. [b] Calculated from $(k_{-1}k_{-2}...k_{-n})/(k_1k_2...k_n)$. [c] Using TNS as reporter. [d] Calculated from the data of Shiao (1970) and Foster & Niemann (1955).

However, most of the possibilities may be eliminated by consideration of the rates reported for these reactions and the expected concentration dependence of the reactions and/or comparison with results for *N*-methyl-α-chymotrypsin. Havsteen (1967) observed only one transient for the binding of proflavin to α-chymotrypsin at pH 5 but two transients for this same reaction at pH 9.0. The pH 5.0 data fit the equation for a simple association–dissociation mechanism, while at pH 9.0 the second transient apparently resulted from an isomerization of the enzyme–inhibitor complex following association. A third very fast transient ($\tau \simeq 10$ μs) was detected with polarization optics in solutions of α-chymotrypsin alone, indicating a rapid equilibration of two enzyme species as shown in step 1 of eq 3. Mechanisms where proflavin binding is preceded by a protein conformational change should give decreasing relaxation rates with increasing concentrations of proflavin (Gutfreund, 1972; Fersht, 1977). In our experiments, τ_I^{-1} was observed to increase and τ_{II}^{-1} was observed to saturate, thus ruling out this explanation for our data.

The rates of α-chymotrypsin dimerization [step 2; $k_2 = 3.7 \times 10^3$ M^{-1} s^{-1}, $k_{-2} = 0.7$ s^{-1} (Koren & Hammes, 1976); $\tau >$ 150 ms at enzyme concentrations used here] and change to an inactive form [step 3; $k_3 \simeq 3.1$ s^{-1}, $k_{-3} = 0.1$ s^{-1} (Fersht & Requena, 1971b); $\tau >$ 300 ms at concentrations used here] are far too slow to produce the observed transient while the rate of dimerization of proflavin [step 4; $k_4 = 7.9 \times 10^8$ M^{-1} s^{-1}, $k_{-4} = 2 \times 10^6$ s^{-1} (Turner et al., 1972); $\tau < 0.5$ μs at our proflavin concentration] is much too fast. Two transients were observed for binding of proflavin to *N*-methyl-α-chymotrypsin, which does not dimerize (Byers & Koshland, 1978); this is

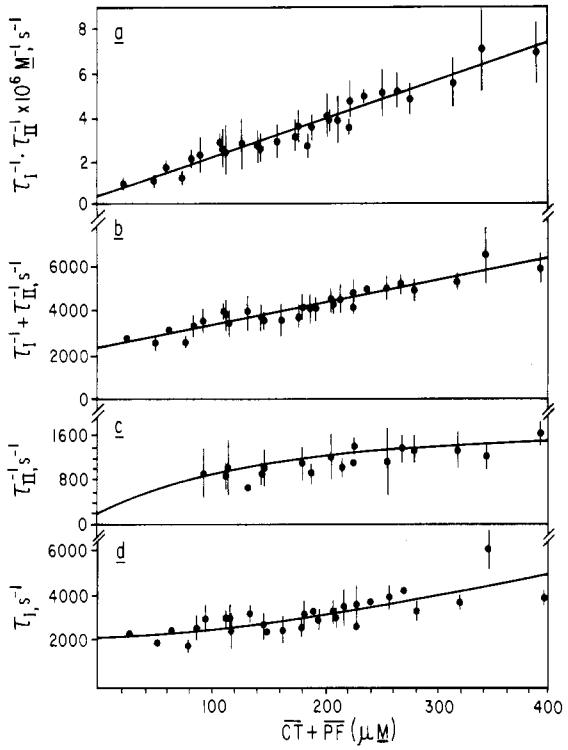


FIGURE 2: Determination of rate constants for chymotrypsin and proflavin complexation. The relaxation rates for proflavin binding to α-chymotrypsin, evaluated as shown in Figure 1, are plotted in accordance with eq 2. Evaluation of τ_{II}^{-1} at all concentrations was not possible due to its small amplitude. Therefore, a reiterative procedure was used wherein τ_I^{-1} and τ_{II}^{-1} values were calculated at each concentration from a set of assumed rate constants. The calculated values were coupled with observed τ's to construct plots similar to those in (a) and (b), which were then used to derive a new set of rate constants, and the procedure was repeated until the assumed and derived set of rate constants agreed. The rate constants reported in Table II resulted from the least-squares fit to plots a and b; the lines in (c) and (d) show the fit to the observed data.

additional proof that step 2 is not responsible for one of the observed transients.

Although nonspecific proflavin binding to chymotrypsin is thought to be negligible (Brandt et al., 1967; Bernhard et al., 1966; I'Haya et al., 1980), the possibility that a second molecule of proflavin can bind nonspecifically, and noncompetitively with the first proflavin bound in step 5, to α-chymotrypsin is shown in step 6. If τ_{II}, the slower of the two observed transients (Figure 1), is considered to be due to step 6 while τ_I is due to step 5, then k_6 and k_{-6} are calculated to be 4×10^6 M^{-1} s^{-1} and 500 s^{-1}, respectively, giving a K_6 of 100 μM. This complex is too tight for nonspecific binding. The second transient also cannot be the result of proflavin dimer binding to chymotrypsin as this apparently does not occur (I'Haya et al., 1980). The fact that the equilibrium constant (K_s^{PF} = 50 ± 40 μM) determined by temperature jump according to eq 2 is in agreement with that determined by titration (K_s^{PF} = 65 ± 10 μM; Table III) lends further support to the mechanism proposed in eq 2.

Data from preliminary experiments in which proflavin was used as a reporter to monitor changes on adding substrate or substrate-like compounds to α-chymotrypsin plus proflavin (or *N*-methyl-α-chymotrypsin) were complex. This might be expected since the minimum mechanism is likely to be that shown in eq 4. Also, it is unlikely that proflavin can report

$$E{\cdot}S \overset{S}{\rightleftharpoons} E \overset{PF}{\rightleftharpoons} E{\cdot}PF \rightleftharpoons (E{\cdot}PF)' \qquad (4)$$

subsequent reactions following initial complexation of substrate with *N*-methyl-α-chymotrypsin since detection of all substrate binding steps requires perturbation of proflavin equilibria and resolution from the second step in proflavin binding.

In summary, proflavin binding at the active site of either α-chymotrypsin or *N*-methyl-α-chymotrypsin gives two transients; the slower transient is apparently due to a conformational change of the enzyme as the proflavin is better accommodated at the active site.

Rates of Binding of 2-p-Toluidinylnaphthalene-6-sulfonate (TNS) to α-Chymotrypsin, N-Methyl-α-chymotrypsin, and Chymotrypsinogen A. McClure & Edelman (1967) reported that TNS fluoresces on binding to a hydrophobic site on α-chymotrypsin distinct from the active site and behaves as a noncompetitive inhibitor in α-chymotrypsin-catalyzed reactions. Quenching of the fluorescence of the TNS·α-chymotrypsin complex on binding of ligands at the active site has been used successfully to determine their binding constants (McClure & Edelman, 1967; Gabel et al., 1971). Therefore, we have explored its utility for measuring the rates of ligand association–dissociation at the active site of α-chymotrypsin and its derivatives.

Temperature perturbation of an equilibrium mixture of TNS with either α-chymotrypsin, *N*-methyl-α-chymotrypsin, or chymotrypsinogen A at pH 5 or 7.8 resulted in a single transient as observed either by absorbance or by fluorescence. These data are shown in Figures 3a–d and 4. The rate constants, k_1 and k_{-1}, shown in Table II are similar for all three proteins. k_1 appears to be the fastest for *N*-methyl-α-chymotrypsin and the slowest for chymotrypsinogen A.

The dissociation constant, K_s^{TNS}, of 49 ± 16 μM derived from k_1 and k_{-1} for α-chymotrypsin determined from the temperature-jump experiments at pH 5 does not agree with the K_s^{TNS} of 280 ± 50 μM determined from titration of α-chymotrypsin at pH 5 (Table III). This may indicate the involvement of other equilibria. However, no "bursts" of fluorescence preceded the measured transient, no slower transients followed it, and no significant deviations from linearity were apparent in the plotted data. The agreement of K_s values at pH 7.8 is somewhat better (Table III; 64 vs. 120 μM).

Some potential possibilities for other equilibria at pH 5 are shown in eq 5 where E_2 is the α-chymotrypsin dimer (Gilleland

$$\begin{array}{ccccc} E_2 + TNS & \rightleftharpoons & E_2{\cdot}TNS & \overset{TNS}{\rightleftharpoons} & E_2{\cdot}TNS_2 \\ \downarrow\uparrow & & \downarrow\uparrow & & \\ E + TNS & \rightleftharpoons & E{\cdot}TNS & & \\ \downarrow\uparrow & & \downarrow\uparrow & & \\ E_I + TNS & \rightleftharpoons & E_I{\cdot}TNS & & \end{array} \qquad (5)$$

& Bender, 1976; Horbett & Teller, 1974) and E_I is the inactive form (Stoesz & Lumry, 1978; Fersht & Requena, 1971b; Kim & Lumry, 1971).

While there is no experimental evidence for TNS binding to E_I or to E_2, it may occur because (1) E_I resembles chymotrypsinogen A which binds TNS and (2) if one of the monomers in E_2 is thought of as a bound substrate, then the noncompetitive inhibitor TNS would be expected to bind. Evidence against the kinetic importance of reactions involving E_2 and E_I in the observed binding of TNS includes the following: (1) E_I is a minor component at pH 5; (2) similar results were observed with *N*-methyl-α-chymotrypsin and chymotrypsinogen A, which do not dimerize, and with α-chymotrypsin at pH 7.8 where E_2 is essentially zero; (3) equilibration among the different enzyme species (i.e., $E_2 \rightleftharpoons$

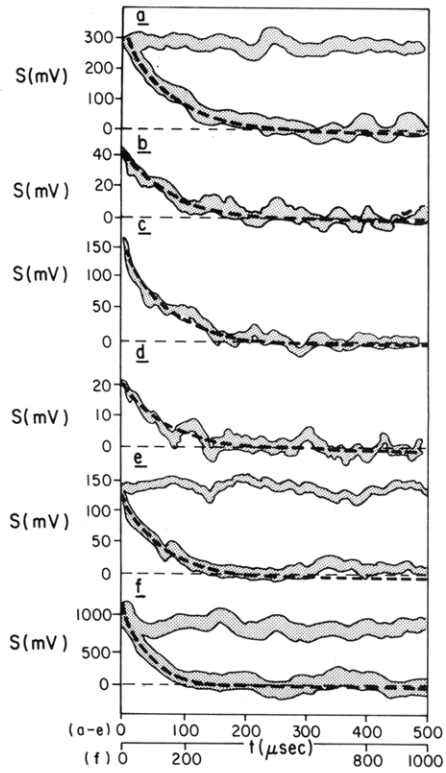


FIGURE 3: Oscilloscope traces for fluorescence monitoring of 2-*p*-toluidinylnaphthalene-6-sulfonate and indole binding to protein. The reactions were monitored through a cutoff filter (A_{340} = 2; A_{370} = 0.1) with excitation at 350 nm at either pH 5.00 [0.2 M acetate–Cl^-, μ = 0.2 M; (b)–(e)] or pH 7.8 [0.1 M Tris, 0.1 M KCl, 0.03 M $CaCl_2$; (a) and (f)]. The dashed lines pictured within each trace indicate the fit to rate and amplitude values determined from logarithm plots. The horizontal trace in all cases represents the control trace. CT = chymotrypsin; CTng = chymotrypsinogen; TNS = 2-*p*-toluidinylnaphthalene-6-sulfonate; I = indole. (a) CT + TNS, pH 7.8. The transient shown fits the equation $S = 310 \exp(-12.0 \times 10^3 t)$, indicating a relaxation rate of 12×10^3 s^{-1} at 143 μM $\overline{CT}$ + $\overline{TNS}$. (b) CT + TNS, pH 5.0. The transient shown fits the equation $S = 44 \exp(-14.8 \times 10^3 t)$, indicating a relaxation rate of 14.8×10^3 s^{-1} at 331 μM $\overline{CT}$ + $\overline{TNS}$. (c) *N*-MCT + TNS, pH 5.0. The transient shown fits the equation $S = 160 \exp(-15.0 \times 10^3 t)$, indicating a relaxation rate of 15×10^3 s^{-1} at 144 μM $\overline{N\text{-MCT}}$ + $\overline{TNS}$. (d) CTng + TNS, pH 5.0. The transient shown fits the equation $S = 21 \exp(-14 \times 10^3 t)$, indicating a relaxation rate of 14×10^3 s^{-1} at 802 μM $\overline{CTng}$ + $\overline{TNS}$. (e) CT + I, pH 5.0. TNS quenching was used to report indole binding. The transient shown fits the equation $S = 125 \exp(-16.5 \times 10^3 t)$, indicating a relaxation rate of 16.5×10^3 s^{-1} for 430 μM $\overline{CT{\cdot}TNS}$ + $\bar{I}$. (f) CT + I, pH 7.8. TNS quenching was used to report indole binding. The transient shown fits the equation $S = 1400 \exp(-12.5 \times 10^3 t)$, indicating a relaxation rate of 12.5×10^3 s^{-1} at 520 μM $\overline{CT{\cdot}TNS}$ + $\bar{I}$.

$E \rightleftharpoons E_I$ and $E_2{\cdot}TNS \rightleftharpoons E{\cdot}TNS \rightleftharpoons E_I{\cdot}TNS$) is too slow to be observed on the temperature-jump time scale or to couple to any of the other equilibria. Therefore, the transient detected most probably results from the reaction of interest, i.e., TNS binding to the protein.

In summary, it appears that TNS should act as an excellent reporter for the monitoring of events occurring at the active site on ligand binding because of its simple relaxation spectra and the high sensitivity of detecting fluorescence changes.

Rate of Binding of Indole to α-Chymotrypsin As Monitored with 2-p-Toluidinylnaphthalene-6-sulfonate (TNS). Indole is a competitive inhibitor of α-chymotrypsin that binds at the active site (Foster & Niemann, 1955; Steitz et al., 1969). Its binding at different pHs and temperatures has been thoroughly investigated (Shiao, 1970; Yapel et al., 1966; Fersht & Requena, 1971a). Therefore, it was chosen as a model substrate-like compound to explore the feasibility of using TNS·α-chymotrypsin complex quenching to report the number of changes and their rates on binding of ligands at the active site.

As discussed above, the system TNS + α-chymotrypsin gave a single relaxation transient by temperature jump at pH 5 and 7.8. When the system TNS + α-chymotrypsin + indole was observed under the same conditions, two transients were observed. The slower transient was shown to be due to TNS + E ⇌ TNS·E on the basis of its numerical similarity to the transient in the absence of indole, its dependence on the TNS + E concentrations, and its relative independence of indole + E concentrations. On the other hand, the faster transient (Figure 3e,f) was dependent on the indole + E concentration and relatively independent of the TNS + E concentration. Therefore, by adjustment of the concentrations such that the indole + E·TNS relaxation remained faster than the TNS + E relaxation, the kinetic complexity was reduced, and the rate of indole binding was determined from the concentration (indole + E) dependence of τ_1 (Figure 5a,b). In practice, this was accomplished through use of low TNS concentrations that were easily detected by fluorescence.

The data were evaluated according to eq 6 where E·TNS*

$$E + TNS \overset{\tau_{II}}{\rightleftharpoons} E{\cdot}TNS^* \overset{I,\ \tau_I}{\rightleftharpoons} I{\cdot}E{\cdot}TNS \qquad (6)$$

is the fluorescent species, I·E·TNS and E + TNS are nonfluorescent species, and I is indole. Other variations of eq 6 can be written, including the equilibria shown in eq 5. Equation 6 fits all the experimental data while other variations of eq 6 do not. The K_s^I value of 430 μM at pH 5 calculated from the rate constants k_1 and k_{-1} determined by temperature jump agrees well with values derived from titration data (Shiao, 1970).

The faster relaxation observed, τ_1, could be associated with an isomerization of I·E·TNS* ⇌ I·E·TNS rather than the binding of indole to E·TNS* (eq 6). The mechanism of fluorescence quenching presumably involves a conformational change in enzyme (McClure & Edelman, 1967; Gabel et al., 1971), which, according to the data presented here, occurs concomitantly with substrate binding. Alternatively, a fluorescent precomplex (I·E·TNS*) could be formed at diffusion controlled rates that does not build up to detectable levels.[2] Gutfreund (personal communication) has suggested that initial enzyme–substrate encounter rates should be about 2 orders of magnitude faster than those generally observed.

Use of the TNS·α-chymotrypsin complex to monitor the rates of substrate and competitive inhibitor binding appears promising. Fluorescence detection, coupled with the fact that binding is observed directly without requisite coupling to another equilibrium, yields excellent sensitivity. Furthermore, the upper concentration ranges of $\bar{E}$ + $\bar{S}$ accessible to investigation are only limited by the response of the instrument rather than by the necessity to keep the reaction of interest resolvable from other faster transients.

Application of this technique to monitor slower conformational steps subsequent to initial substrate–enzyme complexation seems possible but requires further investigation. According to the data presented here, one would not expect further enzyme–substrate conformational changes to yield fluorescence changes directly (unless they have increased fluorescence) because the I·E·TNS complex (eq 6) is quenched.

[2] Existence of the intermediate could be suggested by saturation of the τ_I^{-1} vs. [$\bar{E}$] + [$\bar{S}$] plot as well as by detection of additional transients.

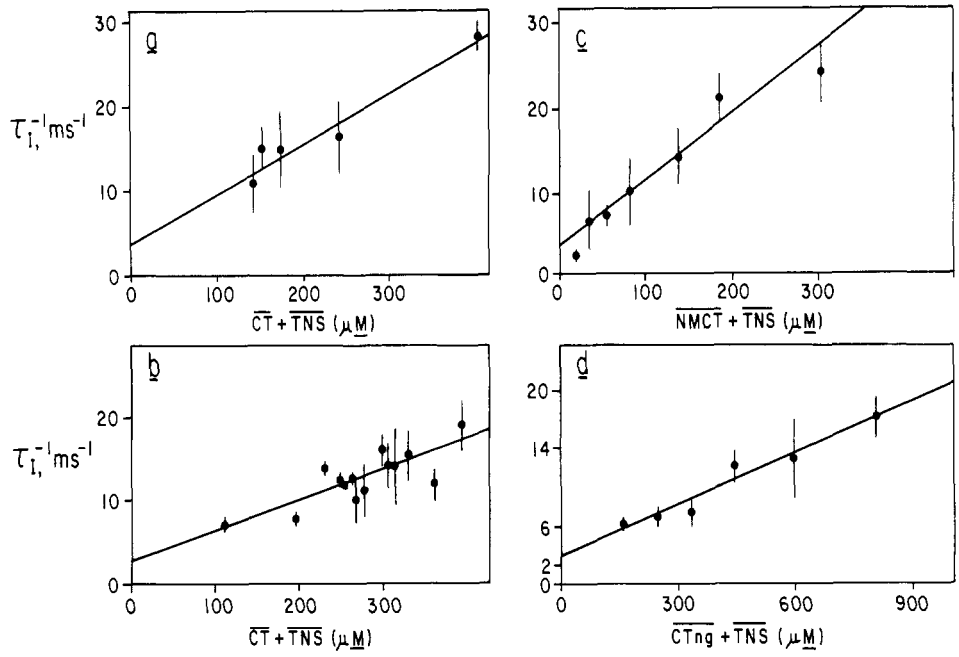


FIGURE 4: Determination of rate constants for 2-*p*-toluidinylnaphthalene-6-sulfonate and chymotrypsin complexation at pH 7.8. The relaxation rates, evaluated as shown in Figure 3, for TNS binding to α-chymotrypsin [at pH 7.8 in (a), at pH 5.0 in (b)], to *N*-methyl-α-chymotrypsin (c), and to chymotrypsinogen A (d) are plotted in accordance with a simple association–dissociation mechanism. In calculating equilibrium concentrations, the binding constant for TNS with *N*-methyl-α-chymotrypsin was assumed to be equivalent to that for binding with α-chymotrypsin (Table I). Reiteration of the temperature-jump data was used to derive the binding constant for chymotrypsinogen A. Rate constants derived from the least-squares fit to the plots are reported in Table II.

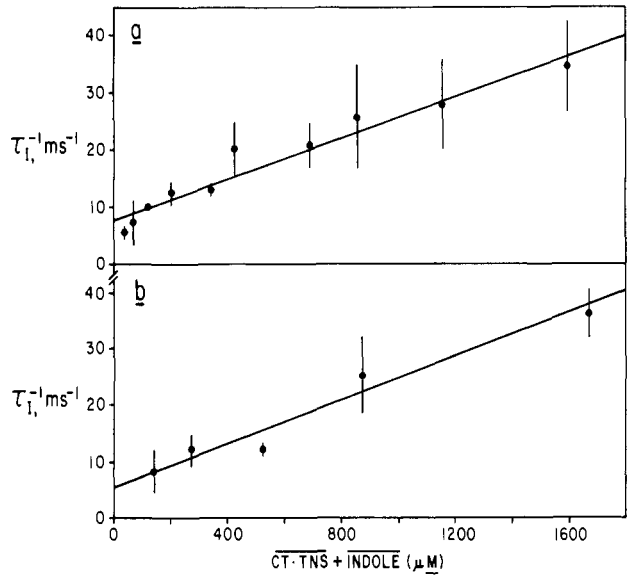


FIGURE 5: Determination of rate constants for indole and chymotrypsin complexation at pH 5.0 and 7.8. The relaxation rates for indole binding (τ_I^{-1} in eq 6), evaluated as shown in Figure 3e,f, are plotted in accordance with a simple association–dissociation mechanism. Noncompetitive binding by indole (K_s^{indole} = 400 μM; Shiao, 1970) and TNS was assumed in calculating equilibrium concentrations. Rate constants derived from least-squares fitting of the data are reported in Table II.

Detection of additional reactions might be accomplished by reaction coupling or by the dependence of the observed transients on the concentration of the reactants. For example, it should be possible to look for reactions subsequent to formation of I·E·TNS by increasing the TNS, thereby moving the transient due to E·TNS complexation from the time region of interest.

*Rate of Binding of N-Acetyl-*L*-3,5-dinitrotyrosine Ethyl Ester to N-Methyl-α-chymotrypsin.* The reporter of events occurring at the active site of an enzyme on binding of substrate can be the substrate itself. This can be accomplished by proper selection of the leaving group (i.e., *p*-nitrophenyl), of the *N*-acyl group (i.e., furylacryloyl), or of the side chain of the amino acid providing the scissile bond. The latter choice is preferred with α-chymotrypsin to avoid nonproductive binding (Fastrez & Fersht, 1973). Since N^{α}-acetyl-L-3,5-dinitrotyrosine ethyl ester (DNTEE) has all the requisite components for forming the critical substrate–enzyme interactions required of a substrate, and since the kinetics of binding have previously been investigated by other methods (Fink, 1976b), it was of great interest to study the rate of binding of this compound to α-chymotrypsin by the temperature-jump method. In order to eliminate the turnover steps, we used *N*-methyl-α-chymotrypsin as a model for α-chymotrypsin.

Temperature perturbation of *N*-methyl-α-chymotrypsin plus DNTEE at pH 5.0 yielded four observable transients by absorbance changes at 480 nm (Figure 6a,c). The first transient was an essentially instantaneous decrease in absorbance, which was also observed on perturbation of solutions of DNTEE plus chymotrypsinogen A and with DNTEE alone. Therefore, this transient of unknown cause is not treated below.

The second transient was also very fast, yet it could be resolved from the faster initial transient instrumentally (Figure 6a) and was only evident when *N*-methyl-α-chymotrypsin was also present. The third and fourth transients were much slower than the second transient but similar to each other; they were resolved graphically (Figure 6c). Temperature perturbation of DNTEE and chymotrypsinogen A solutions under the same conditions gave only the first transient.

While the presence of other forms of *N*-methyl-α-chymotrypsin could conceivably give rise to one or more of the observed transients, we believe this possibility can be ruled out for the reasons already presented above with other reporter compounds. It should be noted that *N*-methyl-α-chymotrypsin does not form a dimer. Since transients I, II, and III (not counting the fastest change) (Figure 6a) were not observed

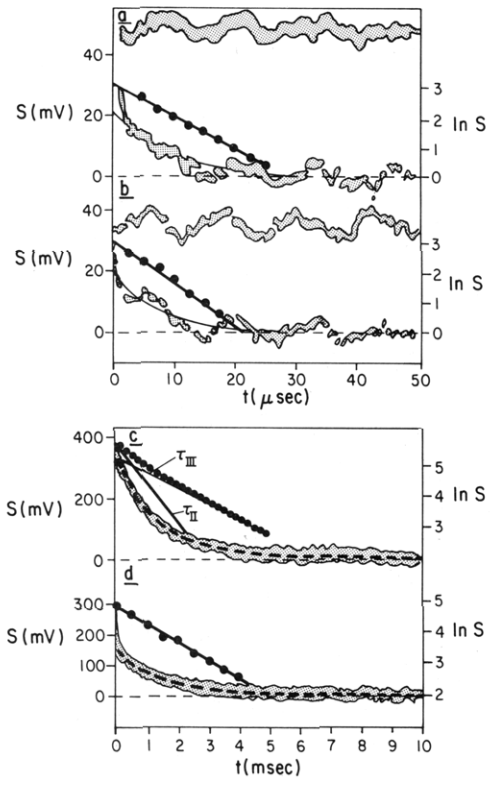


FIGURE 6: Oscilloscope traces for binding of *N*-acetyl-L-3,5-dinitrotyrosine ethyl ester (DNTEE) to *N*-methyl-α-chymotrypsin (*N*-MCT) and (methionine sulfoxide)-*N*-methyl-α-chymotrypsin (MS-*N*-MCT). A solution buffered at pH 5.00 (0.2 M acetate–Cl^-, μ = 0.2 M) containing DNTEE and protein was monitored spectrophotometrically at 490 nm after jumping from 4 to 12 °C. The logarithm of points from the oscilloscope displays are plotted (●), and the fit to the data is indicated by the smooth curves drawn through the transients in parts a and b or by the dashed lines in parts c and d. (a) τ_I for *N*-MCT. The initial rapid change was observed in solutions lacking protein and was therefore neglected. The linear plot fits the equation $\ln S = 3.1 - (10.6 \times 10^4)t$, indicating a relaxation rate of 10.6×10^4 s^{-1} at 1.9 mM $\overline{\text{DNTEE}} + \overline{N\text{-MCT}}$. (b) τ_I for MS-*N*-MCT. The linear plot fits the equation $\ln S = 3.0 - (14 \times 10^4)t$, indicating a relaxation rate of 14×10^4 s^{-1} at 2.5 mM $\overline{\text{MS-}N\text{-MCT}} + \overline{\text{DNTEE}}$. (c) τ_{II} and τ_{III} for *N*-MCT. The nonlinear logarithm plot was "peeled" into two linear portions. The sum of the exponentials is given by the equation $S = 145 \exp(-1260t) + 220 \exp(-520t)$, indicating relaxation rates of 1260 s^{-1} and 520 s^{-1} for τ_{II} and τ_{III}, respectively, at 1.9 mM $\overline{\text{DNTEE}} + \overline{N\text{-MCT}}$. (d) τ_{II} for MS-*N*-MCT. The linear plot fits the equation $\ln S = 5.1 - 590t$, indicating a relaxation rate of 590 s^{-1} at 2.5 mM $\overline{\text{MS-}N\text{-MCT}} + \overline{\text{DNTEE}}$.

with chymotrypsinogen A, observable transients due to nonspecific binding of DNTEE to *N*-methyl-α-chymotrypsin appear to be excluded.

The most reasonable mechanism involves binding of DNTEE to *N*-methyl-α-chymotrypsin, followed by two enzyme–substrate conformational changes that may occur by one of two possible pathways (eq 7 and 8). Since τ_I^{-1} was

$$E + S \underset{}{\overset{\tau_I}{\rightleftharpoons}} ES_I \overset{\tau_{II}}{\rightleftharpoons} ES_{II} \overset{\tau_{III}}{\rightleftharpoons} ES_{III} \qquad (7)$$

$$\begin{array}{ccccc} E + S & \overset{\tau_I}{\rightleftharpoons} & ES_I & \overset{\tau_{III}}{\rightleftharpoons} & ES_{III} \\ & & \Big\Updownarrow \tau_{II} & & \\ & & ES_{II} & & \end{array} \qquad (8)$$

extremely fast and linearly dependent on the sum of the equilibrium enzyme and substrate concentrations (Figure 7a), it was interpreted as resulting from enzyme–substrate complexation. Transients τ_{II}^{-1} and τ_{III}^{-1} had dissimilar concentration dependencies (Figure 7b,c) that best fit the mechanism depicted in eq 7 and conflict with those predicted by eq 8. Rate constants for τ_I, τ_{II}, and τ_{III} in eq 7 are reported in Table II as k_1 and k_{-1}, k_2 and k_{-2}, and k_3 and k_{-3}, respectively.

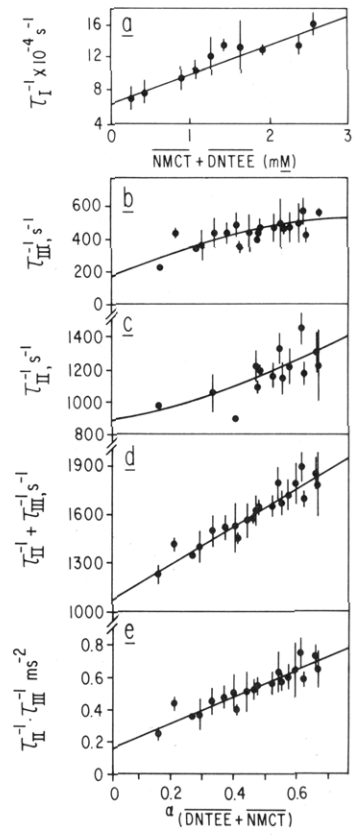


FIGURE 7: Determination of rate constants for complexation of *N*-acetyl-L-3,5-dinitrotyrosine ethyl ester (DNTEE) with *N*-methyl-α-chymotrypsin (*N*-MCT). Relaxation rates for DNTEE binding to *N*-methyl-α-chymotrypsin, evaluated as shown in Figure 6, are plotted according to eq 7. The initial binding, represented by τ_I^{-1}, is much faster than other steps and therefore considered as an uncoupled, simple association–dissociation reaction (plot a). Since subsequent reactions are in rapid equilibrium with initial binding, they are plotted vs. the equilibration term, $\alpha = ([\bar{E}] + [\overline{\text{DNTEE}}])/([\bar{E}] + [\overline{\text{DNTEE}}] + K_1)$, where $K_1 = k_{-1}/k_1$, determined in plot a. Direct evaluation of τ_{II} and τ_{III} was not always possible so procedures identical with those described in Figure 2 were used in determining plots d and e. Plots b and c show the fit to the observed data. The rate constants reported in Table II resulted from least-squares fitting of plots a, d, and e.

The k_1 value determined for DNTEE and *N*-methyl-α-chymotrypsin is similar to k_1 values determined for indole, proflavin, and TNS and those reported for (trifluoroacetyl)-D-tryptophan (Smallcombe et al., 1972), proflavin (Havsteen, 1967), and (furylacryloyl)-L-tryptophanamide (Hess et al., 1970). The k_{-1} value determined for DNTEE is about 1 order of magnitude faster than that for the compounds above but is very close to the predicted value for *N*-acetyl-L-tryptophan *p*-nitrophenyl ester (Renard & Fersht, 1973). The k_2 and k_{-2} values for DNTEE are similar to the values observed for proflavin binding to α-chymotrypsin. The K_s values for DNTEE and *N*-methyl-α-chymotrypsin by temperature-jump perturbation and titration were 0.7 mM and 1.8 mM, respectively (Table III).

The data reported here, obtained by temperature-jump perturbation of the system DNTEE and *N*-methyl-α-chymotrypsin, are consistent both mechanistically and ratewise with that obtained with α-chymotrypsin and *N*-acetyl-L-phenyl-

alanine *p*-nitroanilide at subzero temperatures (Fink, 1976a; based on Fink's extrapolation of his data to 25 °C and 0% dimethyl sulfoxide with an estimated error of ±50%; Table II). This is the first direct indication that the several intermediates observed in organic solvent at subzero temperatures are actually viable intermediates in aqueous solution. Unlike the data at subzero temperatures, the present work with temperature-jump perturbation provides the forward and backward rate constants for the three observed transients.

The DNTEE and *N*-methyl-α-chymotrypsin system would appear to be near ideal for detailed investigation of the pH and temperature dependencies, as well as other aspects of enzyme–substrate interactions. Unfortunately, absorbance of DNTEE alone at 480 nm places practical limits on the usable concentration ranges of E + DNTEE. This problem is compounded because K_m is 4.6 mM (k_{cat} = 0.03 s^{-1}; pH 5.0, 26.5 °C, acetate buffer of ionic strength 0.2) compared to 0.5 mM for *N*-acetyl-L-tyrosine ethyl ester (Coll & Whitaker, 1979). Nevertheless, further use of this method should provide much additional data on the steps involved in the accommodation of enzyme to substrate. One such possibility is presented below.

Rate of Binding of N-Acetyl-L-3,5-dinitrotyrosine Ethyl Ester (DNTEE) to (Methionine sulfoxide)$_{192}$-N-methyl-α-chymotrypsin (MS-N-MCT). Data from several investigations have been interpreted to indicate that Met_{192} of α-chymotrypsin undergoes relatively large movements when substrate is bound, resulting in Met_{192} either acting as a lid to the hydrophobic pocket or alternatively compressing the large leaving group of substrates such as dipeptides (Treadway & Schultz, 1976; Fersht et al., 1973; Landis & Berliner, 1980a,b; Matta et al., 1980). Therefore, since Met_{192} has been implicated in both substrate binding and conformational changes, it was of interest to determine if MS-*N*-MCT gives different results from *N*-MCT on binding of DNTEE.

Limited data for the temperature perturbation of the DNTEE and MS-*N*-MCT system at pH 5 gave similar transients to those for DNTEE + *N*-MCT except that the slow relaxation (transients II and III for DNTEE + *N*-MCT) was not resolvable into two relaxations for DNTEE + MS-*N*-MCT (Figure 6b,d). In order to be more certain that a second slow relaxation does not exist, it will be necessary to investigate the system at higher concentrations of DNTEE + MS-*N*-MCT.

The data, analyzed according to eq 6, are shown in Figure

$$\text{MS-}N\text{-MCT} + \text{DNTEE} \underset{}{\overset{\tau_I}{\rightleftharpoons}}$$

$$\text{MS-}N\text{-MCT·DNTEE}_I \overset{\tau_{II}}{\rightleftharpoons} \text{MS-}N\text{-MCT·DNTEE}_{II} \quad (9)$$

8 and the calculated rate constants are given in Table II. The k_1 value is about half that of k_1 for DNTEE and *N*-MCT while the k_{-1} value is about twice that for DNTEE and *N*-MCT. Thus, k_{-1}/k_1 is 7 mM for DNTEE and MS-*N*-MCT as compared with 1.7 mM for DNTEE and *N*-MCT. These results are in agreement with those of Weiner et al. (1966) and Treadway & Schultz (1976) for α-chymotrypsin and MS-α-chymotrypsin.

Therefore, it appears that temperature-jump investigations of the system DNTEE and chemical modifications of *N*-methyl-α-chymotrypsin will be a useful tool in revealing the enzyme groups involved in the multiple "association–activation" steps observed.

Conclusions. The data presented here, as well as published data, indicate that *N*-methyl-α-chymotrypsin is an appropriate model for α-chymotrypsin with respect to enzyme–substrate binding (Tables I and II). Acceptance of this premise will permit detailed investigation of the steps and rates involved

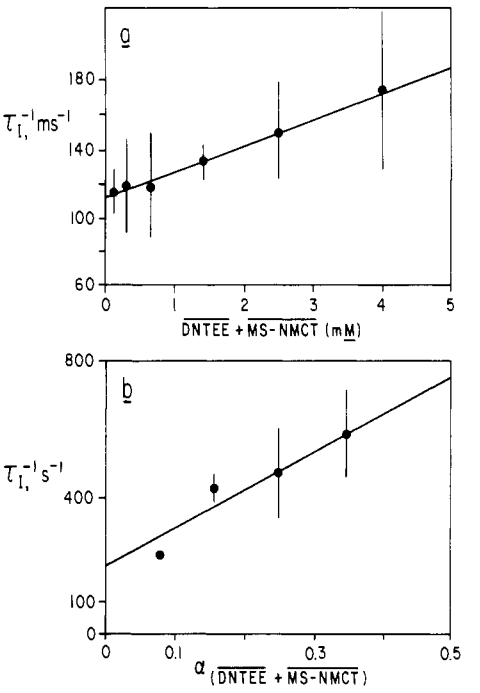


FIGURE 8: Determination of rate constants for complexation of *N*-acetyl-L-3,5-dinitrotyrosine ethyl ester (DNTEE) with (methionine sulfoxide)-*N*-methyl-α-chymotrypsin (MS-*N*-MCT). Relaxation rates for DNTEE binding to MS-*N*-MCT, evaluated as shown in Figure 6, are plotted according to eq 9. Treatment of the data is the same as reported in Figure 7, except that only a single transient subsequent to initial complexation was observed, which is evaluated in plot b. Approximate rate constants derived from the limited data shown are reported in Table II.

in the complexation of substrates with at least one enzyme, α-chymotrypsin. In our work we have investigated the rates of binding of a noncompetitive inhibitor, two competitive inhibitors, and a substrate to *N*-methyl-α-chymotrypsin and of the inhibitors to α-chymotrypsin. Data for these rates make a considerable contribution to what is known about the rates of binding of substrate to α-chymotrypsin in aqueous systems at temperatures above 0 °C (Table II).

The noncompetitive inhibitor, TNS, and the small competitive inhibitor, indole, were observed to bind to α-chymotrypsin in a single step. The larger competitive inhibitor, proflavin, which contains more of the structure found in α-chymotrypsin substrates, bound more tightly to *N*-methyl-α-chymotrypsin and α-chymotrypsin than did TNS and indole and exhibited a protein–ligand conformational change subsequent to initial complexation. The substrate, N^{α}-acetyl-L-dinitrotyrosine ethyl ester, with all the requisite enzyme interactions for efficient hydrolysis, showed two conformational changes following initial complexation. Thus, the number of intermediate steps prior to covalent bond formation was found to be dependent on the complexity of the ligand. Conceivably, more than three steps would be associated with the complexation of α-chymotrypsin with a larger substrate, such as a protein.

The data presented (Table II) indicate a substantial difference between the dissociation rates (k_{-1}) of a substrate, DNTEE, and of inhibitors (TNS, proflavin, and indole). The more rapid (10–100 times) dissociation rate of DNTEE is in agreement with proposals of induced strain in bound substrates, which leads toward the transition state complex between E·S and E·P (the Circe effect).

The present data, obtained in aqueous systems near room temperature, have increased the assurance that results obtained at subzero temperatures in 65% dimethyl sulfoxide have va-

lidity for enzyme-catalyzed reactions. They further argue strongly for Fink's proposal that each of the observed changes subsequent to initial enzyme–substrate complexation are indicative of subtle conformational changes in the enzyme–substrate complex that maximize interactions between the enzyme and substrate thereby leading toward the transition state complex between E·S and E·P. They further offer direct proof of induced fit between substrate and enzyme in an enzyme once considered to have a rigidly fixed active site (Bender et al., 1966).

Temperature-jump perturbation appears to offer the means to determine directly much important information about the dynamics of enzyme–substrate complexation, a process that heretofore has been studied by indirect methods and/or at equilibrium. Such studies should permit one to determine the contribution of the complexation and subsequent conformational changes to the overall activation energy of the catalytic process (the Circe effect).

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Rose Bengal Mediated Inhibition of DNA Polymerases: Mechanism of Inhibition of Avian Myeloblastosis Virus Reverse Transcriptase under Nonoxidative Conditions†

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ABSTRACT: DNA polymerases from eukaryotic, prokaryotic, and retroviral sources exhibit strong sensitivity (I_{50} = 5–8 μM) to Rose Bengal dye under dark (nonoxidative) conditions. The mechanism of inhibition by Rose Bengal was investigated by using avian myeloblastosis virus reverse transcriptase as a test enzyme. Rose Bengal has been found to inhibit both polymerization and reverse transcriptase associated ribonuclease H activity. The inhibition by Rose Bengal is completely reversible, the degree of inhibition being dependent on the final concentration of Rose Bengal in the reaction mixture. The kinetic analyses indicated that the inhibition is competitive with respect to substrate deoxynucleoside triphosphate and noncompetitive with respect to template-primer. The protection against Rose Bengal inhibition is afforded strictly by deoxynucleoside triphosphate that is complementary to template nucleotide. The addition of Rose Bengal to an ongoing reaction consistently inhibited DNA synthesis after a short time lag. Subsequently, studies on the kinetics of polymerization carried out by varying template to primer ratio and incubation temperature indicated that the primary action of Rose Bengal on the ongoing reaction is the prevention of reinitiation, while elongation of chains that were already initiated was unaffected. The effect of Rose Bengal on the template binding function of reverse transcriptase, using a Millipore filter binding assay procedure, provided further insight into its mechanism of inhibition, for pretreatment of enzyme with dye completely abolished the ability of the enzyme to bind to the template-primer while the stability of preformed enzyme–template complex was unaffected by Rose Bengal addition. The preliminary spectrophotofluorometric analyses of enzyme–Rose Bengal complexes indicated that the major site of Rose Bengal reactivity resides in a hydrophobic domain of the enzyme molecule, implicating this region as being responsible for stabilizing the binding of enzyme to template.

The enzymatic synthesis of DNA is a complex process and can be divided into the following steps: (a) the binding of enzyme to template-primer, (b) the binding of complementary deoxyribonucleoside triphosphate (dNTP)[1] residue to enzyme followed by the first phosphodiester bond formation between the primer terminus and bound dNTP (a process called initiation), and (c) the subsequent linking of complementary dNTPs, to form a polydeoxynucleotide (a process called elongation). In order to better understand the mechanism of catalysis of DNA synthesis and to define the structure–function relationship of DNA polymerases, we have begun an extensive characterization of various catalytic reactions excecuted by DNA polymerases with avian myeloblastosis virus (AMV) reverse transcriptase (RT) as a model DNA polymerase (Modak & Marcus, 1977a,b; Marcus et al., 1978; Modak & Srivastava, 1979; Srivastava & Modak, 1980a,b). We have developed reagents for the site-specific labeling of DNA polymerases that not only serve as active-site probes but also aid in unraveling intricacies of the enzymatic process of DNA synthesis (Srivastava et al., 1981; Modak et al., 1980). We have shown that pyridoxal 5′-phosphate is a specific inhibitor of dNTP binding and that it may be linked to a lysine residue

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[1] Abbreviations: AMV, avian myeloblastosis virus; dNTP, deoxynucleoside triphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; RB, Rose Bengal; RNase H, ribonuclease H; RT, reverse transcriptase.

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