Catalytic Competence of Human α- and γ-Thrombin in the Activation of Fibrinogen and Factor XIII†

Sidney D. Lewis,‡ Laszlo Lorand,§ John W. Fenton II,‖ and Jules A. Shafer*,‡

Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109, Department of Biochemistry and Molecular and Cell Biology, Northwestern University, Evanston, Illinois 60201, and Wadsworth Center for Laboratories and Research, New York State Department of Health, Empire State Plaza, Albany, New York 12201

Received May 7, 1987; Revised Manuscript Received July 10, 1987

ABSTRACT: Steady-state kinetic parameters were compared for the action of α- and γ-thrombin on the physiologically important thrombin substrates fibrinogen and factor XIII at 37 °C, pH 7.4, and 0.14 M NaCl. γ-Thrombin, an α-thrombin derivative proteolytically cleaved at R-B73 and K-B154, was observed to catalyze the release of fibrinopeptide A (FPA) from fibrinogen with a specificity constant (k_{cat}/K_m) of 5×10^3 M^{-1} s^{-1}. This value was ~2400-fold lower than the specificity constant for the corresponding α-thrombin-catalyzed reaction. The low specificity constant was attributed to an increase in K_m and a decrease in k_{cat} for γ-thrombin-catalyzed release of FPA from fibrinogen. Conversion of α-thrombin to γ-thrombin also resulted in an ~800-fold reduction in the specificity constant for thrombin-catalyzed release of fibrinopeptide B (FPB) from fibrin I, as well as a loss in discriminatory power. Whereas α-thrombin preferentially released FPA from intact fibrinogen, γ-thrombin released FPA and FPB from intact fibrinogen at similar rates. In contrast to the large difference in specificity constants observed for α- and γ-thrombin catalysis with fibrin(ogen) as substrate, the specificity constant (2.6×10^4 M^{-1} s^{-1}) observed for γ-thrombin-catalyzed release of activation peptide from factor XIII was only 5-fold lower than the corresponding value for the α-thrombin-catalyzed reaction. Additionally, the promotion of factor XIII activation by fibrin characteristic of the α-thrombin-catalyzed reaction did not occur in the γ-thrombin-catalyzed reaction. These observations suggested that little of the ~2400-fold reduction in specificity constant for the γ-thrombin-catalyzed release of FPA from fibrinogen was due to decreased access of the active site of γ-thrombin to polypeptide substrates per se, but rather the low activity of γ-thrombin toward fibrinogen and fibrin I probably reflected a disrupted fibrin(ogen) recognition domain.

α-Thrombin is the serine proteinase in the blood-clotting cascade responsible for converting circulating fibrinogen to the insoluble fibrin matrix of blood clots. [See Blombäck (1979), Doolittle (1984), and Shafer and Higgins (1988) for reviews of thrombin-catalyzed activation of fibrinogen during the terminal stage of the blood-clotting cascade.] Human α-thrombin is comprised of a 36-aminoacyl residue A chain linked via a disulfide bond to a 259 aminoacyl residue B chain (Walz et al., 1974; Butkowski et al., 1977; Thompson et al., 1977; Degen et al., 1983).

Treatment of human α-thrombin with immobilized bovine trypsin results in limited proteolysis in the B chain at R-B73 and K-B154 (as judged by Edman degradation of the resulting protein) to yield γ-thrombin, which is comprised of three noncovalently associated polypeptide segments (Fenton et al., 1977b). These polypeptide segments consist of B1–73, B74–154 disulfide linked to A1–36, and B155–259, assuming that the segment comprising B1–73 was not truncated by as yet undetected further tryptic cleavages in the R- and K-rich region delineated by B62–73. Interestingly, autolysis of α-thrombin at pH >7 yields thrombin derivatives similar in structure and catalytic properties to the derivative obtained by treatment of α-thrombin with immobilized trypsin (Fenton et al., 1977b, 1979; Thompson et al., 1977). Autolysis at pH 8.0 in 50 mM ammonium bicarbonate solution at 25 °C has been shown to yield as primary products the γ-thrombin derivative proteolytically cleaved at R-B73 and K-B154 and the γ-thrombin derivative proteolytically cleaved at R-B70 and K-B154 (Chang, 1986). A γ-thrombin derivative corresponding to α-thrombin (des-B63–73, des-B124–154) has been isolated after autolysis of α-thrombin at pH 8.6 (Boissel et al., 1984).

Despite the fact that each residue comprising the serine proteinase HDS-catalytic triad (H-B43, D-B97, and S-B205) in the γ-thrombin derivatives resides in a different polypeptide segment, the catalytic activity of γ-thrombin is similar to that of α-thrombin toward many small ester and amide substrates [e.g., Chang et al. (1979), Lottenberg et al. (1982), and Sonder and Fenton (1986)]. This observation suggests that the relative spatial proximity of the catalytic triad is maintained in γ-thrombin. Human γ-thrombin, however, is less active than α-thrombin by a factor of well over 1000 in the conversion of fibrinogen to fibrin (Fenton et al., 1977b, 1979).

Conversion of fibrinogen to fibrin is initiated by α-thrombin-catalyzed hydrolysis at R-Aα16 in the two Aα-chains of fibrinogen to form fibrinopeptide A (FPA)[1,2] and fibrin I

† This research was supported by National Institutes of Health Grants HL 32006 (J.A.S.), HL 02212 (L.L.), and HL 13160 (J.W.F.) and by U.S. Public Health Service Career Award HL 03512 (L.L.).

‡ The University of Michigan.
§ Northwestern University.
‖ New York State Department of Health.

[1] Abbreviations: AP, activation peptide, the 37 aminoacyl residue peptide released when thrombin cleaves factor XIII at R-a37; FPA, fibrinopeptide A, the 16 aminoacyl residue peptide released when thrombin cleaves fibrinogen at R-Aα16; FPB, fibrinopeptide B, the 14 aminoacyl residue peptide released when thrombin cleaves fibrinogen at R-Bβ14; fibrin I, des-FPA-fibrinogen; fibrin II, des-FPA, des-FPB-fibrinogen; PEG, poly(ethylene glycol); pna, *p*-nitroanilide; tos, *p*-toluenesulfonyl; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

0006-2960/87/0426-7597$01.50/0

monomer (Blombäck et al. 1978; Hantgan & Hermans, 1979; Lewis et al., 1985b, and work cited therein). Upon formation, fibrin I monomers rapidly assemble in an end to end fashion to fibrin I protofibrils. Formation of fibrin I protofibrils is followed by α-thrombin-catalyzed hydrolysis at R-Bβ14 to release fibrinopeptide B (FPB) from the amino terminus of each of the two Bβ-chains of fibrin. Although both fibrin I and fibrin II form fibrous gels, they are unstable as blood clots. Fibrin fibers are stabilized by factor XIIIa-catalyzed formation of γ-glutamyl-ϵ-lysine peptide cross-links between adjacent fibrin units as well as between fibrin and other plasma proteins. Factor XIIIa itself is produced from its zymogen via a multistep reaction initiated by thrombin-catalyzed hydrolysis at R-a37 near the amino terminus of each of the two a-chains of factor XIII zymogen (Lorand & Konishi, 1964; Takagi & Doolittle, 1974; Lorand et al., 1974; Curtis et al., 1974; Ichinose et al., 1986; Takahashi et al., 1986; Grundmann et al., 1986).

In the present study, kinetic parameters were determined for the α- and γ-thrombin-catalyzed hydrolyses of fibrinogen and factor XIII in the blood-clotting cascade. The kinetic parameters reported in the present study are consistent with the notion that a structural domain altered in the conversion of α-thrombin to γ-thrombin (by treatment with immobilized bovine trypsin) is an important determinant of the affinity of thrombin for fibrinogen as well as the ability of thrombin to discriminate between the scissile bonds at R-Aα16 and R-Bβ14 in fibrinogen.

Experimental Procedures

Materials. Previously described procedures were used to prepare human fibrinogen (Lewis & Shafer, 1984), fibrin I monomer (Lewis et al., 1985a), and factor XIII (Lorand et al., 1981). Human α-thrombin (99.35% α, 0.65% β, 0% γ), 25 thrombin units/μg [assayed according to the method of Lewis and Shafer (1984)], and γ-thrombin (0.01% α, 2.67% β, 97.32% γ), 0.01 thrombin unit/μg, were prepared as described elsewhere (Fenton et al., 1977a; Bing et al., 1977). Values of kinetic parameters reported in this work are based upon the concentration of α- and γ-thrombin as determined from absorbance at 280 nm with an $E^{1\%}_{280}$ of 18.3 in 0.1 N NaOH and an M_r of 36500 (Fenton et al., 1977a). No corrections were made for the fact that active-site titrations (Chase & Shaw, 1970) of α- and γ-thrombin yielded concentrations that were respectively 93% and 77.5% of that expected from absorbance measurements. Hirudin (100 units/mL) and aprotinin (13 TIU/mg) were from Sigma. Trypsin–TPCK (270 units/mg) was from Worthington. tos-GPR-pna (Chromzym-TH) was from Boehringer Mannheim.

Reaction Kinetics. The rates of release of FPA and FPB and activation peptide (AP) were monitored at 37 °C, pH 7.4, 9.5 mM sodium phosphate, 137 mM NaCl, 2.5 mM KCl, and 0.1% PEG-6000 as described previously (Higgins et al., 1983; Janus et al., 1983). PEG-6000 (0.1%) was used in all kinetic studies to retard adsorption of thrombin on surfaces (Wasiewski et al., 1976; Latallo & Hall, 1986). In experiments wherein hirudin and aprotinin were used, the inhibitors were preincubated for ≥20 min with the enzyme before the enzyme–inhibitor solution was added to thermally equilibrated (37 °C) reaction mixtures. Initial rates of release of *p*-nitroaniline from the tos-GPR-pna (Chromzym-TH) were measured under the conditions of Lottenberg et al. (1982). In a typical experiment the release of *p*-nitroaniline was followed at 405 nm at 25 °C in a buffered solution (pH 7.8) containing 178 μM substrate after addition of a solution of α-thrombin, γ-thrombin, or trypsin at a final enzyme concentration of 0.19 nM.

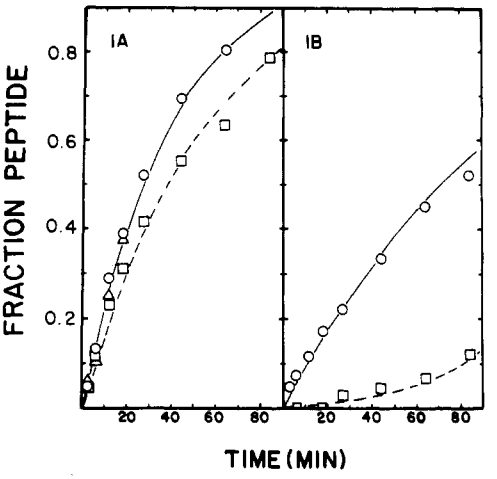

FIGURE 1: Comparison of γ-thrombin-catalyzed (A) and α-thrombin-catalyzed (B) release of FPA and FPB from fibrin(ogen) at 37 °C, pH 7.4, 9.5 mM sodium phosphate, 137 mM NaCl, 2.5 mM KCl, and 0.1% PEG-6000. (A) 89.4 nM γ-thrombin with the following substrates: 0.30 μM fibrinogen (○, FPA; □, FPB), 0.1 μM fibrin I (△, FPB). The solid and dashed lines are those predicted from eq 3 with k_{cat}/K_m set at 4.8×10^3 M^{-1} s^{-1} (solid line) and 3.6×10^3 M^{-1} s^{-1} (dashed line). (B) 0.0151 nM α-thrombin with 0.30 μM fibrinogen (○, FPA; □, FPB). The solid lines are those predicted by eq 1 and 2 for the release of FPA and FPB with $k_{catA}/K_{mA} = 1.2 \times 10^7$ M^{-1} s^{-1} and $k_{catB}/K_{mB} = 4.2 \times 10^6$ M^{-1} s^{-1}.

Results and Discussion

Figure 1A depicts the time course for γ-thrombin-catalyzed hydrolysis of fibrinogen at R-Aα16 and R-Bβ14 and concomitant release of FPA and FPB. The corresponding α-thrombin-catalyzed process determined in a parallel experiment is illustrated in Figure 1B to facilitate comparison of the activity of these enzymes. In comparing the γ-thrombin- and α-thrombin-catalyzed release of fibrinopeptides, it is important to note that the concentration of γ-thrombin (Figure 1A) was 6000 times that of α-thrombin (Figure 1B). It was necessary to use a higher concentration of γ-thrombin to compensate for its lower catalytic activity toward fibrinogen so that similar time scales could be used to monitor fibrinopeptide release. Control experiments (data not shown) indicated that rates of release of fibrinopeptides were proportional to the enzyme concentration with both α- and γ-thrombin, as reflected by the fact that samples differing only with respect to the concentration of a particular form of thrombin exhibited equivalent extents of release of fibrinopeptide when the product obtained by multiplying the reaction time by the thrombin concentration was equal for both samples.

Previous studies (Higgins et al., 1983; Lewis et al., 1985b) of α-thrombin-catalyzed activation of fibrinogen have dem-

[2] Although FPA is preferentially released from intact fibrinogen, good evidence has been presented indicating that thrombin also catalyzes the release of FPB from intact human fibrinogen (Hanna et al., 1984). The specificity constant for α-thrombin-catalyzed release of FPB from intact fibrinogen has been reported as being >30 times lower than that for release of FPA under the conditions (37 °C, pH 7.4, 9.5 mM sodium phosphate, 137 mM NaCl, 2.5 mM KCl) used for the present experiments (Higgins et al., 1983). Hanna et al. (1984) have reported a value of 5 for the ratio of the specificity constants for the release of FPA and FPB from intact fibrinogen at 25 °C, pH 8.0, 0.05 M sodium phosphate, and 0.15 M KCl. The difference between the reported values for the specificity constant ratios might be a consequence of differences in experimental conditions.

onstrated that the concentrations of ≤0.6 μM fibrinogen Aα-chains used for the experiments depicted in Figure 1 are well below K_m, so that the α-thrombin-catalyzed release of FPA should follow the first-order rate law

$$[FPA]/[FPA]_f = 1 - \exp(-k_1 t) \quad (1)$$

The earlier studies with α-thrombin also demonstrated that the α-thrombin-catalyzed release of FPB is characterized by a lag (see also Figure 1B), occurs predominantly after FPA release, and follows the rate law

$$\frac{[FPB]}{[FPB]_f} = 1 + \frac{k_2 \exp(-k_1 t)}{k_1 - k_2} - \frac{k_1 \exp(-k_2 t)}{k_1 - k_2} \quad (2)$$

for the appearance of the second product of a consecutive first-order reaction of the type

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

In eq 1 and 2, the subscript f denotes concentrations when the reaction is complete and k_1 and k_2 are the products of the thrombin concentration and the specificity constant (k_{catA}/K_{mA}, k_{catB}/K_{mB}) for α-thrombin-catalyzed hydrolysis at R-Aα16 and R-Bβ14, respectively. As shown in Figure 1B, the previously reported values (Higgins et al., 1983) of 1.2×10^7 and 4.2×10^6 M^{-1} s^{-1} for k_{catA}/K_{mA} and k_{catb}/K_{mB}, respectively, yield good fits (solid lines) of the experimental data to eq 1 and 2. The lag in the α-thrombin-catalyzed release of FPB (but not FPA) was shown by Higgins et al. (1983) and Lewis et al. (1985b) to indicate that the scissile bond at R-Bβ14 in fibrin I is a better substrate for α-thrombin than the scissile bond at R-Bβ14 in intact fibrinogen.[2] In particular, the specificity constant for α-thrombin-catalyzed release of FPB from fibrin I polymer (4.2×10^6 M^{-1} s^{-1}) is >10 times larger than that for the release of FPB from intact fibrinogen. The fact that the γ-thrombin-catalyzed release of FPB does not exhibit a lag (Figure 1A) implies that γ-thrombin does not significantly distinguish between the R-Bβ14 residues in fibrin I and intact fibrinogen. This conclusion was verified in an experiment wherein a solution of fibrin I monomer in 0.02 M acetic acid was brought to pH 7.4 (where fibrin I rapidly polymerizes) and incubated with γ-thrombin. As shown in Figure 1A (triangles), FPB is released from this sample of fibrin I polymer <1.4-fold faster than it is released from intact fibrinogen (squares).

The data in Figure 1A also indicate that the rates of γ-thrombin-catalyzed release of FPA and FPB from intact fibrinogen are within a factor of 1.4 of each other. The observation of similar rates of γ-thrombin-catalyzed release of FPA and FPB from fibrinogen contrasts with the finding that the rate of the α-thrombin-catalyzed release of FPA from intact fibrinogen is at least 30-fold greater than that of FPB under these experimental conditions (Higgins et al., 1983).[2] Thus, γ-thrombin also has lost much of its ability to discriminate between R-Aα16 and R-Bβ14 in intact fibrinogen.

The similar catalytic activity of γ-thrombin toward fibrinogen and fibrin I is consistent with the first-order release of fibrinopeptides, i.e.

$$\text{fraction peptide} = 1 - \exp(k_{cat} e t / K_m) \quad (3)$$

depicted in Figure 1A, since the fibrinogen concentration used for these experiments was much smaller than K_m (see below). The fit of the time-dependent release of fibrinopeptides to eq 3 yielded values of 4.8×10^3, 4.8×10^3, and 3.6×10^3 M^{-1} s^{-1} for k_{cat}/K_m for the γ-thrombin-catalyzed release of FPA from fibrinogen, of FPB from fibrin I, and of FPB from fibrinogen, respectively. These values are 2–3 orders of magnitude lower than the values of the specificity constants for the corresponding α-thrombin-catalyzed reactions (Table I).

Table I: Kinetic Parameters for Thrombin-Catalyzed Release of FPA and AP[a]

substrate	enzyme	k_{cat}/K_m (M^{-1} s^{-1})
Fgn		
R-Aα16	α-T	1.2×10^7 [b]
R-Aα16	γ-T	4.8×10^3 [c]
R-Bβ14	α-T	$\leq 4.0 \times 10^5$
R-Bβ14	γ-T	3.6×10^3
Fbn I		
R-Bβ14	α-T	4.2×10^6
R-Bβ14	γ-T	4.8×10^3
FXIII		
R-a37	α-T	1.4×10^5 [d]
R-a37	γ-T	2.6×10^4

[a] Kinetic constants were determined at 37 °C, pH 7.4, 9.5 mM sodium phosphate, 137 mM NaCl, 2.5 mM KCl, and 0.1% PEG-6000. Values of K_m are based on the concentration of Aα-chains of fibrinogen and a-chains of factor XIII. Except where indicated by ~, a less than 10% error is estimated from standard deviations for the listed rate constants. Fgn, α-T, γ-T, Fbn I, and FXIII, respectively, denote fibrinogen, α-thrombin, γ-thrombin, fibrin I, and factor XIII. [b] For this reaction $K_m = 7.2$ μM and $k_{cat} = 84$ s^{-1}. [c] For this reaction $K_m \sim 330$ μM and $k_{cat} \sim 1.6$ s^{-1}.[e] [d] For this reaction $K_m \sim 84$ μM and $k_{cat} \sim 10$ s^{-1}.[e] [e] Due to the infeasibility of obtaining substrate concentrations greater than the value of K_m, for these determinations the listed individual values of K_m and k_{cat} should be viewed respectively as approximate lower and approximate upper limits. Since initial velocities are proportional to k_{cat}/K_m when $[S] \ll K_m$, accurate values for k_{cat}/K_m could be obtained.

In contrast to its low activity toward fibrinogen, γ-thrombin exhibited its well-documented high activity toward low molecular weight substrates. For example, when the low molecular weight peptide substrate tos-GPR-pna was used, γ-thrombin exhibited a catalytic efficiency similar to that of α-thrombin as previously reported (Lottenberg et al., 1982; Sonder & Fenton, 1986). The k_{cat} value of 1.7×10^2 s^{-1} we observed for both α- and γ-thrombin was in tolerable agreement with the k_{cat} values of 1.3×10^2 and 1.6×10^2 s^{-1} reported previously with this substrate (Lottenberg et al., 1982). Under our routine assay conditions (178 μM tos-GPR-pna) trypsin yielded a velocity of *p*-nitroaniline release about half that observed with an equivalent concentration of α-thrombin.

The γ-thrombin used in the present experiments was obtained from a solution of α-thrombin that had been passed through a column of immobilized trypsin. Thus, it was important to exclude the possibility that the putative activity of γ-thrombin toward fibrinogen was not due to contamination by unconverted α-thrombin or by trypsin adventitiously released from the resin. To resolve this issue, we determined the effect of the protease inhibitors, aprotinin and hirudin, on the catalytic activity of γ-thrombin. Studies of the effect of aprotinin and hirudin on the activity of trypsin, α-thrombin, and γ-thrombin toward tos-GPR-pna (a good substrate for all of these enzymes) established that aprotinin inhibited trypsin without significantly affecting the activity of γ-thrombin, and hirudin (under the assay conditions) inhibited α-thrombin without significantly affecting the activity of γ-thrombin. For example, a concentration of aprotinin that completely inactivated 0.19 nM trypsin or a concentration of hirudin that 95% inactivated 0.19 nM α-thrombin had no detectable effect (<5%) on the activity of 0.19 nM γ-thrombin toward tos-GPR-pna. Our ability to inactivate α-thrombin with hirudin without inactivating γ-thrombin is in accord with previous reports of a decreased inhibitory potency of hirudin toward γ-thrombin (Fenton, 1979).

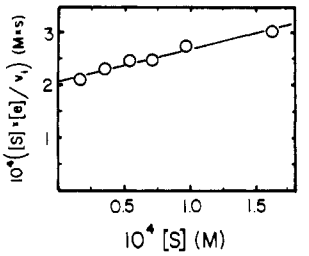

FIGURE 2: Dependence of initial velocity on substrate concentration for the γ-thrombin-catalyzed release of FPA from fibrinogen. Plotted on the x axis as [S] is the concentration of fibrinogen Aα-chains (18–160 μM). The solid line represents a least-squares fit to the equation $[S]e/V = ([S]/k_{cat}) + K_m/k_{cat}$ with $k_{cat} = 1.6\ s^{-1}$ and $K_m = 330\ \mu M$. See the legend to Figure 1 for experimental conditions.

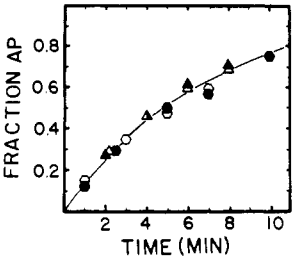

FIGURE 3: γ-Thrombin (93 nM) catalyzed release of AP from plasma factor XIII (0.1 μM). In the absence of fibrinogen (▲) or in the presence of 0.1 μM fibrinogen (△), 0.1 μM fibrin I (○), and 0.1 μM fibrin I and 1 mM $CaCl_2$ (●). The solid line represents the best fit of the data to the equation fraction AP = $1 - \exp(-k_{cat}et/K_m)$ with k_{cat}/K_m set at $2.6 \times 10^4\ M^{-1}\ s^{-1}$. See legend to Figure 1 for experimental conditions.

Having established that aprotinin and hirudin do not (under the assay conditions) significantly affect the activity of γ-thrombin toward tos-GPR-pna, we examined their effects on the activity of γ-thrombin toward fibrinogen. Incubation of γ-thrombin (94 nM) with either aprotinin (enough to neutralize 24 nM trypsin) or hirudin (enough to neutralize 47 nM α-thrombin) had no effect on the rate of γ-thrombin-catalyzed release of fibrinopeptides from 8.3 μM fibrinogen. In contrast to γ-thrombin, trypsin did not release FPA from fibrinogen but released another peptide with chromatographic properties distinct from those of FPA.[3] This peptide was not obtained when fibrinogen was treated with γ-thrombin. These observations (together with control experiments showing that aprotinin and hirudin, respectively, inhibit trypsin-catalyzed and α-thrombin-catalyzed release of peptides from fibrinogen) make it unlikely that the activity attributed to γ-thrombin is due to contaminating trypsin or α-thrombin.

To determine the reason for the large reduction in catalytic activity of γ-thrombin relative to that of α-thrombin, an attempt was made to evaluate the steady-state kinetic parameters K_m and k_{cat} from the dependence of the initial velocity of FPA release on the concentration of fibrinogen Aα-chains. Interpretation of the initial rate of FPA release on the concentration of Aα-chains in terms of the Michaelis–Menten equation predicts the linear relationship

$$S/V = K_m/(k_{cat}e) + S/(k_{cat}e) \qquad (4)$$

which, when used to fit the observed dependence of initial rate of FPA release on the concentration of fibrinogen Aα-chains, yielded estimates for k_{cat} and K_m (Figure 2). These steady-state kinetic parameters are compared in Table I (and the table footnotes) to those for α-thrombin. It is important to note that the value of K_m of 330 μM determined from the data in Figure 2 must be considered to be an approximate lower limit, since it was not feasible to obtain substrate concentrations high enough to permit measurements of initial velocities at substrate concentrations greater than 0.5 K_m. The data clearly indicate, however, that the conversion of α-thrombin to γ-thrombin is associated with a marked increase in K_m (~46-fold) for fibrinogen, suggesting that γ-thrombin has a lower affinity for fibrinogen than does α-thrombin.

Although it was not possible to obtain accurate values for k_{cat} and K_m, the ratio of these constants (k_{cat}/K_m) could be obtained since initial velocities are proportional to k_{cat}/K_m when $[S] \ll K_m$, so that the reciprocal of the Y intercept of the plot in Figure 2 should yield a reliable estimate of k_{cat}/K_m. This value of k_{cat}/K_m ($4.8 \times 10^3\ M^{-1}\ s^{-1}$) agrees with the value obtained for k_{cat}/K_m from the pseudo-first-order γ-thrombin-catalyzed release of FPA shown in Figure 1A. The extent to which the greater than 2400-fold reduction in k_{cat}/K_m on going from α-thrombin to γ-thrombin reflects a decrease in k_{cat} can only be approximated because of the uncertainty in our estimates of K_m and k_{cat}. The estimated value of 330 μM observed for K_m indicates that γ-thrombin-catalyzed release of FPA is characterized by a k_{cat} which could be as much as ~50-fold lower than the k_{cat} for the α-thrombin-catalyzed reaction. The decreased k_{cat} together with the increased K_m for fibrinogen suggests that γ-thrombin not only binds fibrinogen more weakly than does α-thrombin but that γ-thrombin also binds fibrinogen less productively. This conclusion is consistent with the notion that important determinants of both the strength and geometry of fibrinogen binding are lost in the conversion of α-thrombin to γ-thrombin. If we imagine that the fibrinogen binding modes are such that γ-thrombin expresses full activity (k_{cat} equals that for α-thrombin) or no activity ($k_{cat} = 0$), then a 50-fold decrease in k_{cat} estimated for γ-thrombin would imply that only 2% of the γ-thrombin–fibrinogen complexes are catalytically active and that the true K_m for formation of the catalytically active complex between γ-thrombin and fibrinogen is actually 50-fold higher than the value of 330 μM observed for the apparent K_m.[4] An equally plausible explanation for the decreased k_{cat} observed with γ-thrombin is that γ-thrombin binds fibrinogen in a single suboptimal orientation wherein the reactivity of the scissile bond at R-Aα16 is only 2% as reactive as it is when fibrinogen is bound to α-thrombin. The observation that γ-thrombin does not significantly discriminate between R-Aα16 and R-Bβ14 in intact fibrinogen and that γ-thrombin does not significantly discriminate between R-Bβ14 in fibrinogen and fibrin I also supports the view that an important part of a fibrin(ogen) recognition domain is disrupted in the conversion of α-thrombin to γ-thrombin.

To determine the extent to which the large difference in the activities of α- and γ-thrombin toward fibrinogen might reflect an intrinsic inability of γ-thrombin to process polypeptides the size of fibrinogen, we determined the activity of γ-thrombin toward plasma factor XIII, another physiologically important macromolecular thrombin substrate with a molecular weight similar to that of fibrinogen. Lorand and Credo (1977) demonstrated that both α- and γ-thrombin can activate factor XIII. No rates or kinetic parameters were reported, however, for the thrombin-catalyzed step in the activation pathway. The

[3] Studies by Hessel et al. (1982) of the effect of trypsin on fibrinogen indicate that trypsin probably releases a peptide corresponding to Aα1–19 instead of FPA. In addition to the peptide released in place of FPA, we observed release of FPB, as well as small quantities of other peptides, upon treatment of fibrinogen with trypsin.

[4] A discussion of the effects of nonproductive binding on steady-state kinetic parameters is given by Fersht (1985).

closed triangles in Figure 3 illustrate the time dependence for γ-thrombin-catalyzed hydrolysis at R-a37 near the amino terminus of each of the two a-chains of plasma factor XIII to release activation peptide (AP) comprised of residues a1–37 of plasma factor XIII zymogen. The best fit of the time dependence of AP release, shown by the solid line in Figure 3, to the relationship fraction peptide = $1 - \exp(-k_{cat}et/K_m)$ yielded a value of 2.6×10^4 M^{-1} s^{-1} for k_{cat}/K_m. Control experiments (data not shown) at a 2-fold higher factor XIII concentration than that used for the experiment depicted in Figure 3 (0.1 μM) gave a similar value for k_{cat}/K_m, suggesting that $K_m \gg 0.1$ μM for this reaction and that the analysis used to determine k_{cat}/K_m is appropriate. The value of 2.6×10^4 M^{-1} s^{-1} determined for the specificity constant for γ-thrombin-catalyzed release of activation peptide is ~5-fold less than the value of 1.4×10^5 M^{-1} s^{-1} reported (Janus et al., 1983) for α-thrombin-catalyzed release of AP. The fact that the specificity constant for γ-thrombin acting on factor XIII is only 5-fold lower than that of α-thrombin suggests that little of the ~2400-fold decrease in the specificity constant (for FPA release) produced upon conversion of α-thrombin to γ-thrombin can be ascribed to contraction in the active-site cleft and a concomitant decrease in the access of polypeptide substrates to the active site of γ-thrombin.

This conclusion is also supported by the observations of less than a 5-fold difference in rates for the reaction of α- and γ-thrombin with antithrombin III, a M_r 65000 protein (Chang et al., 1979; Latallo & Jackson, 1986). Thus, the ~46-fold increase in K_m for fibrinogen associated with the conversion of α-thrombin to γ-thrombin is in accord with previous proposals that α-thrombin contains a fibrinogen binding site distinct from the catalytic site and that this second site is lost when α-thrombin is converted to γ-thrombin (Magnusson, 1972; Fenton, 1981, 1986; Conery & Berliner, 1983; Berliner et al., 1985; Fenton & Bing, 1986). One might argue, however, that the structural reorganization associated with the conversion of α-thrombin to γ-thrombin constrains the specificity pocket at the active site so as to uniquely block the interaction of thrombin with the aminoacyl residues in the vicinity of the scissile bond of R-Aα16 and R-Bβ14 of fibrinogen.[5] Although the extent and effect of putative structural alterations in the fibrinopeptide binding domain of γ-thrombin remain to be evaluated, studies (see below) of the effects of fibrin on the thrombin-catalyzed activation of factor XIII suggest that a fibrin(ogen) recognition domain in α-thrombin distinct from the fibrinopeptide binding domain is altered in γ-thrombin.

The α-thrombin-catalyzed hydrolysis at R-a37 in factor XIII which exhibits a K_m value ~4000-fold higher than the plasma concentration of factor XIII (~20 nM) would be inefficient if it were not promoted by non-cross-linked fibrin (Janus et al., 1983; Lewis et al., 1985a). Evidence has been presented suggesting that the promoter activity of non-cross-linked fibrin probably resides in its ability to reduce the K_m for catalysis (Lewis et al., 1985a). Promotion of α-thrombin-catalyzed activation of factor XIII by fibrin has been proposed to serve the function of ensuring that factor XIII is not converted to factor XIIIa until the factor XIIIa substrate, non-cross-linked fibrin, has formed (Lewis et al., 1985a).

Examination of the data in Figure 3 reveals that neither fibrin I nor fibrinogen promotes the γ-thrombin-catalyzed release of AP from factor XIII zymogen. Fibrin I, however, promotes α-thrombin-catalyzed release of AP, and fibrinogen, after being converted to fibrin I, promotes the α-thrombin-catalyzed reaction (Lewis et al., 1985). The data in Figure 3 also show that Ca^{2+}, a cofactor for factor XIIIa, had no effect on γ-thrombin-catalyzed release of AP in the presence of non-cross-linked fibrin I. Ca^{2+}, however, inhibits α-thrombin-catalyzed release of AP from factor XIII in the presence of non-cross-linked fibrin I (Lewis et al., 1985a). This inhibition appears to be a consequence of the fact that factor XIIIa formed in the early phase of the reaction is catalytically competent when Ca^{2+} is present. Thus, upon its formation, factor XIIIa cross-links the fibrin and thereby inactivates it as a promoter of the α-thrombin-catalyzed reaction (Lewis et al., 1985a). This mode of control may serve to ensure that production of additional factor XIIIa does not occur after factor XIIIa activity is expressed.

The failure of non-cross-linked fibrin I to promote γ-thrombin-catalyzed release of AP is consistent with the recent report (Greenberg et al., 1987) that fibrin II has no effect on the time dependence of the appearance of factor XIIIa activity when factor XIII is treated with γ-thrombin, since fibrin I and II are equally efficient in promoting α-thrombin-catalyzed release of AP from factor XIII (Lewis et al., 1985a). The simplest explanation for the inability of the γ-thrombin-catalyzed release of AP from factor XIII to be promoted by non-cross-linked fibrin is that a fibrin(ogen) recognition domain present in α-thrombin (but not in γ-thrombin) interacts with the fibrin in the fibrin–factor XIII complex, thereby reducing the K_m for α-thrombin-catalyzed hydrolysis at R-a37. Since the FPA and FPB domains of fibrinogen are absent in fibrin II, this fibrin(ogen) recognition domain is probably distinct from the fibrinopeptide binding domain of thrombin.

Studies of thrombin derivatives spin-labeled in the vicinity of S-B205 indicate that certain domains of the active site of γ-thrombin may be more constrained than the corresponding domains in α-thrombin (Berliner et al., 1981). This difference might account for the 5-fold reduction in activity of γ-thrombin toward factor XIII, a macromolecular substrate that presumably does not interact with the fibrin(ogen) recognition domain of α-thrombin. Although the trypsin-catalyzed conversion of human α-thrombin to γ-thrombin is associated with a substantial change in secondary structure (as reflected by circular dichroism spectra) (Villanueva, 1981), it is not clear whether the altered structure and specificity of γ-thrombin reported in the present study reflect proteolysis at R-B73, K-B154, or both sites. In this regard, it should be noted that the fibrin(ogen) recognition domain of α-thrombin appears to be positively charged (Chang, 1983; Conery & Berliner, 1983; Berliner et al., 1985; Fenton, 1986; Fenton & Bing, 1986) and that Chang (1986) has proposed that this site is composed of the cationic clusters of R and K residues in the region (B62–73) adjacent to R-B73.

Interestingly, bovine β-thrombin (prepared by treating bovine α-thrombin with immobilized trypsin) has a similar activity toward small amide substrates but less than 0.1% clotting activity relative to that of intact bovine α-thrombin. Since bovine β-thrombin lacks a residue homologous to K-B154 and is presumably cleaved only in the vicinity of R-B73 (Sonder & Fenton, 1986), the low clotting activity of bovine β-thrombin is consistent with the view that the region adjacent to R-B73 is an important part of the fibrinogen recognition domain of

[5] The extent to which interactions with these regions of fibrinogen are altered in γ-thrombin might be assessed by comparison of steady-state kinetic parameters for α-thrombin-catalyzed hydrolysis of fibrinogen fragments (containing the R-Aα16 and/or R-Bβ14 domains), reported by Scheraga and his co-workers [see Hanna et al. (1984) and work cited therein], to the steady-state kinetic parameters for the analogous γ-thrombin-catalyzed reactions.

α-thrombin. Surprisingly, however, an autolyzed form of bovine β-thrombin, shown by Lundblad et al. (1979) to be homologous to human des(R-B66–73)-α-thrombin (i.e., α-thrombin with a truncated A chain), has been observed to have a K_m for fibrinogen similar to that of bovine α-thrombin (Lundblad et al., 1984). This result contrasts with the observation reported in the present work that a ~46-fold increase in K_m for fibrinogen is associated with the conversion of human α-thrombin to γ-thrombin. The different kinetic properties between bovine β-thrombin and human γ-thrombin suggest that the fibrin(ogen) recognition domain of α-thrombin may not be restricted to the vicinity of R-B73. The finding that the cleavage of human α-thrombin at A-B150 by human neutraphil elastase yields a thrombin derivative having a near normal activity toward a small amide substrate, but a substantially reduced clotting activity relative to that of intact α-thrombin (Brower et al., 1987), also suggests that the fibrin(ogen) recognition domain of human α-thrombin may not be restricted to B62–73.

Registry No. Thrombin, 9002-04-4; blood coagulation factor XIII, 9013-56-3.

REFERENCES

Berliner, L. J., Bauer, R. S., Chang, T.-L., Fenton, J. W., II, & Shen, Y. L. (1981) *Biochemistry 20*, 1831–1837.

Berliner, L. J., Sugawara, Y., & Fenton, J. W., II (1985) *Biochemistry 24*, 7005–7009.

Bing, D. H., Cory, M., & Fenton, J. W., II (1977) *J. Biol. Chem. 252*, 8027–8034.

Blombäck, B. (1979) in *Plasma Proteins* (Blombäck, B., & Hanson, L. A., Eds.) pp 223–253, Wiley, New York.

Blombäck, B., Hessel, B., Hogg, D., & Therkildsen, L. (1978) *Nature (London) 275*, 501–505.

Boissel, J. P., LeBonniec, B., Rabiet, M. J., Labie, D., & Elion, J. (1984) *J. Biol. Chem. 259*, 5691–5697.

Brower, M. S., Walz, D. A., Garry, K. E., & Fenton, J. W., II (1987) *Blood 69*, 813–819.

Butkowski, R. J., Elion, J., Downing, M. R., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 4942–4957.

Chang, J.-Y. (1983) *FEBS Lett. 164*, 307–313.

Chang, J.-Y. (1986) *Biochem. J. 240*, 797–802.

Chang, T.-L., Feinman, R. D., Landis, B. H., & Fenton, J. W., II (1979) *Biochemistry 18*, 113–119.

Chase, T., Jr., & Shaw, E. (1970) *Methods Enzymol. 19*, 20–27.

Conery, B. G., & Berliner, L. J. (1983) *Biochemistry 22*, 369–375.

Curtis, C. G., Brown, K. L., Credo, R. B., Domanik, R. A., Gray, A., Stenberg, P., & Lorand, L. (1974) *Biochemistry 13*, 3774–3780.

Degen, S. J. F., McGillivray, R. T. A., & Davie, E. W. (1983) *Biochemistry 22*, 2087–2097.

Doolittle, R. F. (1984) *Annu. Rev. Biochem. 53*, 195–229.

Fenton, J. W., II (1981) *Ann. N.Y. Acad. Sci. 370*, 468–495.

Fenton, J. W., II (1986) *Ann. N.Y. Acad. Sci. 485*, 5–15.

Fenton, J. W., II, & Bing, D. H. (1986) *Semin. Thromb. Hemostasis 12*, 200–208.

Fenton, J. W., II, Fasco, M. J., Stackrow, A. B., Aronson, D. L., Young, A. M., & Finlayson, J. S. (1977a) *J. Biol. Chem. 252*, 3587–3598.

Fenton, J. W., II, Landis, B. H., Walz, D. A., & Finlayson, J. S. (1977b) in *Chemistry and Biology of Thrombin* (Lundblad, R. L., Fenton, J. W., II, & Mann, K. G., Eds.) pp 43–70, Ann Arbor Science, Ann Arbor, MI.

Fenton, J. W., II, Landis, B. H., Walz, D. A., Bing, D. H., Feinman, R. D., Zabinski, M. P., Sonder, S. A., Berliner, L. J., & Finlayson, J. S. (1979) in *The Chemistry and Physiology of Human Plasma Proteins* (Bing, D. H., Ed.) pp 151–183, Pergamon, Elmsford, NY.

Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., pp 109–111, W. H. Freeman, New York.

Greenberg, C. S., Achyuthan, K. E., & Fenton, J. W., II (1987) *Blood 69*, 867–871.

Grundmann, U., Amann, E., Zettlmeissl, G., & Kupper, H. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8024–8028.

Hanna, L. S., Scheraga, H. A., Francis, C. W., & Marder, V. J. (1984) *Biochemistry 23*, 4681–4687.

Hantgan, R. R., & Hermans, J. (1979) *J. Biol. Chem. 254*, 11272–11281.

Hessel, B., Blombäck, B., & Soderman, S. (1982) in *Fibrinogen: Recent Biochemical and Medical Aspects* (Henschen, A., Graeff, H., & Lottspeich, F., Eds.) pp 101–108, de Gruyter, Berlin.

Higgins, D. L., Lewis, S. D., & Shafer, J. A. (1983) *J. Biol. Chem. 258*, 9276–9282.

Ichinose, A., Hendrickson, L. E., Fujikawa, K., & Davie, E. W. (1986) *Biochemistry 25*, 6900–6906.

Janus, T. J., Lewis, S. D., Lorand, L. L., & Shafer, J. A. (1983) *Biochemistry 22*, 6269–6272.

Latallo, Z. S., & Hall, J. A. (1986) *Thromb. Res. 43*, 507–521.

Latallo, Z. S., & Jackson, C. M. (1986) *Thromb. Res. 43*, 523–537.

Lewis, S. D., & Shafer, J. A. (1984) *Thromb. Res. 35*, 111–120.

Lewis, S. D., Janus, T. J., Lorand, L. L., & Shafer, J. A. (1985a) *Biochemistry 24*, 6772–6777.

Lewis, S. D., Shields, P. P., & Shafer, J. A. (1985b) *J. Biol. Chem. 260*, 10192–10199.

Lorand, L., & Konishi, K. (1964) *Arch. Biochem. Biophys. 105*, 58–67.

Lorand, L., & Credo, R. B. (1977) in *Chemistry and Biology of Thrombin* (Lundblad, R. L., Fenton, J. W., II, & Mann, K. G., Eds.) pp 311–323, Ann Arbor Science, Ann Arbor, MI.

Lorand, L., Gray, A. J., Brown, K. Credo, R. B., Curtis, C. G., Domanik, R. A., & Stenberg, P. (1974) *Biochem. Biophys. Res. Commun. 56*, 914–922.

Lorand, L., Credo, R. B., & Janus, T. J. (1981) *Methods Enzymol. 55*, 333–341.

Lottenberg, R., Hall, J. A., Fenton, J. W., II, & Jackson, C. M. (1982) *Thromb. Res. 28*, 313–332.

Lundblad, R. L., Noyes, C. M., Mann, K. G., & Kingdon, H. S. (1979) *J. Biol. Chem. 254*, 8524–8528.

Lundblad, R. L., Nesheim, M. E., Straight, D. L., Sailor, S., Bowie, J., Jenzano, J. W., Roberts, J. D., & Mann, K. G. (1984) *J. Biol. Chem. 259*, 6991–6995.

Magnusson, S. (1971) *Enzymes (3rd Ed.) 3*, 277, 321.

Shafer, J. A., & Higgins, D. L. (1988) *CRC Crit. Rev. Clin. Lab. Sci.* (in press).

Sonder, S. A., & Fenton, J. W., II (1986) *Clin. Chem. (Winston-Salem, N.C.) 32*, 934–937.

Takagi, T., & Doolittle, R. F. (1974) *Biochemistry 13*, 750–756.

Takahashi, N., Takahashi, Y., & Putnam, F. W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8019–8023.

Thompson, A. R., Enfield, D. L., Ericsson, L. M., Legaz, M. E., & Fenton, J. W., II (1977) *Arch. Biochem. Biophys. 178*, 356–367.
Villanueva, G. B. (1981) *Biochemistry 20*, 6519–6525.
Walz, D. A., & Seegers, W. H. (1974) *Biochem. Biophys. Res. Commun. 60*, 717–722.
Wasiewski, W., Fasco, M. J., Martin, B. M., Detwiler, T. C., & Fenton, J. W., II (1976) *Thromb. Res. 8*, 881–886.

Complex of α-Chymotrypsin and *N*-Acetyl-L-leucyl-L-phenylalanyl Trifluoromethyl Ketone: Structural Studies with NMR Spectroscopy†

Tzyy-Chyau Liang and Robert H. Abeles*
Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254
Received May 6, 1987; Revised Manuscript Received July 8, 1987

ABSTRACT: A dipeptidyl trifluoromethyl ketone, *N*-acetyl-L-leucyl-L-[1-^{13}C]phenylalanyl trifluoromethyl ketone, was synthesized. This compound inhibits chymotrypsin with K_i = 1.2 μM [Imperiali B., & Abeles, R. H. (1986) *Biochemistry 25*, 3760–3767]. The complex formed between this inhibitor and α-chymotrypsin was examined with ^{1}H, ^{13}C, and ^{19}F NMR spectroscopy to establish its structure in solution. The keto group of the trifluoro ketone is present as an ionized hemiketal group as deduced from the comparison of its ^{13}C chemical shift with those of model hemiketals. The pK_a of the hemiketal hydroxyl in the complex is approximately 4.9, which is about 4.2 units lower than the pK_a of model hemiketals. This observation provides direct evidence that serine proteases are able to stabilize the oxyanions of tetrahedral adducts. Evidence is also presented for the presence of an Asp–His H bond and protonation of the imidazole group of His-57 in the tetrahedral adduct. The pK_a of His-57 is higher than 10. This observation directly indicates that the pK_a of His-57 is elevated in a complex containing a tetrahedral adduct.

N-Acetyl-L-leucyl-L-phenylalanyl trifluoromethyl ketone (**1**) is a slow binding inhibitor of chymotrypsin (K_i = 1.2 μM; Imperiali & Abeles, 1986). It has been proposed, on the basis of analogy with aldehydic peptides, that peptidyl trifluoromethyl ketones react with the active site serine to form a hemiketal. The resemblance of this hemiketal to the tetrahedral adduct formed during the catalytic reaction is probably a major factor contributing to the tight binding. In order to obtain direct information concerning the structure of the chymotrypsin–inhibitor adduct, we have carried out NMR studies of the enzyme–inhibitor complex.

Materials and Methods

α-Chymotrypsin (type II, 3× crystallized, from bovine pancreas) obtained from Sigma Chemical Co. was used without further purification. Trifluoroacetic acid silver salt and (2-bromoethyl)benzene were purchased from Aldrich Chemical Co. Barium [^{13}C]carbonate (99 atom % ^{13}C) was obtained from Cambridge Isotope Laboratories (Woburn, MA).

Synthesis of 4-Phenyl-1,1,1-trifluoro[2-^{13}C]butan-2-ol. This trifluoromethyl alcohol was synthesized from [1-^{13}C]-hydrocinnamaldehyde according to the procedure of Kitazume and Ishikawa (1981). The labeled hydrocinnamaldehyde was prepared as follows: (2-phenylethyl)magnesium bromide was prepared from (2-bromoethyl)benzene and magnesium turnings in dry ether. To this ether solution at 0 °C was added $^{13}CO_2$ generated from $Ba^{13}CO_3$ and sulfuric acid (Ott, 1981). [1-^{13}C]Hydrocinnamic acid thus obtained was reduced with excess lithium aluminum hydride in ether at 0 °C to afford [1-^{13}C]hydrocinnamyl alcohol, which was then oxidized to give [1-^{13}C]hydrocinnamaldehyde with pyridinium chlorochromate (Corey & Suggs, 1975).

Synthesis of 4-Phenyl-1,1,1-trifluoro[2-^{13}C]butan-2-one. A solution of the trifluoromethyl alcohol (2.0 g), obtained from the previous reaction, in 10 mL of dioxane was stirred in a cold-water bath. To this solution was added 33 mL of 0.3 N $KMnO_4$ solution containing 0.5 N NaOH. The reaction was quenched after 5 min by addition of solid sodium bisulfite and dilute HCl solution. Ethyl acetate (30 mL) was added to the resultant solution. The aqueous layer was separated and extracted with ethyl acetate (20 mL two times). The combined organic extracts were washed with water (30 mL), washed with brine (20 mL), dried ($MgSO_4$), and evaporated to give 1.7 g of product. ^{1}H NMR[1] ($CDCl_3$) δ: 7.31–7.15 (m, 5 H) and 2.97 (m, 4 H). ^{13}C NMR ($CDCl_3$) δ: 190.68 (q, J_{CF} = 35 Hz).

Synthesis of 3-Bromo-4-phenyl-1,1,1-trifluoro[2-^{13}C]butan-2-one. The trifluoromethyl ketone obtained from the previous reaction was converted into the corresponding silyl enol ether following a literature procedure (House et al., 1969). This silyl enol ether was brominated in $CHCl_3$ at −78 °C according to the procedure of Reuss and Hassner (1974).

Synthesis of 3-Amino-4-phenyl-1,1,1-trifluoro[2-^{13}C]butan-2-ol. The trifluoromethyl bromo ketone (0.7 g) from the previous reaction was dissolved in 5 mL of dioxane and added to an aqueous solution (5 mL) containing 0.35 g of NaN_3. The solution was stirred at room temperature for 2 h. At this time,

† This is Publication No. 1634 from the Graduate Department of Biochemistry, Brandeis University. This work was supported by National Institutes of Health Grant GM12633-24 and Tobacco Research Grant 1778R1.

[1] Abbreviations: NMR, nuclear magnetic resonance; ZLCK, 1-chloro-3-(carbobenzyloxyamino)-7-aminoheptan-2-one; FID, free induction decay; DMSO, dimethyl sulfoxide.

0006-2960/87/0426-7603$01.50/0