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On the Reaction of Purified Bovine Thrombin with *N*-Acetylimidazole†

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ABSTRACT: The reaction of thrombin with the mild acetylating agent, *N*-acetylimidazole, has been studied to further elucidate the relationship between structure and function in this highly specific proteolytic enzyme. Contrary to previous findings from other laboratories, the reaction of *N*-acetylimidazole with purified bovine thrombin resulted in an enzyme preparation with markedly decreased fibrinogen-clotting activity but apparently unchanged activity in the hydrolysis of tosyl-L-arginine methyl ester. The enzyme was reactivated upon treatment with either neutral hydroxylamine or exposure to mild alkaline conditions. The partial inactivation of the fibrinogen-clotting activity was associated with the acetylation of four to five tyrosyl residues as determined by spectrophotometric techniques or by estimation of hydroxamate formation after treatment with alkaline hydroxylamine. The enzyme was partially protected from inactivation by the presence of the competitive inhibitor, benzamidine hydrochloride, or by ester substrates. The inactivation by *N*-acetylimidazole was also prevented by tosyl-L-arginine and thus differed from the previously observed inactivation by phenylmethanesulfonyl fluoride. This information is used to support the hypothesis that inactivation of thrombin by *N*-acetylimidazole occurs at the macromolecular substrate binding site. Inactivation to a lesser degree has been observed with "aged" preparations of thrombin. The reaction of "aged" thrombin with *N*-acetylimidazole was associated with the acetylation of six to seven tyrosyl residues, suggesting a change in conformation since the process of "aging" is not apparently associated with autolysis.

The nature of the factors governing the highly specific catalytic action of thrombin (EC 3.4.4.13) are only poorly understood at this time. The use of diisopropyl phosphorofluoridate (Gladner and Laki, 1956; Miller and van Vunakis, 1956) and phenylmethanesulfonyl fluoride (Lundblad, 1971) has indicated the presence of a seryl residue at the catalytic site of thrombin. Glover and Shaw (1971) utilized an active-site-directed inhibitor, 1-chloro-3-tosylamido-7-amino-2-heptanone, to demonstrate the catalytic essentiality of a single histidyl residue. Since the above reagents diminish equally both the esterase and proteinase (fibrinogen-clotting) activity of highly purified preparations of thrombin, it is assumed that the affected residues participate in the actual catalytic reaction rather than in substrate binding or maintenance of proper conformation.

There are, however, conditions where differential stabilities of thrombin esterase and proteinase activity are observed. Sokolovsky and Riordan (1969) reacted crude preparations of bovine thrombin with tetranitromethane and observed that clotting activity was lost more rapidly and to a greater extent than esterase activity. This observation was extended by Lundblad and Harrison (1971) who utilized a highly purified preparation of bovine thrombin. These investigators reported a loss in fibrinogen-clotting activity upon treatment of thrombin with tetranitromethane but the esterase activity was apparently unchanged. The effect of acetylation of throm-

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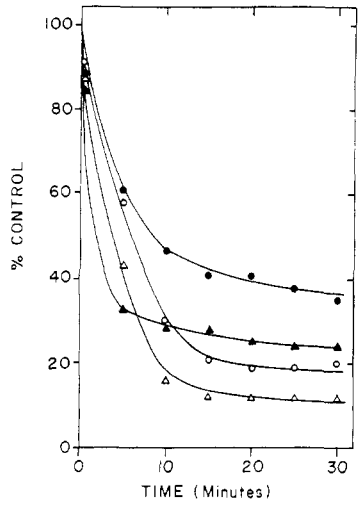

FIGURE 1: The inactivation of thrombin fibrinogen-clotting activity by *N*-acetylimidazole. The *N*-acetylimidazole was added as a benzene solution and evaporated to dryness prior to the addition of 0.5 ml of thrombin (0.12 mg of protein) and 0.5 ml of 0.1 M sodium phosphate buffer. The reactions were maintained at 23° and 0.1-ml portions were removed at the indicated times, diluted with 0.1 ml of 0.06 M Tris (pH 7.5)–0.09 M NaCl, and assayed for fibrinogen-clotting activity. The results are expressed as percent of a control reaction in the absence of *N*-acetylimidazole. The reactions were 4.5×10^{-4} M in *N*-acetylimidazole at pH 7.5 (○) and 8.0 (●) or 9.0×10^{-4} M in *N*-acetylimidazole at pH 7.5 (△) and 8.0 (▲).

bin on catalytic activity has been investigated by several groups. Landaburu and Seegers (1959) used acetic anhydride in saturated sodium acetate to acetylate thrombin. These workers observed that upon acetylation of the amino groups fibrinogen-clotting activity was rapidly lost whereas esterase (Tos-L-ArgOMe)[1] activity increased. The purity of the thrombin preparations used in these experiments is questionable as both the native (thrombin C) and partially acetylated (thrombin E) preparations could "lyse" fibrin clots, a property generally attributed to the presence of plasmin (EC 3.4.4.14). More recently, Kotaku *et al.* (1970) observed that four tyrosyl residues in equine thrombin reacted with *N*-acetylimidazole with little change in the clotting activity but with a rapid initial decrease in esterase activity followed by a gradual return of this activity as the reaction proceeded.

It is the purpose of the present communication to report on studies of the reaction of purified bovine thrombin with *N*-acetylimidazole, and to further elucidate the effects of mild acetylation on catalytic activity.

Materials and Methods

N-Acetylimidazole was either obtained from the Pierce Chemical Co. or synthesized after the method of Boyer (1952) from isopropenyl acetate (Eastman 6324) and recrystallized imidazole. Benzamidine hydrochloride was a product of Eastman Organic Chemicals. Tos-L-ArgOMe was purchased from Schwarz BioResearch. Sulfopropyl-Sephadex C-50 and Sephadex G-25 (coarse) were obtained from Pharmacia. Bovine fibrinogen was a product of Sigma Chemical Corp. and crude bioactivated bovine topical thrombin (lots 995508C and 997712A) was obtained from Parke-Davis. Sodium dodecyl sulfate was obtained from Schwarz-Mann. Acrylamide and *N,N'*-methylenebisacrylamide were obtained from the Eastman Chemical Co. and recrystallized from chloroform and 1-propanol, respectively, prior to use. Purified bovine thrombin was prepared by chromatography on sulfopropyl-Sephadex C-50 as previously described (Lundblad, 1971). The dialyzed material resulting from the chromatographic fractionation of 100,000 unit batches required centrifugation (105,000*g*, for 1 hr at 4°) to remove insoluble material which interfered with the spectral studies. No activity was lost during this procedure and there was no significant increase in specific activity. The preparation of thrombin utilized in this study had a specific activity of 1800–2200 NIH units/mg.

"Aged" thrombin was prepared by allowing purified thrombin in 0.05 M NaCl to stand for 24–36 hr at 37° such that 30–50% of the fibrinogen-clotting activity had been lost. Occasionally a second centrifugation step was required to remove insoluble material of unknown origin which appeared during aging.

Reaction with N-Acetylimidazole. The reaction of *N*-acetylimidazole with thrombin was performed essentially as described by Riordan and Vallee (1967). For the kinetic studies described, components of the reaction mixture other than thrombin were rapidly introduced into the reaction vessel and purified bovine thrombin (0.24 mg/ml) previously dialyzed against 0.05 M NaCl was added to initiate the reaction. Portions were then removed at the indicated time intervals and assayed for fibrinogen-clotting activity and esterase activity using Tos-L-ArgOMe as previously described (Lundblad, 1971).

In the majority of the work described, the determination of the extent of tyrosine modification was estimated directly from the decrease in absorbance at 278 nm (Myers and Glazer, 1971). Occasionally the extent of tyrosine modification was determined by the increase in absorbance at 278 nm upon the addition of neutral hydroxylamine (Riordan and Vallee, 1967). The total amount of O-acetylation was determined by the modification of the alkaline hydroxamate method as described by Tildon and Ogilvie (1972). The value of 34,000 for the molecular weight of thrombin was used in the calculations, and the value of 1210 M^{-1} cm^{-1} for the molar absorptivity difference between *N*-acetyltyrosine and *N,O*-diacetyltyrosine at 278 nm (Myers and Glazer, 1971). The protein concentration was estimated by ninhydrin reaction following alkaline hydrolysis (Fruchter and Crestfield, 1965) using the dimethyl sulfoxide reagent of Moore (1968). Bovine serum albumin was used as the standard. Electrophoresis in sodium dodecyl sulfate was conducted according to the modified method of Weber and Osburn (1969) described previously (Mann *et al.*, 1971a). Following electrophoresis, gels were stained with either Coomassie Blue (for protein) or by means of the Schiff periodate method (Fairbanks *et al.*, 1971) for carbohydrate. Quantitative estimates of the relative amounts of the different thrombin forms were obtained by means of scans of the gels with a Gilford Model 222A spectrophotometer equipped with a 2410 S linear transport, and a disc integrator equipped Barber Coleman recorder. Coomassie Blue stained gels were scanned at 600 nm and Schiff-stained gels at 560 nm.

Results

The results of the incubation of purified bovine thrombin treated with *N*-acetylimidazole on fibrinogen-clotting activity at pH 7.5 and 8.0 are shown in Figure 1. As would be ex-

[1] Abbreviations used are: Tos-L-ArgOMe, tosyl-L-arginine methyl ester; Tos-L-Arg, tosyl-L-arginine.

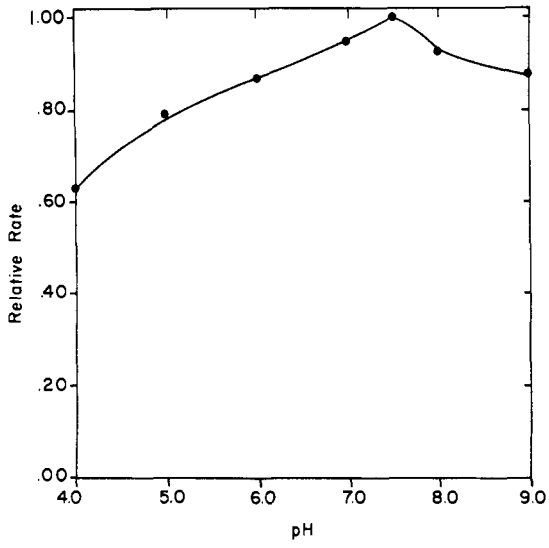

FIGURE 2: The pH dependence of the inactivation of thrombic fibrinogen-clotting activity by *N*-acetylimidazole. The reactions were 9×10^{-4} M with respect to *N*-acetylimidazole added as a benzene solution which was subsequently evaporated to dryness and contained 0.5 ml of thrombin (0.12 mg of protein) and 0.5 ml of a buffer composed of 0.02 M Tris–0.02 M imidazole–0.02 M acetate at the indicated pH. Assays against fibrinogen were performed on 1:10 dilution of the reaction mixture which was maintained at 23°. Data were calculated from initial reaction rates.

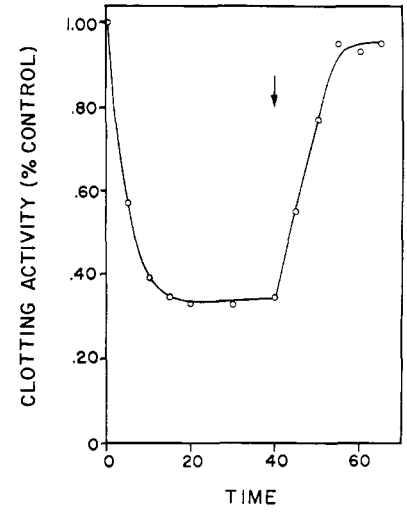

FIGURE 3: The reactivation of acylated thrombin by neutral hydroxylamine. The reaction was 9.0×10^{-4} M with respect to *N*-acetylimidazole and performed at pH 7.5 as described under Figure 1.

pected, increasing the concentration of *N*-acetylimidazole results in an elevated rate of loss of the fibrinogen-clotting activity. It can also be observed that there is little difference in the rates of inactivation at the two pH values. Esterase activity was not lost under these conditions (Table I). The reaction of *N*-acetylimidazole proceeded equally well in phosphate, barbital, or borate buffer. It was not possible to utilize Tris buffers in these studies because of the lability of *N*-acetylimidazole in this medium (Riordan and Vallee, 1967; Connellan and Shaw, 1970). Sodium phosphate buffers were utilized for future experimentation to remain consistent with the solvent conditions utilized in previous work on tyrosine modification in this laboratory (Lundblad and Harrison, 1971). It is recognized that acetylphosphate synthesis (Stadtman, 1954) may occur under our reaction conditions. Acetyl phosphate has been used as a specific acetylating agent for 3-phosphoglyceraldehyde dehydrogenase but showed negligible activity with other proteins (Park *et al.*, 1970). Figure 2 shows the pH dependence of the inactivation of thrombic fibrinogen-clotting activity by *N*-acetylimidazole. The decrease in the rate of inactivation observed under both more acid and alkaline conditions is probably a function of reagent stability (Stadtman, 1954) rather than a reflection of the ionization of a functional group on the protein.

The inactivation of the fibrinogen-clotting activity of thrombin by *N*-acetylimidazole could be readily reversed by the addition of neutral hydroxylamine. The reversal of the inhibition of fibrinogen-clotting activity by neutral hydroxylamine is shown in Figure 3. The recovery of activity to control values is quite rapid, being essentially complete within 10 min after the addition of hydroxylamine. Factors influencing deacylation were not examined in the current study.

Thrombin may be protected from inactivation by either benzamidine, a competative inhibitor, the ester substrate, Tos-L-ArgOMe, or by tosyl-L-arginine, a product of esterolytic cleavage. The results of these experiments are shown in Table II.

The degree to which thrombin can be inhibited by *N*-acetylimidazole is apparently dependent on the conformational integrity of the enzyme molecule. If bovine thrombin is incubated under conditions where the fibrinogen-clotting activity is gradually lost but the esterase activity is main-

TABLE I: Effect of *N*-Acetylimidazole on Thrombic Esterase Activity.[a]

Conditions	ΔA_{247}/min per ml			
	0 min	10 min	20 min	30 min
N-Acetylimidazole, 9×10^{-4} M, pH 7.5	0.42	0.42	0.40	0.43
Control, pH 7.5	0.39	0.42	0.40	0.39

[a] The reaction mixture composition was that described under Figure 1. At the indicated times 0.1-ml portions were removed and assayed for the ability to catalyze the hydrolysis of Tos-L-ArgOMe.

TABLE II: Protection of Thrombin from Inactivation by *N*-Acetylimidazole.

Reaction Conditions[a]	% Inhibn[b]
Control	0
+*N*-Acetylimidazole[c]	74
+*N*-Acetylimidazole[c] + Tos-ArgOMe[d]	23
+*N*-Acetylimidazole[c] + Tos-L-Arg[e]	50
+*N*-Acetylimidazole[c] + Tos-L-Arg[d]	60
+*N*-Acetylimidazole[c] + benzamidine[d]	18

[a] The reactions were performed as described under Figure 1. [b] This was determined from the initial rates of inactivation. [c] 1.8 mM. [d] 1.0 mM. [e] 2.0 mM.

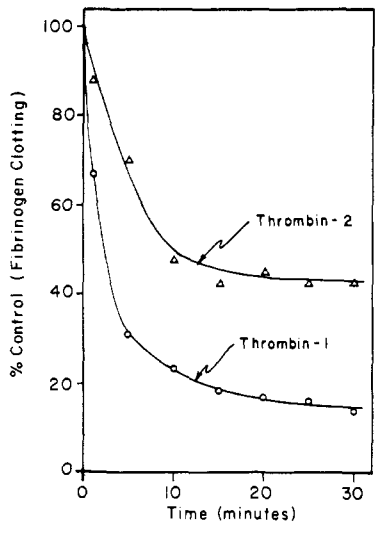

FIGURE 4: The effect of aging on the response of thrombin to *N*-acetylimidazole. The reactions were performed as described under Figure 1 and were 9.0×10^{-4} M with respect to *N*-acetylimidazole. The thrombin utilized was either freshly thawed (thrombin 1, ○) or aged at 37° until 30% of the fibrinogen-clotting activity had been lost (thrombin 2, △). Each thrombin is used as its respective control.

tained (Lundblad, 1971), the sensitivity of the enzyme to *N*-acetylimidazole is lost. This is demonstrated in Figure 4. Thrombin 2 was incubated at 37° for 24 hr and had lost approximately 30% of its fibrinogen-clotting activity when compared to a freshly thawed control; the esterase activity of this preparation toward Tos-L-ArgOMe remained unchanged. Upon treatment of the "aged" enzyme preparation with *N*-acetylimidazole fibrinogen-clotting activity is lost at a slower rate and to a lesser extent than that observed for a freshly thawed preparation of the same batch of thrombin (thrombin 1). It should be mentioned that after the reaction with *N*-acetylimidazole both preparations of thrombin have approximately the same activity toward fibrinogen. Table III shows that under these conditions of "aging" where fibrinogen-clotting activity is lost there is no change in the distribution of high and low molecular weight species of thrombin or in component peptide chains as described by Mann and coworkers (1971a,b). Even following 40 hr of aging in 0.05 M NaCl, 37°, little change is observed in the relative proportions of large (mol wt 39,000) and small (mol wt 28,000) thrombin species. In the sample studies in this case, the mol wt 39,000 thrombin accounted for 66% of the total protein and 71% of the total carbohydrate.[2] Following 24 hr of aging, 63% of the total protein and 70% of the total carbohydrate was associated with the mol wt 39,000 species. The factors influencing "aging" of thrombin are only poorly understood at this time. The time necessary appears to vary among different thrombin preparations with some samples taking as long as 36 hr to lose an appreciable amount of fibrinogen-clotting activity.

TABLE III: Thrombin Species Distribution as a Function of Aging.[a]

Preparation[b]	% 39,000	% CHO[c]	% 28,000	% CHO[c]
Thrombin 1 (fresh)	66	70	34	30
Thrombin 2 ("aged")	63	71	37	29

[a] The gel electrophoresis experiments were performed as described in the text. [b] The "fresh" thrombin was thawed and immediately dialyzed into 0.2 N acetic acid and subsequently lyophilized. The "aged" thrombin was allowed to stand at 37° for 40 hr prior to dialysis *vs.* acetic acid. [c] Refers to the per cent of total carbohydrate in electrophoresis sample.

TABLE IV: Tyrosine Modification by *N*-Acetylimidazole in Thrombin.

Reaction Conditions[a]	O-Ac-Tyr[b]	O-Ac-Tyr[c]	O-Ac[d]	% Inactivn
Thrombin, *N*-acetylimidazole	4.2	4.4	4.7	83
Thrombin, *N*-acetylimidazole, benzamidine	3.9	4.4	3.2	36
"Aged" thrombin, *N*-acetylimidazole	6.1		5.7	43

[a] The reaction consisted of 2.0 ml of purified thrombin (1.3 mg/ml) and 0.5 ml of 0.1 M sodium phosphate (pH 7.5). These components were mixed and added to 1.0 mg of *N*-acetylimidazole (evaporated from benzene) and the reaction was allowed to proceed for 20 min at room temperature (23°). The reactions were terminated by removal of reagents with a G-25 Sephadex column. [b] Tyrosine acetylation estimated by the method of Myers and Glazer (1971). [c] Tyrosine acetylation determined by the method of Riordan and Vallee (1967). [d] The total O-acetylation of thrombin was determined by the alkaline hydroxyamate method of Tildon and Ogilvie (1972).

Studies were then performed to determine the extent of modification of thrombin by *N*-acetylimidazole under our reaction conditions. The results obtained in these studies are shown in Table IV. In the absence of an inhibitor such as benzamidine approximately four tyrosyl residues in thrombin are acetylated by *N*-acetylimidazole. In the presence of the competitive inhibitor, benzamidine, a similar degree of acetylation of tyrosyl residues is observed. The results obtained with "aged" thrombin preparations show that these preparations incorporated a somewhat larger number of acetyl groups than freshly thawed preparations.

Discussion

Treatment of purified bovine thrombin with *N*-acetylimidazole induces changes in the catalytic activity of thrombin similar to those observed upon nitration of the enzyme with tetranitromethane. The observation that approximately four tyrosyl residues out of a total of 12 in thrombin are modified in the course of this reaction is consistent with our earlier report that 4–5 tyrosyl residues are converted to 3-nitrotyrosine upon treatment of thrombin with tetranitromethane under similar reaction conditions (Lundblad and Harrison,

[2] A complete description of the nature of the carbohydrate attachment to large and small thrombin is in preparation.

1971). However, benzamidine was observed to protect thrombin from inactivation by *N*-acetylimidazole where it had no effect on inactivation by tetranitromethane. This would suggest that these two reagents might react with different tyrosyl residues in thrombin. Work is currently in progress to further study the reaction of tetranitromethane with thrombin and to identify the sites of nitration in this enzyme. Furthermore the agreement observed between the extent of tyrosine modification as determined by spectral methods and the extent of total O-acetylation as determined by the alkaline hydroxamate reaction would preclude the existence of an acetylated seryl residue. Thus we feel that the observed changes in the catalytic activity of bovine thrombin upon reaction with *N*-acetylimidazole are due to the modification of tyrosine. In any event, the modification of the seryl or histidyl residue at the active site as observed in the reaction of trypsin with *N*-acetylimidazole (Houston and Walsh, 1969) should have resulted in equivalent inactivation of fibrinogen-clotting activity and esterase activity. Similarities in the reaction of *N*-acetylimidazole with thrombin and trypsin might have been expected from the suggested homology between these two enzymes (Hartley, 1969). However, it should be noted that the reaction conditions utilized in the present investigation differed from those used in the trypsin study.

The reason for the differences in changes in the catalytic activity of thrombin upon acetylation reported here and those previously reported by Kotaku *et al.* (1970) is not clear at this time. These workers reported the modification of four tyrosyl residues in equine thrombin with little significant change in fibrinogen-clotting activity but a rapid initial decrease in esterase activity followed by a slow regain of this activity. It is possible that equine thrombin is significantly different from bovine thrombin in terms of the factors governing enzyme specificity.

Although it is not possible to associate the reaction of a single tyrosyl residue with observed changes in catalytic activity, the protection of thrombin from *N*-acetylimidazole inactivation by Tos-L-ArgOMe and Tos-L-Arg would suggest that there is a specific tyrosyl residue critical to the binding of protein substrates but somewhat less important in esterolytic processes. It is recalled that whereas Tos-L-ArgOMe protected the enzyme from inactivation by phenylmethanesulfonyl fluoride, Tos-L-Arg had no effect (Lundblad, 1971). It was then suggested that presence of a free carboxyl group prevented effective binding. It is, however, possible that the bulk of the methyl group may prevent ready access for phenylmethanesulfonyl fluoride to the active-site seryl residue. With respect to the present experiments, the bulk of the tosyl group would appear to be sufficient to hinder acetylation at a tyrosyl residue in the substrate binding site. There are, no doubt, differences in the binding of Tos-L-ArgOMe and Tos-L-Arg as reflected by the decreased ability of Tos-L-Arg to protect the enzyme from inactivation by *N*-acetylimidazole.

The increase in the degree of acetylation of an "aged" thrombin preparation compared with a "fresh" preparation is consistent with the "aging" causing a change from a "tight" conformation to a "loose" conformation with concomitant increased proclivity of tyrosyl residues to react with *N*-acetylimidazole. A change in conformation is also supported by the failure to find changes in the molecular size or of the chain distribution within the respective thrombin preparations. Our results suggest that two preparations of thrombin may be identical from the polypeptide composition and yet show vastly different clotting activities. Our observations clearly eliminate gross autolysis as the basis for the changes in chemical modification which occur in aging. They do not, of course, rule out, or for that matter test, the influence of possible multiple aggregation states of thrombin. The fact that thrombin is an aggregating protein is well known; however, our procedures would not distinguish, and for that matter recognize, thrombin polymers.[3]

Acknowledgments

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[3] Preliminary studies of the sedimentation behavior of aged and fresh thrombin in 0.25 M sodium phosphate (pH 7.5) indicate that no change in the aggregate state of the molecule is occurring, at least under these circumstances, during aging.