CHANGES IN THE EXPOSURE OF TRYPTOPHYL AND TYROSYL RESIDUES IN THROMBIN DUE TO DIISOPROPYLPHOSPHOROFLUORIDATE AND BENZAMIDINE INHIBITIONS*

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Summary. Chemical modifications of tyrosine and tryptophan residues in diisopropylphosphoryl-thrombin (DIP-thrombin) and benzamidine-inhibited thrombin (BA-thrombin) by N-acetylimidazole and hydrogen peroxide-dioxane mixture indicate the burial of two tyrosyl and two tryptophyl residues relative to the active enzyme. During inhibition the circular dichroism spectra in the peptide-absorbing region is apparently unchanged while small detectable changes are observed in the aromatic region. It is concluded that tryptophan and tyrosine residues are part of the structural features of the active center of thrombin but they do not play active roles in the catalytic process.

Thrombin (EC 3.4.4.13) is a highly specific serine proteinase. It is like trypsin in side chain specificity (1) and they both show reasonably high sequence homology (2). Undoubtedly, they should also show some homology in three-dimensional folding and catalytic mechanism, even if they differ in substrate specificity. The X-ray and difference spectral studies have shown the presence of tryptophan and tyrosine in the catalytic center of trypsin (3,4). In thrombin, it has been shown that the substrate, N-benzoyl-L-arginine ethyl ester (BAEE) partially protects the enzyme from N-bromosuccinamide oxidation (5). It has

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also been reported that benzamidine, a competitive inhibitor, partially protects thrombin from inactivation by N-acetylimidazole (6) but not from inactivation by tetranitromethane (7). In our present study, we have investigated the effect of benzamidine and diisopropylphosphorofluoridate inhibitions on the exposure of tryptophan and tyrosine residues in thrombin in order to establish that these chromophores are located in the catalytic center. Since the X-ray crystallographic study of thrombin is in its initial stages, we hope that by knowing some of the amino acid side chains in the catalytic center and analogy with a closely related and well-studied enzyme will aid in the elucidation of the topology of the active center of thrombin.

Materials and Methods

Bovine thrombin was obtained from Parke-Davis and purified by the method of Lundblad (8). The N-acetylimidazole was purchased from Sigma Chemical Company, stored in *vacuo* over phosphorous pentoxide and used without further purification. Dioxane was distilled from sodium and stored under nitrogen. DIP-thrombin was prepared from freshly opened diisopropylphosphorofluoridate according to the method of Markland (9). The esterase activity was determined using tosyl-L-arginine methyl ester as substrate. Protein concentrations were based on spectrophotometric measurements using an extinction coefficient, $E^{1\%}_{1cm} = 19.5$ (10) and a molecular weight of 34,000. All sbsorbances were corrected for light scattering by subtracting 1.7 times the apparent absorption at 320 nm (11).

The oxidation by hydrogen peroxide was performed as described by Hachimori *et al.* (12) except that in some cases the reactions were quenched by making the reaction mixtures 1 ug/ml in catalase. For the calculation of the number of oxidized tryptophan in the protein, a value for ΔE_{282} of 3490 for the oxidation of a single tryptophan was used (12). The acetylation by N-acetylimidazole was performed as described by Riordan *et al.* (13). The value, $\Delta E = 1160$ at 275 nm was used as the change in molar extinction coefficient due to the acetylation of one mole of tyrosine (13).

Circular dichroism (CD) was made on a Cary 60 recording spectropolarimeter equipped with a Cary 6002 circular dichroism attachment. Amino acid analysis was carried out in a Technicon TSM Automatic Analyzer.

Results

Reactivity of Tryptophan Residues with H_2O_2-Dioxane. The oxida-

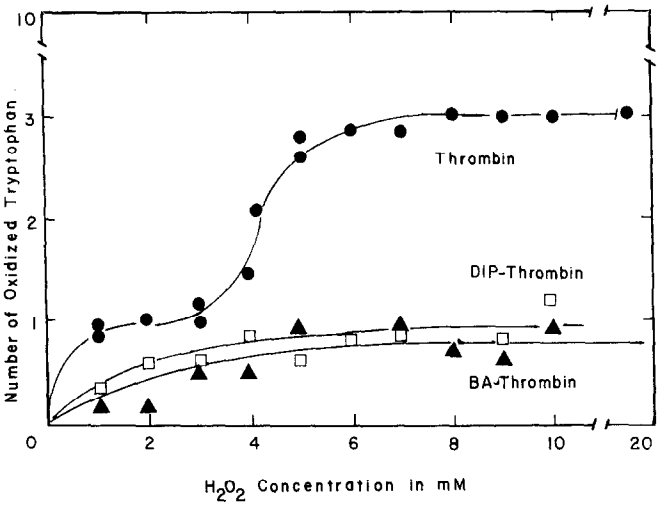

Figure 1. Oxidation of Tryptophan in Thrombin by H_2O_2-Dioxane. (●-●) Thrombin; (□-□) DIP-thrombin; and (▲-▲) BA-thrombin. Protein solutions were prepared in 0.5 M bicarbonate-carbonate buffer, pH 8.7. Protein concentrations ranged from 7.5×10^{-6} to 1.1×10^{-5} M.

tion curve of thrombin is shown in Figure 1. There is a leveling off for up to 3 mM H_2O_2 corresponding to the oxidation of one mole of tryptophan per mole of protein. Two additional residues were oxidized at 5 mM which is retained up to 10 mM. The concentration of the reagent was raised to 20 mM but no further oxidation took place indicating that only three out of ten tryptophans could be oxidized. In a disulfide-cleaved urea-denatured thrombin all ten residues were oxidized. Table I shows the verification of the oxidation data by tryptophan determination as described by Edelhoch (14). The oxidation is accompanied by gradual loss of esterase activity while the clotting activity drops exponentially and at 1 mM H_2O_2 no clotting activity remains. In both DIP-thrombin and BA-thrombin only one residue appears to be oxidized even up to 10 mM H_2O_2. Thus, relative to the active enzyme, two tryptophan residues become unreactive or buried in the presence of either the covalent or ionic inhibitor. Amino acid

TABLE I

Oxidation of Tryptophan Residues in Thrombin

H_2O_2 in mM	Tryptophan oxidized[1]	Tryptophan not oxidized[2]
0	0	10.0
2	1	8.5
7	3	6.7

1 based on the data in Figure 1.
2 based on Edelhoch method (14).

analysis of the oxidized thrombin showed two methionine and one histidine were also destroyed. One tyrosine was destroyed at higher concentration of the reagent (7 mM).

Reactivity of Tyrosine with N-acetylimidazole. The acetylation of thrombin is shown in Figure 2. The reactivity curve shows a plateau corresponding to eight acetylated residues per molecule of thrombin. The acetylation has very little effect on esterase activity while the clotting activity decreased to 40% of the control. It should be noted that the concentration of N-acetylimidazole is higher than that necessary to acetylate serine residues (15) and it is possible that the active-site serine is also acetylated under this condition. The acetylation of BA-thrombin is similar to the acetylation of active thrombin during the first fifteen to twenty-five minutes but increases rapidly beyond that time until all the eleven residues in the molecule are acetylated. The acetylation of DIP-thrombin is relatively slow during the first seventy-five minutes and beyond that time acetylation also increases rapidly to expose the remaining residues. When compared with the active enzyme the slow initial acetylation in DIP-thrombin corresponds to about two less reactive residues.

The circular dichroism spectra of thrombin, DIP-thrombin

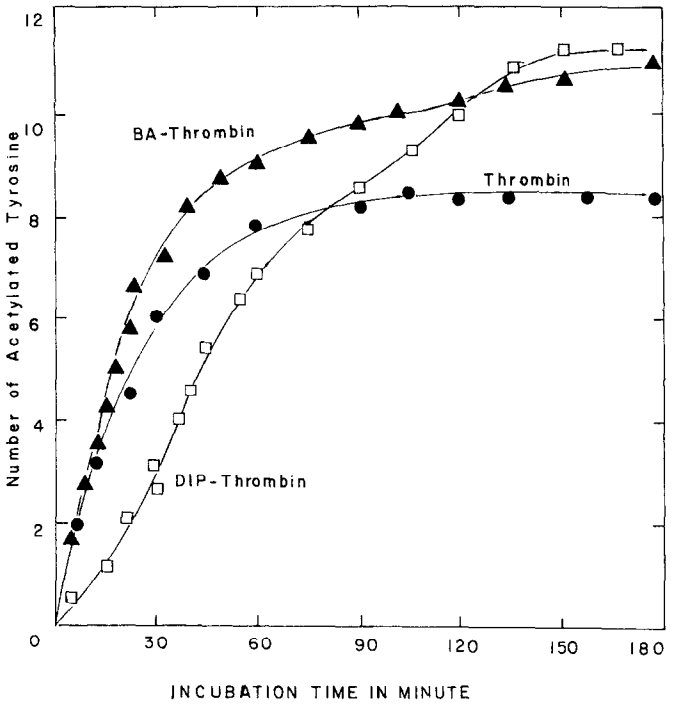

Figure 2. Acetylation of Tyrosine in Thrombin by N-acetylimidazole. Legends are the same as in Figure 1. Protein solutions were prepared in 0.05 M borate buffer, pH 7.5 in 115-fold molar excess of the reagent.

and BA-thrombin in the peptide-absorbing region are shown in Figure 3A. No significant changes are observed in the presence of the inhibitors. The Cotton effect in the aromatic region (Figure 3B) is characterized by two positive peaks at 288-289 nm and 292-294 nm which may be confidently assigned to tryptophan (16). The two peaks are less resolved and of lower intensities in DIP-thrombin and BA-thrombin. Similarly, the complex bands at 250-280 nm tyrosine region have changed slightly during inhibition.

Discussion

The evidence presented in this study indicates that about two tryptophan and two tyrosyl residues in thrombin become buried as a result of inhibition by diisopropylphosphorofluori-

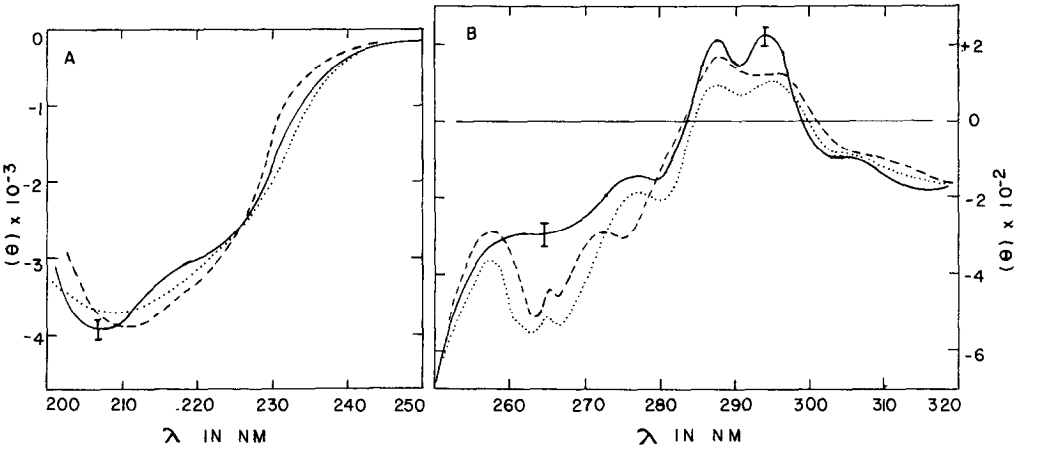

Figure 3. Circular Dichroism Spectra of Thrombin. (A) Far-ultraviolet peptide region. (B) Near ultraviolet aromatic side chain region. Thrombin (solid lines); DIP-thrombin (dashed lines); and BA-thrombin (dotted lines). All solutions were buffered in 0.125 M phosphate buffer, pH 7.1.

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date. Two tryptophan and no tyrosine residues were buried due to benzamidine inhibition. Since the polypeptide conformation is observed to change very little or not at all during inhibition we believe that this burial is due to the combination of direct masking of these chromophores by the bulk of the inhibitor, together with subtle side chain reorientation and reinteraction associated with the flexibility of the active site. It is difficult to associate the loss of some of the enzymatic activity to the oxidation of tryptophan or acetylation of tyrosine because oxidation could have partially destroyed histidine or acetylation could have reversibly acetylated serine. There is some experimental evidence to support this contention. Chulkova et al. (5) reported that BAEE which protected thrombin from inactivation in the presence of N-bromosuccinamide, protected only histidine from oxidation. The study on equine thrombin (17) showed that acetylation of tyrosine did not appreciably affect the enzymatic

activity and that oxidation of tryptophan caused only gradual inactivation.

We conclude from this study that a tyrosine and possibly a tryptophan residue are part of the structural features of the active center of thrombin but they do not play active roles in the catalytic process.

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References:

1. Sherry, S. and Troll, W. (1954) J. Biol. Chem. 208, 95-105.
2. Magnusson, S. (1970) in Structure-Function Relationships of Proteolytic Enzymes (Desnuelle, P., Neurath, H. and Ottesen, M., Eds) pp. 138-143, Munksgaard, Copenhagen.
3. Villanueva, G.B. and Herskovits, T.T. (1971) Biochemistry 10, 4589-4594.
4. Krieger, M., Kay, L.M. and Stroud, R.M. (1974) J. Mol. Biol. 83, 209-230.
5. Chulkova, T.M. and Orekhovich, V.N. (1968) Biokhimiya 33, 1222-1225.
6. Lundblad, R.L., Harrison, J.H. and Mann, K.G. (1973) Biochemistry 12, 409-413.
7. Lundblad, R.L. and Harrison, J.H. (1971) Biochem. Biophys. Res. Commun. 45, 1344-1349.
8. Lundblad, R.L. (1971) Biochemistry 10, 2501-2506.
9. Markland, F.S. (1969) J. Biol. Chem. 244, 694-700.
10. Winzor, D.J. and Scheraga, H.A. (1964) J. Phys. Chem. 68, 338-343.
11. Baughman, D.J. and Waugh, D.F. (1967) J. Biol. Chem. 242, 5252-5259.
12. Hachimori, Y., Horinishi, H., Kurihara, K. and Shibata, K. (1964) Biochim. Biophys. Acta 93, 346-360.
13. Riordan, J.F., Wacker, W.F.C. and Vallee, B.L. (1965) Biochemistry 4, 1758-1765.
14. Edelhoch, H. (1967) Biochemistry 6, 1948-1952)
15. Houston, L.L. and Walsh, K.A. (1970) Biochemistry 9, 156-165.
16. Strickland, E.H., Wilchek, M., Horwitz, J. and Billups, C. (1970) J. Biol. Chem. 245, 4168-4177.
17. Kotoku, I., Matsushima, A., Bando, M. and Inada, Y. (1970) Biochim. Biophys. Acta 214, 490-497.