A PARTIALLY ACTIVE TRYPSIN INDUCED BY LIMITED ACETYLATION

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The reaction of N-acetylimidazole has been recently proposed as a specific reagent for the determination of "free" tyrosyl residues in proteins (Riordan, Wacker and Vallee, 1965a). In applying this reagent to trypsin, Riordan et al. (1965b) found that the acetylation of 6.7 tyrosines has very little effect on trypsin activity. However, we have recently found that under milder conditions, acetylation with acetylimidazole reduces the activity of trypsin by approximately 65% in a manner which can be reversed by treatment with hydroxylamine or imidazole at neutral pH. Furthermore, the acetylation appears to induce a change in the efficiency of the catalytic site without altering the binding site of the enzyme.

Bovine trypsin (Worthington Lot TR6EA) was acetylated by treatment with N-acetylimidazole in 0.01 M TES[2], 0.01 M $CaCl_2$ at pH 7.5, 0° C. Figure 1 shows the effect of a 465-fold molar excess of acetylimidazole on trypsin activity towards BAEE at 0° and 22°. At 22° C a transient inactivation occurs which reaches a maximum within five minutes and the modified enzyme rapidly reactivates to original activity. At 0° C the rate of inactivation is slower, reaching the minimum activity in about twenty-five minutes. A marked enhancement of the inactivation process results from the inclusion of a competitive inhibitor of the enzyme such as benzamidine. A similar inhibition is observed when the activity is followed with 0.015 M TAME.

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[2]Abbreviations used: TES, N-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid; BAEE, α-N-benzoyl-L-arginine-ethylester; DFP, diisopropylfluorophosphate; TLCK, tosyllysine chloromethyl ketone; TAME, α-N-tosyl-L-arginine methyl ester.

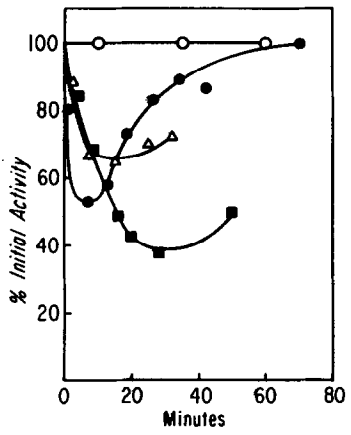


Figure 1. The effect of a 465-fold molar excess of N-acetylimidazole on trypsin activity in the presence and absence of benzamidine. Incubations were carried out at the indicated temperature with 1.6×10^{-4} M trypsin in 0.01 M TES, 0.01 M $CaCl_2$, pH 7.5 and assayed against BAEE. Trypsin at 22° C (O-O); trypsin plus acetylimidazole at 22° C (●-●) and 0° C (△-△); trypsin plus acetylimidazole plus 0.021 M benzamidine (■-■).

The partially active acetyltrypsin was separated from reactants on Sephadex G-50 and its stability examined. It was found to reactivate slowly and spontaneously so that in 48 hours the activity of the derivative had returned to 90% of the original, as illustrated in Figure 2. The inclusion of calcium had no effect on the rate of spontaneous reactivation. Reactivation was promoted by imidazole at pH 7.5 with a loss of approximately one acetyl group from C^{14}-acetyltrypsin. Rapid reactivation occurred with M-hydroxylamine, pH 7.5. The spontaneous reactivation observed in the prolonged acylation reaction in Figure 1 is in all

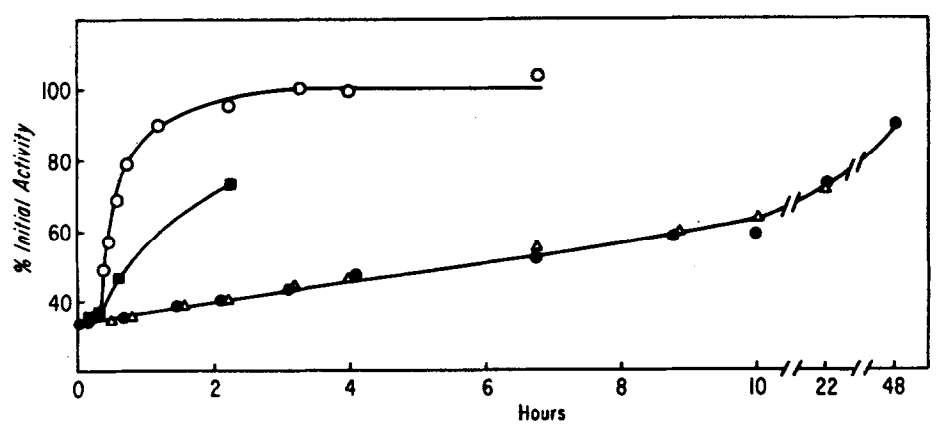
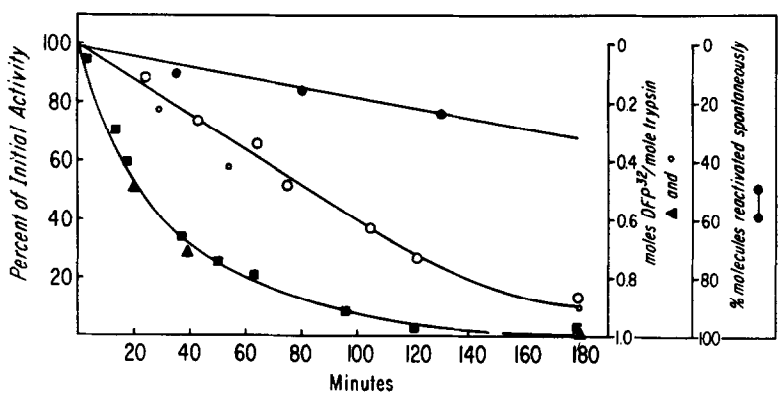


Figure 2. Stability of acetyltrypsin. Trypsin was acetylated in the presence of benzamidine to minimum activity as in Figure 1, using 0.01 M sodium barbital in place of TES. Acetyltrypsin was separated on Sephadex G-50. Spontaneous reactivation in the presence of (△-△) and absence of (●-●) 0.01 M $CaCl_2$ Reactivation promoted by the addition after twenty minutes of imidazole to 0.029 M, pH 7.5 (O-O). Retreatment with 465-fold molar excess of acetylimidazole (■-■).

probability explained by catalysis by the accumulating free imidazole derived from the acetylation reaction and from the hydrolysis of acetylimidazole.

Since retreatment of the purified derivative with acetylimidazole did not decrease the activity further, it appeared that the inhibition induced by acetylation was complete. The question remained whether all of the trypsin molecules were partially active or whether there was a mixture of completely active and completely inactive protein. "All-or-none" experiments were therefore performed. Figure 3 shows that the rate of reaction of trypsin with DFP^{32} is much faster than with the acetyltrypsin derivative. However, the incorporation of DFP^{32} into acetyltrypsin approaches stoichiometry, with proportional loss of activity, indicating that <u>all</u> sites are partially active and that no completely inactive protein is present.



<u>Figure 3</u>. An "all-or-none" assay on trypsin (■-■) and acetyltrypsin (○-○) in 0.01 M TES-$CaCl_2$ at 0°, pH 7.5. Incorporation of DFP^{32} into trypsin (▲-▲) and acetyltrypsin (○-○). Spontaneous reactivation of control acetyltrypsin (●-●).

Similar experiments have been performed with TLCK as the "all-or-none" reagent. Due to the higher temperature required for this reaction, the spontaneous reactivation is much faster than that shown in Figure 3, and it is not yet clear whether the reaction with TLCK is merely very much slowed, or is in fact prevented, by acetylation of the enzyme.

The kinetic parameters in Table I indicate that the binding site for BAEE, TAME and benzamidine of partially-active acetyltrypsin shows equal affinity for these compounds in the modified derivative. The relative velocities for

both substrates, however, are reduced to approximately 37% of that of the native enzyme. This appears to distinguish the present derivative of trypsin from the acetylated derivative recently discussed by Trenholm, Spomer and Wootton (1966).

Table I

Enzyme	Substrate	K_M		Relative[b] Velocity	$K_I \times 10^5$ M Benzamidine	
Trypsin	BAEE	0.90×10^{-5} M		100	5.0	c
	TAME	5.6×10^{-3} M	a	100	2.6	d
Acetyl-trypsin	BAEE	1.05×10^{-5} M		39	5.0	c
	TAME	5.9×10^{-3} M	a	36.5	2.1	d

a. The K_M for the ternary complex of trypsin at high substrate concentrations described by Trowbridge et al. (1963).
b. 0.01 M BAEE at pH 7.8, 26° C, and 0.015 M TAME at pH 7.8, 26° C.
c. Determined by competitive inhibition of BAEE hydrolysis at low substrate concentrations.
d. Determined by competitive inhibition of benzoyl-DL-arginine p-nitroanilide at low substrate concentrations as described by Mares-Guia and Shaw (1965).

Therattil-Antony, Bier and Nord (1961) observed that trypsin is rapidly inactivated by treatment with acetic anhydride. The inactivation was spontaneously reversible at low pH values or promoted by hydroxylamine at higher pH values. They suggested that the site of acetylation was either an aliphatic or an aromatic hydroxyl group but they did not exclude the possibility of acetylation of a primary amino group. Although these observations could be related to the present findings, their derivative differed from the present one in the fact that at pH 8 a completely inactive derivative was obtained.

In the present work the group acetylated cannot be the serine of the active site, because it would undergo a much more rapid deacetylation under the conditions used than is observed. Furthermore, the acetylated enzyme still reacts stoichiometrically with DFP^{32} (Figure 3). Since the binding constant of trypsin does not appear to be affected by acetylation, the inactivation must be due to a modification of the efficiency of the catalytic apparatus. The partial nature

of the inhibition is suggestive of a conformational distortion rather than the acetylation of a residue participating directly in the catalysis, which would lead to a completely inactive species.

There is a growing amount of data that indicates the possibility of substrate-induced conformational changes in enzymes (Grisolia, 1964). Koshland (1958) has presented the concept of the flexibility of the active site contributing to enzyme mechanisms. Recently, Inouye et al. (1966) have shown that several substrates enhance the inactivation of pepsin by diphenylazomethane. Yankeelov and Koshland (1965) have shown that glucose-6-phosphate enhances the reactivity of one sulfhydryl group and decreases the reactivity of another sulfhydryl group and a lysyl and methionyl residue of phosphoglucomutase. It appears that the benzamidine effect in the present case is of the same general type. Conformational changes induced in the benzamidine-trypsin complex could be responsible for making a tyrosine residue more accessible to the acetylating reagent.

Riordan et al. (1965b) have shown that the acetylation of all ten of the tyrosines of trypsin in 8 molar urea or 3.2 molar guanidine eliminates all of the enzyme activity, whereas milder acetylation of only 6.7 of the ten tyrosine residues can be carried out with very little loss of enzyme activity. However, under the present conditions less than 3 tyrosines are acetylated (as judged by preliminary measurements of base-catalyzed deacylation) yet the partial inhibition is observed.

Serine 183 and histidine 46 have been directly implicated in the catalytic mechanism of trypsin. A possible explanation of these acetylation data is that the serine-histidine involvement in the catalytic site has been altered to a less efficient geometry within the active center, without significant distortion of the binding site. Serine has become less effective both in the acylation reaction with DFP^{32} and in the presumably rate-limiting deacylation step of the reaction with BAEE. The slower reaction of the acetyltrypsin with TLCK is consistent with a displacement of histidine 46 within the active

site. Further studies are in progress to determine whether these several modifications of the function of the enzyme are the result of a single simple distortion induced by the acetylation and to identify the site of the critical acetylation.

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