A REEXAMINATION OF THE REACTION OF TPCK WITH α-CHYMOTRYPSIN

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1. Introduction

The use of active-site-directed reagents has done much to enhance our knowledge of the amino acid residues involved in enzymic catalysis [1]. The reaction of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) with α-chymotrypsin established the presence of histidine-57 in the active center of the enzyme [2, 3]. The work of Stevenson and Smillie [4] further showed that the reaction occurred with the $N^{\epsilon 2}$ position of the imidazole ring. Although this evidence is impressive, it is not without ambiguity as it is dependent upon the loss of enzymatic activity during modification by TPCK, which does not necessarily require that the active center be modified [5–7]. The possibility remains that the active-site-directed reagent has not reacted with an active site residue, but shifts there during the various reactions used to localize it. Such intramolecular shifts were considered as possibilities with the diisopropylphosphonofluoride reaction which was initially presumed to react with a residue and to shift to the active serine during hydrolysis [8].

During the examination of several modified proteins with the Pauly reagent, it was observed that TPCK-modified chymotrypsin had more free imidazoyl residues than expected from the work of Ong, Shaw and Schoellman [2, 3]. This observation lead to a further investigation of the nature of the reaction between TPCK and chymotrypsin.

2. Methods

A solution of TPCK (134 mg in 3 ml methanol) was added to 1 g α-chymotrypsin (Armour) in 100 ml water at pH 7.5. After 24 hr at room temp, the material was passed through Sephadex G-25 in the cold. The fractions were combined and lyophilized. TPCK-chymotrypsin, prepared in this way, had less than 1% residual activity when assayed with *N*-acetyl-L-tyrosine ethyl ester (ATEE) at pH 8.0 and 20°.

The Pauly reaction was conducted following the modification of Weiss-Szobolew [9]. Standard curves of histidine, *N*-acetyl histidine, tyrosine and *N*-acetyl-tyrosine ethyl ester were prepared. Recovery experiments with known amounts of these standards in protein solutions also were run. Using the molecular extinction coefficients thus determined, the color yield of a protein is the sum of the tyrosine and histidine residue content.

Diazonium-1-H-tetrazole (DHT) was prepared by the method of Horinishi et al. [10]. Concentration of the reagent was determined by measuring the formation of *N*-acylmonoazotyrosine at a coupling pH 8.8. Standard curves were prepared as for the Pauly reagent. Optical densities were read at 480 and 550 nm for determination of the histidine and tyrosine content.

TPCK-chymotrypsin (2 ml) was denatured by addition to 8 ml 10 M urea, pH 4.3. After 15 min the pH was raised to 8.6 and 0.1 ml β-mercaptoethanol and 0.5 ml EDTA (2 mg/ml) were added. The pH was maintained under nitrogen in a pH-stat for 5 hr. The

liberated sulfhydryl groups were stabilized by reaction with ethylenimine, using three portions (0.2 ml) over a 30 min period. The mixture was then dialyzed against 0.5 M acetic acid. Amino acid analysis was done by the procedure of Moore and Stein on a Technicon amino acid analyzer [11].

3. Results and discussions

Diazonium reagents produce colored products with unsubstituted imidazoles and phenolic groups However, N-substituted imidazoles do not yield colored products [12]. This provides a means by which the unsubstituted imidazole rings of modified chymotrypsins might be estimated. The analysis of several proteins with the reagent diazonium-1-H-tetrazole is shown in fig. 1. The expected number of histidines were found in chymotrypsinogen and α-chymotrypsin while 1.6 residues react in TPCK-chymotrypsin. The failure of 0.4 residues to react need not be interpreted as the presence of 0.4 equivalents of an alkyl substituted histidine since DIP-chymotrypsin, which has both imidazoyl groups free, shows only 1.3 available histidines with the reagent. The reduction in reactive imidazoyl groups for both derivatives may thus merely reflect a steric inhibition effect caused by the presence of the TPCK- or DIP-groupings.

The Pauly reagent, diazotized sulfanilic acid, also yields results agreeing with the DHT procedure. The analysis of lysozyme and α-chymotrypsin shows the presence of 1.0 and 2.0 imidazoyl groups, respectively. For *N*-methyl-His-57-chymotrypsin [13] which has one of the imidazoyl rings methylated and the other free, we obtain 1.0 residue. Thus, on the basis of the known content of free histidine residues in the proteins tested, it can be shown that either the Pauly or Horinishi procedure gives the expected results. In contrast to these findings, TPCK-chymotrypsin has 1.6 imidazoyl groups.

The time course of inactivation of chymotrypsin with TPCK is shown in fig. 2. It can be seen that chymotrypsin is about 35% active when 1.6 imidazoyl groups are still capable of reacting with the Pauly reagent. Therefore only 0.4 histidine residue equivalents have reacted with TPCK at 30 min although the enzyme continues to lose activity with no further loss

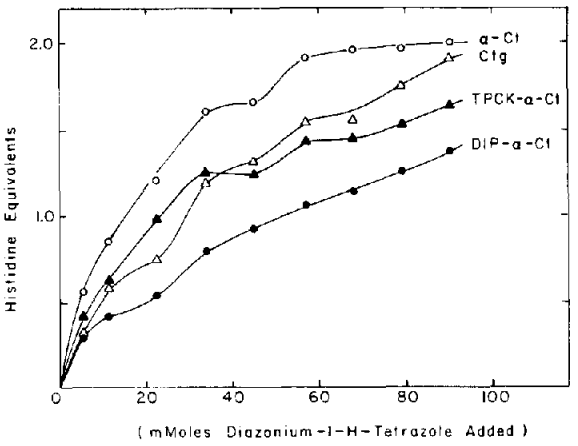

Fig. 1. Titration of the histidine content of several chymotryptic proteins with diazonium-1-H-tetrazole.

in imidazoyl groups. It appears that the rate of the imidazole reaction with TPCK and the loss of activity are not simultaneous events.

To verify the results obtained by the use of diazo-coupling, amino acid analyses were performed. When chymotrypsin and TPCK-chymotrypsin are hydrolyzed by the usual procedures [2, 4], we find 2.0 and 1.3 histidine residues, respectively. These are in agreement with reported values. However, if the proteins are

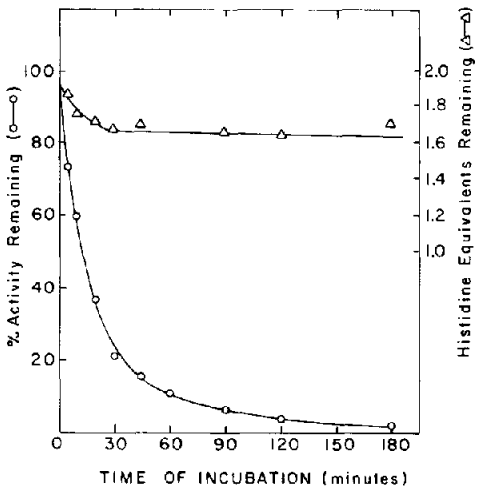

Fig. 2. Comparison of the change in chymotrypsin activity measured at pH 8.0 against ATEE and the reactivity of the histidine residues observed with the Pauly reagent during incubation of the enzyme with TPCK.

denatured and S-aminoethylated prior to hydrolysis, the histidine equivalents found are 2.0 and 1.6, in agreement with the values determined with the diazonium reagents.

The findings would suggest that the reaction of TPCK with chymotrypsin is not solely nor exclusively directed towards His-57. Although there is no reason to doubt the presence of His-57 in or near the active site of chymotrypsin, it must also be true that another residue or residues react with TPCK. Perhaps this finding should not be unexpected since several similar reagents inactivate chymotrypsin but react with residues other than histidine. For example, phenacyl bromide and bromoacetanilide both contain a phenyl ring and the same halomethyl ketone structure as does TPCK, yet they both modify Met-192 with formation of a sulfonium salt [14]. Additionally, TPCK inactivates the thiol protease, ficin, but does so by thioether formation rather than alkylation of an active site histidine as might have been expected using the reaction of TPCK with chymotrypsin as a model [15]. These results emphasize the necessity for critically examining the nature of enzyme inactivation during chemical modification.

Acknowledgements

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