ROLE OF TYROSINE RESIDUES IN CHYMOTRYPSIN ACTION*

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To gain a better understanding of the mechanism of chymotrypsin action, a critical evaluation of the role of each amino acid residue is needed. Previous studies have indicated that a serine (Balls and Jansen, 1952) and a histidine (Weil et al., 1953; Koshland et al., 1962; Schoellman and Shaw, 1962, 1963) residue are involved in the enzyme action and that methionine (Koshland et al., 1962), lysine (Chervenka and Wilcox, 1956) and a number of tryptophan (Viswanatha and Lawson, 1961; Wood and Balls, 1955) residues are not essential to the catalytic action. In the present study, the role of the four tyrosine residues are examined.

Early work was inconclusive (Sizer, 1949; Edman, 1947); but recently Glazer and Sanger (1963) iodinated the C-terminal tyrosine of the B-chain and Labouesse et al. (1964) acetylated two tyrosine residues without elimination of enzyme activity. Dube and coworkers (1963) have indicated that a tyrosine residue is protected by diisopropylfluorophosphate against iodination. In our studies a correlation between activity loss and modification of amino acid residues using iodine monochloride has been utilized for clarifying the role of the four tyrosine residues. The iodination methods and amino acid analyses followed the procedures of McFarlane (1958) and M. E. Koshland et al. (1963). In order to facilitate the interpretation of the data,

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the protein starting material was an oxidized chymotrypsin in which the "surface" methionine residue, 3 residues from the active serine, had been converted to the sulfoxide. The preparation and characterization of this enzyme have been described previously (Koshland et al., 1962) and it will be referred to here as sulfoxide-chymotrypsin. The amount of iodine taken up by the protein was determined by radioactive analysis of the I^{131}. The amounts of unmodified amino acid residues remaining were determined on the amino acid analyzer of Spackman et al. (1958) after acid or alkaline hydrolysis of the protein.

In order to identify the role of the four tyrosines in chymotrypsin, the combination of "efficiency" and "all-or-none" assays was used as described previously (Ray et al., 1960; Koshland et al., 1962; Ray et al., 1963). Briefly stated, an "efficiency" assay is sensitive to partially active enzyme species; consequently, changes in the values of this assay may result from changes in K_m and/or V_m. An all-or-none assay is designed to give 100% reactivity with partially active species, but zero reactivity with inert species. Values obtained by an all-or-none assay will decrease, therefore, only when enzyme molecules without detectable activity have been formed. For the efficiency assay in this case, the acetyl tyrosine ethyl ester procedure of Schwert and Takenaka (1955) was used and for the all-or-none assay, Bender's (Schonbaum et al., 1961) cinnamoyl imidazole reagent was effective.

The results are shown in Figures 1 and 2. During the initial phases of iodination of sulfoxide-chymotrypsin only tyrosine residues are affected. When 3.4 atoms of iodine have been taken up per molecule of chymotrypsin no decrease in activity has occurred as measured by either the all-or-none or the efficiency assays. It is to be noted that the efficiency assay for sulfoxide-chymotrypsin gives a value which is 37% that of the native chymotrypsin. This reflects the fact

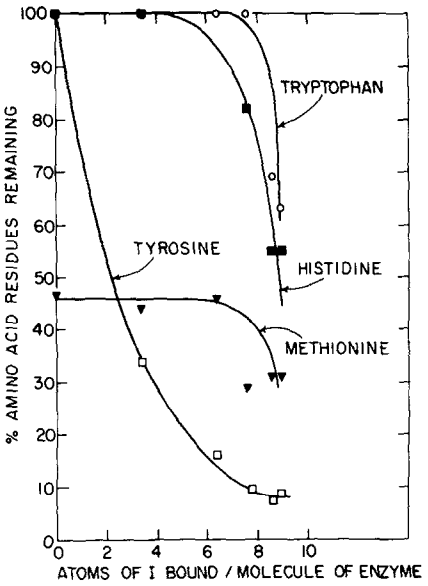

Figure 1. Modification of amino acid residues on iodination of sulfoxide-chymotrypsin. Iodination conditions: 0.2 M glycine buffer, pH 8.5, 0° C and 4 x 10^{-5} M α-chymotrypsin.

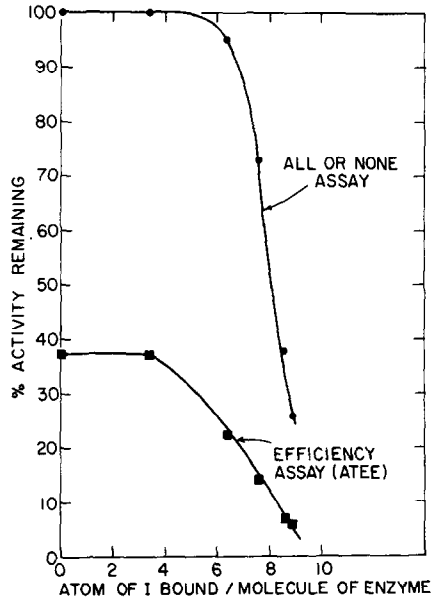

Figure 2. Loss of activity on iodination of sulfoxide-chymotrypsin. Conditions as in Figure 1. The all-or-none assay value is proportional to the amount of enzyme capable of reacting, i.e. partially active enzyme gives the same reading as fully active enzyme. The efficiency assay value decreases when partially active species are produced, i.e. is sensitive to changes in K_m and V_m.

that oxidation of the surface methionine increases the K_m of acetyl tyrosine ethyl ester by a factor of approximately 3 (Koshland et al., 1962). When 3.4 atoms of iodine have been taken up, approximately 2.6 tyrosine residues have been modified. From these data alone, therefore, it appears that at least 3 of the 4 tyrosine residues in chymotrypsin can be iodinated without significant change in either the K_m or V_m for the hydrolysis of acetyl tyrosine ethyl ester. When 6.4 residues of iodine have been covalently bound to the enzyme, 3.4 of the 4 tyrosine residues have been modified. At this stage 95% of the activity as measured by the all-or-none assay remains and a slight decrease in the activity as measured by the efficiency assay is observed. As further iodination occurs, activity is lost abruptly and modifications of histidine, tryptophan and methionine residues are observed.

The fact that no activity loss is observed when 2.6 tyrosine residues have been modified would suggest that these residues are not involved in either binding or catalysis. This conclusion is reasonable but it is not unequivocal. It is possible that a methionine modification has masked a tyrosine effect, i.e. that modification of either tyrosine or methionine affects the tertiary structure in the same way and their effects are competitive rather than additive. However, since methionine modification affects K_m only slightly, it can be concluded that three of the tyrosines have at most a minor effect on K_m and none of them is essential to the catalysis of ester hydrolysis.

The data suggest further that even the fourth tyrosine residue is not essential for the action of chymotrypsin. Assuming the most extreme case, i.e. that 3 residues are iodinate exclusively prior to iodination of the fourth residue, at least 34% of the fourth residue is modified at a point at which only 5% of the all-or-none

assay activity is lost. This could only be true if the chymotrypsin species in which the fourth tyrosine residue was iodinated still retained appreciable activity.

As iodination of tyrosine residues is increased there is a noticeable effect on the efficiency assay. This is confirmed by a separate kinetic study (cf. Figure 3). A chymotrypsin prepara-

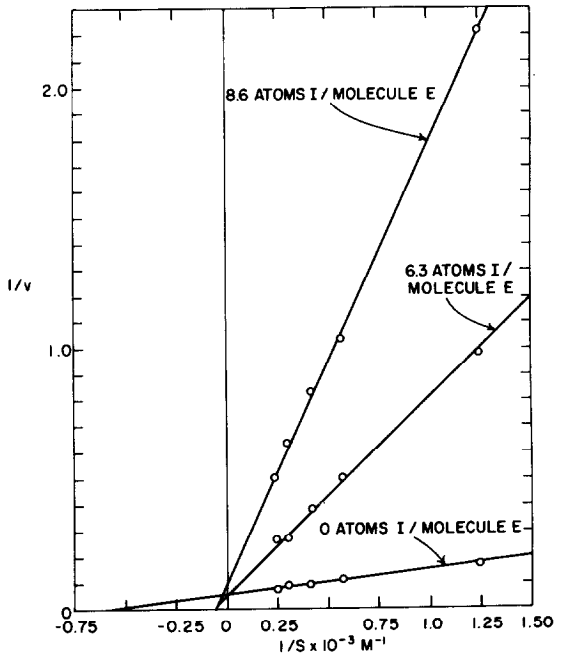

Figure 3. Determination of Michaelis constants for iodinated chymotrypsin samples. ATEE was used as a substrate and assay was done with a pH stat.

tion which contained 6.3 atoms of iodine per molecule had an increase in K_m by a factor of 6 without any change in the maximum velocity. The chymotrypsin preparation containing 8.6 atoms of I per molecule of enzyme shows no further change in the K_m but shows a decrease by a factor of more than 2 in the V_m. This decrease in V_m correlates with a loss in the all-or-none activity and the iodination of histidine. These data indicate that modification of at least 1 of the 4 tyrosine residues probably results in a change in K_m.

In any modification leading to little loss in activity, it must be considered whether the particular property of the residue which is essential to the catalysis has been effected. Thus it is possible that tyrosine plays an essential role but that iodination has not modified the property of tyrosine responsible for its function. Mono-iodination changes the pK of the phenolic hydrogen by about 0.8 pK units and diiodination by 2.8 pK units (Gemmill, 1954). An iodine atom has a Van der Waal's radius of 2.15 Å as compared to a radius of 1.7 Å for the benzene ring. Thus iodination has a dramatic effect on the polarization of the electrons in tyrosine and on its physical size. It seems improbable that these changes leave the essential tyrosine properties unaltered, and therefore the most plausible hypothesis to explain the activity of tyrosine modified enzyme is that none of the tyrosine residues perform an essential role in the catalysis of ester hydrolysis by chymotrypsin.

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