ESTERS OF IODINATED TYROSINE AS INHIBITORS OF CHYMOTRYPSIN

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1. Introduction

Hydrolysis of the isolated A chain of insulin with chymotrypsin will, under carefully controlled conditions, lead to specific cleavage between amino acid residues 14 (Tyr) and 15 (Glu(NH_2)). Each of the two peptides thus formed contains one of the tyrosines of the insulin A chain [1]. Chymotryptic hydrolysis has been used in an attempt to fragment A chains from iodinated insulin preparations in order to study the relative susceptibility of the various tyrosine residues to iodination [2]. In attempting to confirm and extend this work we had reason to doubt that iodinated tyrosine derivatives would act as substrates for chymotrypsin. We have shown that chymotrypsin will not hydrolyse either 3-iodo-*N*-acetyltyrosyl ethyl ester or 3,5-diiodo-*N*-acetyltyrosyl ethyl ester and that these two compounds inhibit the chymotryptic hydrolysis of *N*-acetyltyrosyl ethyl ester.

2. Materials and apparatus

Chymotrypsin was purchased from Sigma Chemical Corp., 12 mg were dissolved in 50 ml of 1 mM HCl.

N-Acetyl-L-tyrosyl ethyl ester (ATEE) was purchased from Sigma Chemical Corp. It was dissolved at a concentration of approximately 0.1 mM in isopropanol and its concentration accurately determined from its extinction coefficient by recording the ultraviolet spectrum of 200 μl diluted to 10 ml with water.

3-Iodo-*N*-acetyltyrosyl ethyl ester (MATEE) and 3,5-diiodo-*N*-acetyltyrosyl ethyl ester (DATEE) were prepared by the slow and careful addition of 101 or 202 mg of iodine dissolved in aqueous KI to 100 mg ATEE dissolved in 20 ml isopropanol adjusted to pH 9 with 0.1 N NaOH. After freeze-drying the solid was dissolved in 90% isopropanol and eluted from a column of Sephadex G-25 with ammonia solution pH 10. The fractions containing the highest concentrations of MATEE and DATEE were pooled and lyophilised. For use in enzyme kinetic studies these iodinated preparations were dissolved in isopropanol and diluted with water to a final concentration of 3% isopropanol. Their ultraviolet spectra were recorded and their concentrations calculated from their molar extinction coefficients [3]. pH was measured using the expanded scale of a Radiometer pH meter model 25.

Small volumes were routinely measured with a syringe microburette obtained from the Micrometric Instrument Co., Cleveland, Ohio.

3. Methods

0–200 μl of 0.1 M ATEE were added to 9.5 ml 3% isopropanol in water with or without MATEE or DATEE. The volume was made up to 10 ml and the pH carefully adjusted to 7.00 by the addition of 10 mM NaOH. The reaction was started by the rapid addition of 25 μl of chymotrypsin solution from a micropipette and the course of the reaction was followed by titrating in 10 mM NaOH to maintain the pH at 7.00.

4. Results

No acid production could be detected for at least 30 min when chymotrypsin was added to three different concentrations of MATEE or DATEE ranging from 0.7–1.8 mM.

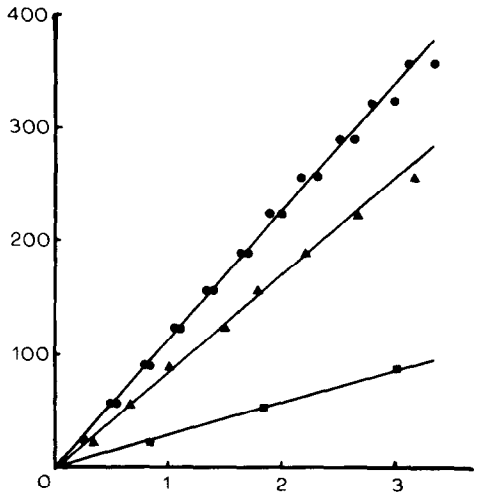

Fig. 1. The hydrolysis of 2 mmoles ATEE by chymotrypsin. • control; ▲ with 0.6 mmole DATEE; ■ with 0.9 mmole DATEE. Abscissa: μmoles ATEE hydrolysed per mg enzyme Ordinate: time in min.

Fig. 1 shows the rate of hydrolysis of 2 mM ATEE compared with the rate in the presence of 0.6 and 0.9 mM DATEE.

Fig. 2 shows Lineweaver Burke plots for the hydrolysis of ATEE in the presence and absence of 1.70 mM MATEE and 0.55 mM DATEE.

5. Conclusions

The apparent K_m of ATEE for chymotrypsin at pH 7.0 obtained from fig. 2 is 0.61 mM which is in reasonable agreement with the literature value obtained at pH 7.8 [5]. Using this value and the apparent K_m values in the presence of MATEE and DATEE K_i values of 0.46 and 0.29 mM respectively are obtained for the iodinated esters. These values are of course true K_i values for the dissociation of the enzyme inhibitor complex [5] whereas the apparent K_m does not give a true value for the dissociation complex (K_s) of the enzyme substrate complex. The true K_s for the ATEE–chymotrypsin complex is almost certainly much higher [6] so that the introduction of iodine into tyrosine residues apparently results in an increase in affinity for the active site of chymotrypsin.

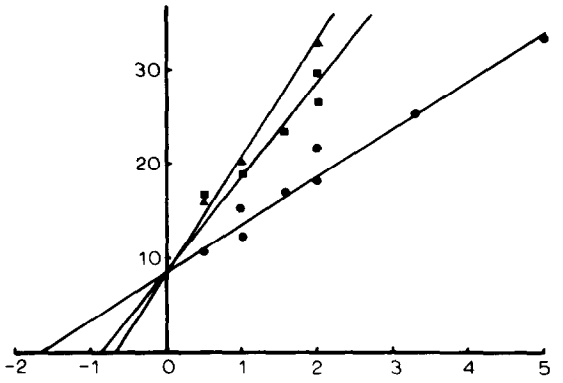

Fig. 2. Lineweaver Burke plot for the chymotryptic hydrolysis of ATEE. • control; ▲ with 1.70 mmoles MATEE; ■ with 0.55 mmole DATEE. Abscissa: $\frac{1}{S}$(litres × mmoles^{-1}) Ordinate: $\frac{1}{V}$(10^3 × μmoles^{-1} × mg enzyme × min).

By studying a series of *N*-acetyl-L-amino acid ester substrates of chymotrypsin Knowles [4] showed that substrates with a lower K_m for chymotrypsin tend to hydrolyse faster. Such a relationship demands that the adsorptive forces which lower K_s and result in better binding of substrate to enzyme are of the same kind as those which affect the subsequent catalysis [7]. Hawkins et al. [7] point out that even though these forces were similar in binding and catalysis faster hydrolysis would not result if the substrate were bound in the "wrong way" as would happen if the substrates were D-amino acid derivatives. But the iodinated tyrosine derivatives which apparently have a high affinity for chymotrypsin but are not hydrolysed are L-amino acid derivatives and presumably cannot be bound in the "wrong way".

It is attractive to postulate that they cannot be hydrolysed because the complex which they form with chymotrypsin cannot undergo an important conformational change without which catalytic activity is not possible [8].

Whatever the reason for the inability of chymotrypsin to hydrolyse MATEE and DATEE it is clear that this enzyme cannot be used to produce peptides from iodinated proteins when the distribution of iodine in these proteins is under study.

Acknowledgements

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References

[1] A.P.Ryle, F.Sanger, L.F.Smith and R.Kitai, Biochem. J. 60 (1955) 541.
[2] L.W. de Zoeten and E.Havinga, Rec. Trav. Chim. 80 (1961) 917.
[3] D.M.Harrison and C.J.Garratt, FEBS Letters 11 (1970) 14.
[4] L.W.Cunningham, J. Biol. Chem. 207 (1954) 443.
[5] J.R.Knowles, J. Theoret. Biol. 9 (1965) 213.
[6] B.Zerner and M.L.Bender, J. Am. Chem. Soc. 86 (1964) 3669.
[7] M.J.Hawkins, J.R.Knowles, L.Wilson and D.Witcher, Biochem. J. 104 (1967) 762.
[8] T.E.Barman and H.Gutfreund, Biochem. J. 101 (1966) 411.