A KINETIC STUDY ON OXYTOCINASE (CYSTINE AMINOPEPTIDASE) THE EFFECT OF REMOVAL OF SIALIC ACID

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Neuraminidase treatment of oxytocinase, resulting in complete removal of sialic acid was performed and its influence on some kinetic properties of the enzyme was studied. Both K_m (L-leucine-*p*-nitroanilide) and K_i H-Cys(Bzl)-Pro-Leu-GlyNH_2 were found to increase in the pH interval 6—8. A relative change in properties was observed at pH values where the conformational change of oxytocinase takes place.

Oxytocinase from human retroplacental serum has been shown to be a glycoprotein[1-3] acting as an aminopeptidase with very broad specificity[1,2,4]. The enzyme molecule contains in addition to other carbohydrates (a total of 44%) about 40 sialic acid residues, all of which can be removed by α-neuraminidase[3] without change in specificity[1]. The sialic acid is involved in a conformational change of the oxytocinase molecule around pH 8·5 (ref.[5,6]) which does not take place in the enzyme when sialic acid is removed. Thus the removal of sialic acid, at physiological pH, will not result only in a decrease in total negative charge and in a change of charge distribution on the molecular surface but also in a change of conformation.

The present communication reports on some studies concerning the influence of the above mentioned changes on the catalytic properties of oxytocinase.

EXPERIMENTAL

Oxytocinase was prepared as described by Yman[3]. A stock solution of pure enzyme (E_{280} = = 0·085 corresponding to a conc. 0·110 mg/ml taking human albumine as the standard in Lowry

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method) in 0·001M sodium phosphate buffer, pH 7·4 was kept at +4°C for a period of several months without loss of activity.

Sialic acid free oxytocinase was prepared by the incubation of 5 ml of stock solution of oxytocinase with 0·4 ml α-neuraminidase (Serva Entwicklungslabor, Heidelberg) at pH 7·4 for 5 hours at 37°C. The incubation mixture was subjected to gel filtration on Sephadex G-200 as described for oxytocinase[3] in order to remove the neuraminidase. The pooled fractions were dialysed against 0·001M sodium phosphate buffer, pH 7·4 for 24 hours and kept at 4°C. The enzyme preparation obtained was homogenous in polyacrylamide gel electrophoresis, giving only one zone both after protein staining and after incubation with leucine-β-naphthylamide as previously described[7].

The substrate, L-leucine-*p*-nitroanilide, was synthetized by Dr E. Kasafírek, Research Institute for Pharmacy and Biochemistry, Prague. The inhibitor, H-Cys(Bzl)-Pro-Leu-GlyNH_2 was prepared by Dr K. Jošt, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague.

The enzymic reaction with L-leucine-*p*-nitroanilide as substrate was carried out at 37°C in thermostatized cuvettes. The incubation mixture in a total volume of 1·0 ml consisted of 0·1M sodium phosphate buffer pH 6·16—7·95, 0·2—1·2 mM substrate and 5 μl of stock solution of intact oxytocinase or 100 μl of neuraminidase — treated oxytocinase (the amount was chosen so as to get approx. the same activity as for the intact oxytocinase). The reaction rate was determined by following the changes of optical density at 406 nm which was read off at one minute intervals for 7—8 minutes.

The inhibitory effect of the peptide was estimated in the above mentioned substrate concentration range. The final concentration of the inhibitor was 0·0125 mM and 0·050 mM.

Michaelis constant (K_m), maximal velocities (V) and inhibition constants (K_i) were determined graphically using a double reciprocal plot[8-10].

RESULTS AND DISCUSSION

As shown by Tuppy and coworkers[1] the substrate specificity of oxytocinase, at least so far the chromogenic substrates used is concerned, is unaffected by treatment by α-neuraminidase. However, when the changes of the enzyme molecule caused by the removal of all the sialic acid residues are taken into account certain alternations of the enzymic properties would be expected.

Fig. 1 shows the pH dependence of the Michaelis constant and maximal velocities for the intact and sialic free enzyme with L-leucine-*p*-nitroanilide as substrate. The curves found for oxytocinase are similar to those obtained earlier by Yman and Sjöholm[6] with L-leucine-β-naphthylamide as substrate but the pH dependence of pK_m is in this case more complex. In the whole pH range studied the pK_m of the neuraminidase-treated enzyme is lower than that of oxytocinase, while the shape of the curve is only moderately changed. For example, at pH 7·4 the K_m values for the oxytocinase and sialic acid free oxytocinase were found to $5 \cdot 10^{-4}$ and $3 \cdot 10^{-3}$M, respectively. As the amount of enzyme in the incubation mixture was chosen to give about the same activity in both cases no information was obtained concerning the effect of removal of sialic acid on the absolute rate of the catalytic reaction. The

identical shape of the curves shows that the same groups are involved in the catalytic reaction in both cases.

The peptide (H-Cys(Bzl)-Pro-Leu-GlyNH$_2$) is not hydrolyzed by oxytocinase and it is as shown by Beránková and coworkers[11,12] a potent inhibitor of the enzyme. Compared to other structural fragments of the oxytocin molecule it is the most effective inhibitor. This tetrapeptide was found to be a competitive inhibitor of both intact and sialic free oxytocinase in the whole pH range studied (*e.g.* Fig. 2). The affinity of oxytocinase for the peptide is about 70 times higher than that for the substrate. At pH 7·4 K_i was found to be 7.10^{-6}M as in the case of K_m, it was increased by neuraminidase treatment of the enzyme (Fig. 3).

The intact enzyme exist in two form in the alkaline pH range, and although only one form is present at neutral and slightly acid pH, the Michaelis-Menten kinetics are too irregular and complex in this range to be evaluated according to Dixon[9]. No conclusions can be therefore drawn from the curves about the nature of the groups important for the formation of the enzyme–substrate complex. When the pH dependence of K_m and K_i of both enzymes is compared it is obvious that at pH

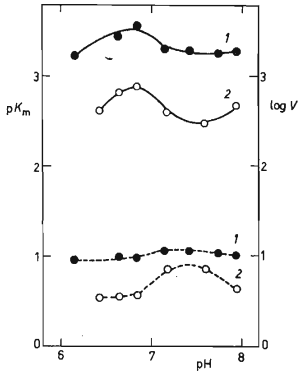


FIG. 1

pH Dependence of Michaelis Constant (— log M, full line) and Maximal Velocities (log M.min^{-1}.10^6, broken line) for Oxytocinase (*1*) and Neuraminidase Treated Enzyme (2)

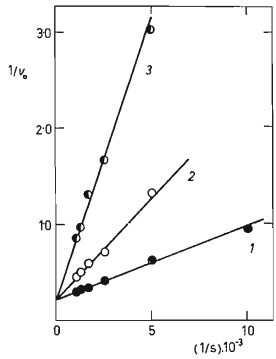


FIG. 2

Double Reciprocal Plot Showing the Competitive Inhibition of Oxytocinase by H-Cys(Bzl)-Pro-Leu-GlyNH$_2$ at pH 6·6

Reaction without inhibitor (*1*), inhibitor concn. $1{\cdot}25.10^{-5}$M (2), inhibitor concn. 5.10^{-5}M (*3*). Abscissa: M^{-1}, ordinate: M^{-1} . min . 10^{-6}.

values lower than 7·5 the main result of the neuraminidase treatment is an increase of both constants. However, at pH values higher than 7·5 the properties of oxytocinase are markedly altered by the removal of sialic acid. Relative to the intact enzyme the K_m value of the neuraminidase treated oxytocinase decreases in this pH range, while K_i on the other hand shows an increase. This behaviour is probably directly

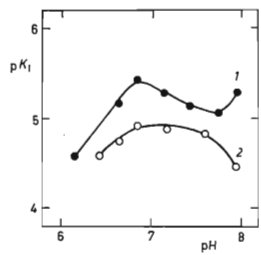
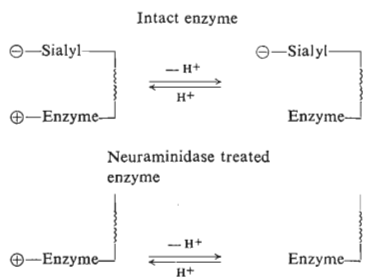


FIG. 3

pH Dependence of Inhibitor Constants for Oxytocinase (*1*) and Neuraminidase Treated Oxytocinase (*2*)

Ordinate: —log M.

connected with conformational change of oxytocinase[3,5,6] which occurs in this range and in which sialic acid plays an important role. Scheme 1 shows on the equilibrium between the two conformational forms of oxytocinase (CAP_1 and CAP_2) at pH values around 8·3 (pK_a of the positively charged group). The intact oxytocinase is compared to the sialic acid free enzyme.



SCHEME 1

According to the present state of knowledge it seems probable that sialic acid has a secondary effect and it is not directly involved either in the formation of the enzyme substrate complex or in the catalytic reaction. However, its presence on the surface of the protein molecule will necessarily influence the distribution of charges in the enzyme molecule which may facilitate the formation and/or improve the stability of the enzyme substrate complex.

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