ON THE MECHANISM OF AMIDE-BOND-CLEAVAGE CATALYZED BY AMINOPEPTIDASE M. KINETIC STUDIES

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1. Materials

Aminopeptidase M, commercial product (Röhm and Haas, Darmstadt) was further purified by repeated gel filtrations (Sephadex G-75 coarse equilibrated with 0.1 M ammonium bicarbonate buffer pH 7.0) up to a specific activity of 66 I.U.

The substrate for kinetic measurements, L-alanine-4-nitranilide was synthesized by the DCCI-procedure (m.p. 97°). All buffer substances were reagents grade.

2. Methods

The pH dependence of the Michaelis constant and the maximal rate were studied in the range between pH 6.0 and pH 10.0 at intervals of 0.1 pH units, using an universal buffer [1] of constant ionic strength.

Substrate cleavage was followed at 25° by measuring the change of absorbance at 405 nm in an Eppendorf Photometer (Netheler and Hinz, Hamburg) equipped with a thermoconstant cell compartment, through at least two half lives of the reaction. In random cases, infinity absorbance readings were taken and were found in all cases to agree within 1% with the value for complete hydrolysis of the substrate. The evaluation of the kinetic parameters resulted from a weighted statistical fit [2].

3. Results and discussion

The results of the measurements are summarized in figs. 1 and 2. Three clear bends are seen in the curves, one near pH 7.2 and the others at pH 8.5 and at pH 9.7.

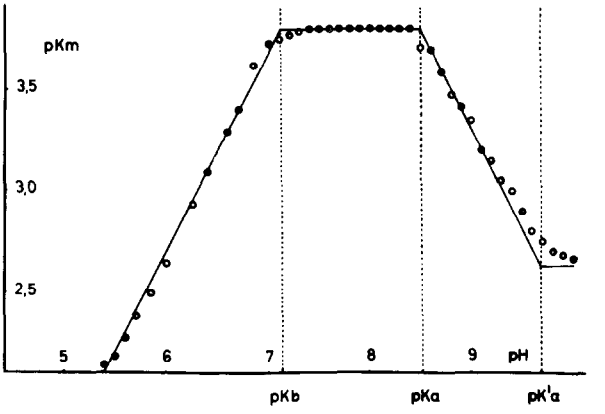

Fig. 1. pH-Dependent changes in the Michaelis constants for the cleavage of L-alanine-4-nitranilide . Enzyme concentration: 4×10^{-8} M; T = 25°

The value at pH 8.5 corresponds to the determined pK_a of the substrate ammonium group; the other values are due to the enzyme protein. Particularly interesting is the pH-dependence of the maximal rate (fig. 2). Ionization of the substrate is obviously the rate determining factor although according to the theory of irreversible one substrate reactions, only the ionizable groups of the enzyme-substrate-complex should be rate limiting [3]. If a progressive deprotonation of the substrate ammonium group occurs above pH 8.5, substrate binding becomes impossible and without formation of a reactive enzyme-substrate-complex, no substrate cleavage will occur. At the acidic site, the maximal rate but *not* the Michaelis constant is independent of the hydroxonium concentration; there is competition between the substrate ammonium groups and hy-

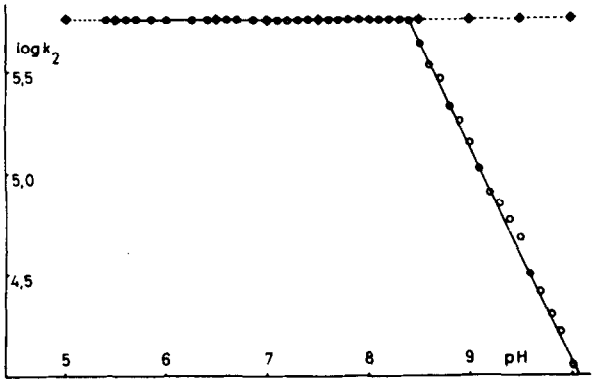

Fig. 2. pH-Dependence of maximal rate for cleavage of L-alanine-4-nitranilide catalyzed by aminopeptidase M. Enzyme concentration 4 X 10^{-8} M (molecular weight 2.8 X 10^5). The filled squares symbolize rate constants for 15 min incubation at the noted pH-values (25°). Measurements were then performed at pH 7.0 (control for denaturation). Beyond pH 6.5 the rate of cleavage could no longer evaluated with sufficient precision under the chosen experimental conditions.

droxonium ions at an anionic binding site at the protein surface. Assuming that all ionizisation steps occur at an identical rate and treating the overall pH-dependence as if there were no ionizisable substrate, i.e. the ammonium group is assumed to be an integral part of the enzyme protein, the problem can be solved kinetically on the basis of a protein ionization, in three steps.

$$E \underset{}{\overset{H^+}{\rightleftharpoons}} EH \overset{H^+}{\rightleftharpoons} EH_2 \text{ (charges omitted)}$$

with only one species, EH, being involved in substrate binding. The charged group of the enzyme-substrate-complex (pK 9.7) is introduced with the assumption that only a definitive state of ionization of this side chain will permit the break down in the direction of product, forming:

$$\begin{array}{ccccc} E & & ES & & \\ \uparrow\downarrow K_a & & \uparrow\downarrow K'_a & & \\ EH & \overset{k_1}{\rightleftharpoons} & EHS & \overset{k_2}{\rightleftharpoons} & EH + P \\ \uparrow\downarrow K_b & & & & \\ EH_2 & & & & \end{array}$$

On steady state treatment, the pH-dependent rate $v = k_2$ [EHS] results as follows:

$$v = \frac{k_2 \text{ [E] total [S]}}{K_m\left(1 + \frac{[H^+]}{K_b} + \frac{K_a}{[H^+]}\right) + [S]\left(1 + \frac{K'_a}{[H^+]}\right)}$$

and on differentiating, the hydroxonium concentration at which the reaction proceeds at maximal rate will be obtained:

$$[H^+]_{opt} = \left(\frac{K_b(K_aK_m + K'_a\,[S])}{K_m}\right)^{\frac{1}{2}}$$

The calculated value for substrate concentrations in the range of the Michaelis constant, pH_{opt} = 7.86, clearly corresponds with the experimental value (fig. 3).

Groups ionizing near neutrality in protein chemistry are usually thought to be imidazole nuclei of histidine side chains. Chemical modification has proved that histidine residues are involved in the reaction mechanism [4]. The group with pK 9.7 is identified, by chemical modification, as a tyrosine side chain [5].

A possible mechanism of amide cleavage by amino-

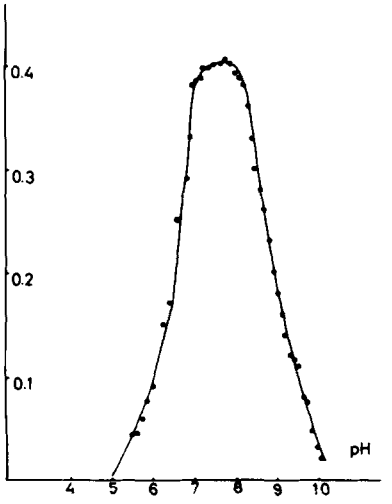

Fig. 3. pH-Optimum for the cleavage of L-alanine-4-nitranilide by aminopeptidase M.
Substrate concentration: 2 X 10^{-4} M
Enzyme concentration: 4 X 10^{-8} M
T = 25°.

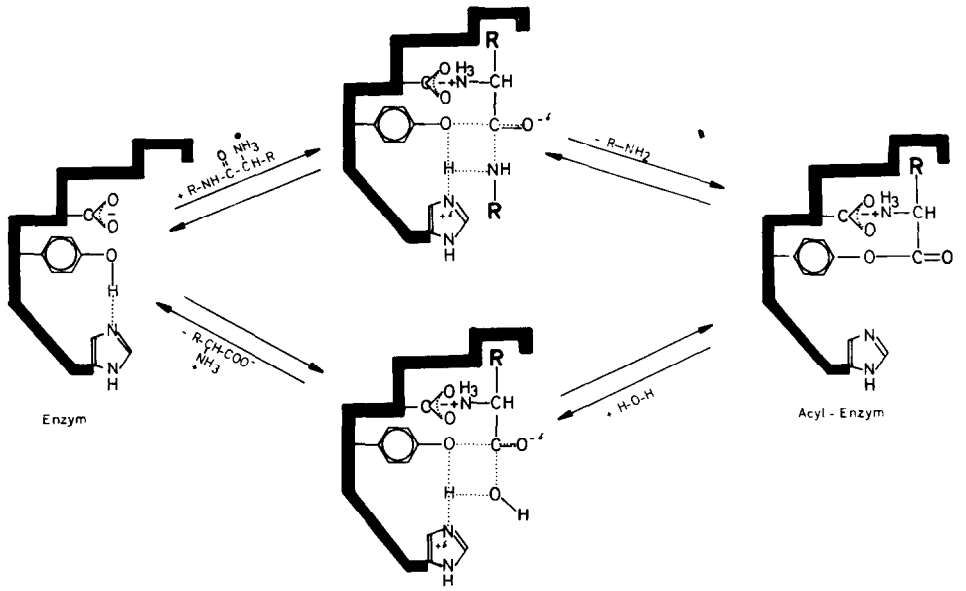

Fig. 4. Cooperative histidine-tyrosine catalysis as a possible mode of action for amide bond cleavage by aminopeptidase M (Explanation see text).

peptidase M is that of cooperative imidazole-tyrosine catalysis (fig. 4). The substrate is bound by ion pair effects in such a strereospecific way that the susceptible amide bond is placed in juxtaposition to a tyrosine-histidine hydrogen bridge of the active center. Facilitated proton transfer along rigid hydrogen bonds occurs during the transformation of the primary absorptive enzyme-substrate-complex to the active form of the complex. Mononitration of the tyrosine active centers leads to disruption of crucial H-bonds; essential protonation of the leaving moiety of the substrate will then be impossible because of absence of facilitated proton transfer.

References

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[4] G.Pfleiderer and U.Emfert, FEBS Letters 4 (1969) 265.
[5] U.Femfert and G.Pfleiderer, FEBS Letters 4 (1969) 262.