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

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1. Materials

The aminopeptidase preparation was the same as described previously [1]. The substrate for the kinetic measurements L-alanine-4-nitroanilide-hydrochloride was purchased from Merck (Darmstadt, Germany). Deuteriumoxide, sodium hydroxide, sodium chloride and boric acid were obtained from the same source. Phosphoric acid was obtained from Fluka (Buchs, Switzerland). All substances were reagent grade.

2. Methods

Solvolysis of the substrate was followed photometrically by monitoring the increase in absorbance at 405 nm in an Eppendorf-Photometer (Netheler und Hinz, Hamburg) equipped with a thermoconstant cell compartment (25°, registered directly inside the cell) through at least two half lives of the reaction. Using a minimum of five substrate concentrations, Michaelis-Menten parameters were calculated from initial rates by Wilkinson's method [2]. 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. No non-random deviations from Michaelis-Menten kinetics were observed. 0.04 M phosphate–acetate–borate buffer of constant ionic strength was used throughout the experiments. Blank rates were checked independently.

3. Results and discussion

Kinetic investigations and results of selective chemical modification lead to a possible mechanism of aminopeptidatic amide bond cleavage consisting of a cooperative imidazole–tyrosine catalysis [1]. Because of its enhanced nucleophility, the tyrosine anion was considered as the attacking nucleophile at the sp^2 carbon atom of the amide bond.

In the optimum pH range of aminopeptidase M catalyzed reactions ($\approx$ pH 8) however the reactive tyrosine of the enzyme is ionized approximately to less than two percent and we assumed that the acidity of the phenolic hydroxyl was enhanced considerably by hydrogen bonding to the active histidine residue.

Evidence on the formation of a hydrogen bridge should be obtained from a study of the effects of deuteriumoxide on the kinetic constants of the enzymatic hydrolysis. It is well known that the velocity of a reaction with rate limiting proton transfer decreases to about one third in deuteriumoxide. The formation of a hydrogen bridge can be considered as a partial proton transfer. Accordingly, if a hydrogen bond were formed between the catalytic groups, the formation of an acyl–enzyme intermediate during the hydrolysis of substrate would not be markedly affected in deuteriumoxide.

Fig. 1 shows the differences in the rate of substrate cleavage in deuteriumoxide and in water. The following water/deuteriumoxide quotients for the maximum rates of overall hydrolysis and the Michaelis constants can be calculated from fig. 2:

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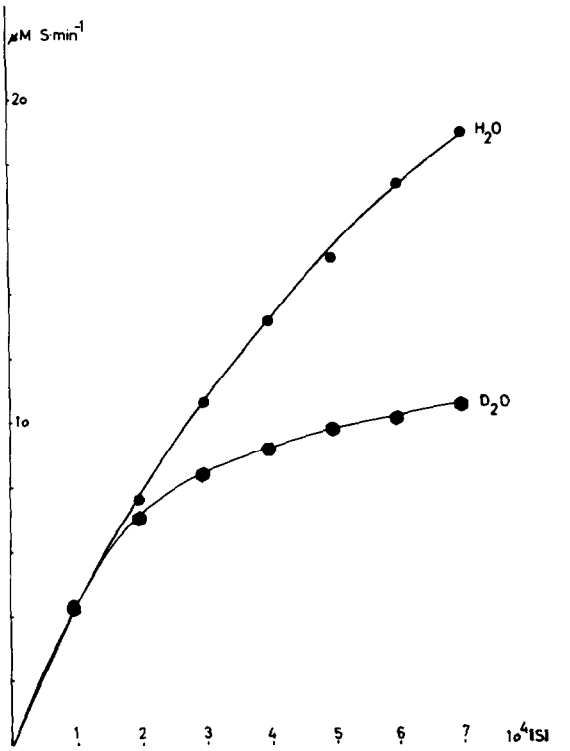

Fig. 1. Michaelis-Menten plot of the initial velocity of solvolysis of L-alanine-4-nitranilide catalyzed by aminopeptidase M in 0.15 M phosphate–acetate–borate buffer pH 8.15 and pH 8.15, 25°.

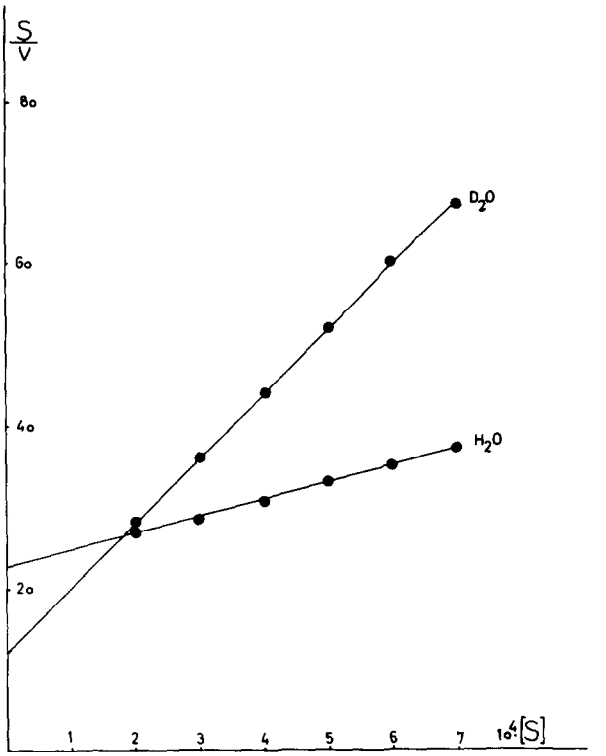

Fig. 2. Hanes plots of the aminopeptidase M catalyzed solvolysis of L-alanine-4-nitranilide in water (pH 8) and deuteriumoxide (pH 8).

$$\frac{(V_{max})_{H_2O}}{(V_{max})_{D_2O}} = 3.94 \qquad \frac{(K_m)_{H_2O}}{(K_m)_{D_2O}} = 7.36$$

The kinetic solvent isotope effect (KSIE) on the maximum rate is of the expected order of magnitude for normal solvolytic reactions with a rate limiting proton transfer step. The KSIE on the Michaelis constant is more informative. If the Michaelis constant were merely a binding constant and if the isotope effects were caused only by differences of zero point energy, it might be expected that there would be no isotope effects on Michaelis constants. Since none of these assumptions is correct, it is not surprising that the existence of isotope effects on Michaelis constants is more the rule than the exception [3]. The observed KSIE on the Michaelis constant in the aminopeptidase reaction does not merely reflect changes in the ionization behavior of the active form of the enzyme or the substrate (fig. 3). The rate maxima agree to corresponding values of pH and p*D* (Glass electrode correction of [4] is used for deuteriumoxide solutions).

If the overall reaction is written schematically without the implication of an acyl–enzyme intermediate in the simplest Michaelis-Menten form:

$$E + S \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} ES \xrightarrow{k_{23}} E + P$$

the Michaelis constant results with

$$K_m = \frac{k_{21} + k_{23}}{k_{12}}$$

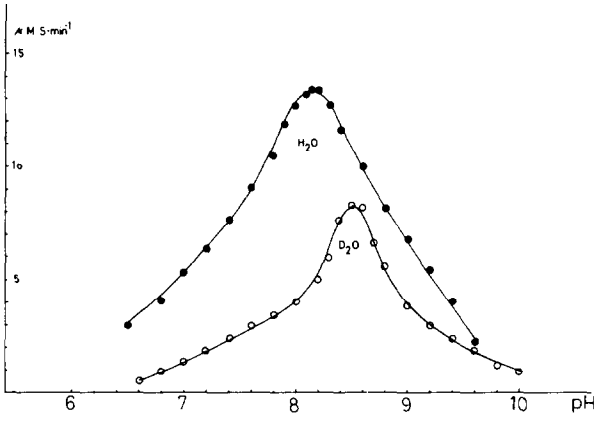

Fig. 3. pH-rate profiles of the aminopeptidatic solvolysis of L-alanine-4-nitranilide in water and deuteriumoxide.

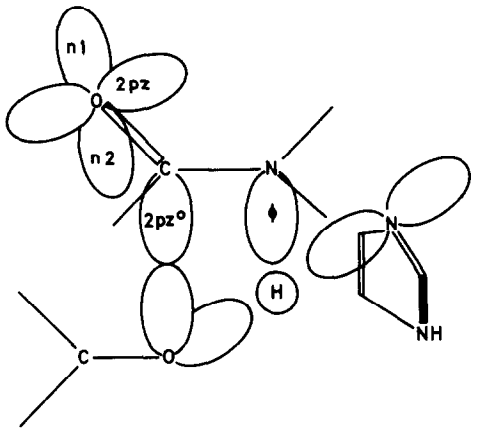

Fig. 4. Mechanism of tyrosine–imidazole cooperation. For explanation see text.

The constant k_{23}, a complex value, corresponds to both acylation and deacylation. If the nucleophilic attack of the tyrosyl anion proceeds via a SN_1cA or SN_2cA mechanism, i.e. the protonation of the leaving group results from a pre-equilibrium step (and anilide hydrolysis as a matter of fact will not proceed without protonation of the leaving moiety because $ArNH^-$ is a poor leaving group compared to the entering nucleophile) then the quotient $(k_{23})_{H_2O}/(k_{23})_{D_2O}$ corresponds to the quotient $(V_{max})_{H_2O}/(V_{max})_{D_2O}$ An existing hydrogen bridge causes the quotient $(k_{12})_{H_2O}/(k_{12})_{D_2O}$ to approach unity. Because of rapid exchange of the protonated species with the solvent, the back reaction, k_{21}, however would show a normal KSIE.

Considering the following conditions:

$$\frac{(K_m)_{H_2O}}{(K_m)_{D_2O}} = 7.36 \qquad \frac{(k_{23})_{H_2O}}{(k_{23})_{D_2O}} = 3.94 \qquad \frac{(k_{12})_{H_2O}}{(k_{12})_{D_2O}} = 1$$

the KSIE on the rate constants of the backward reaction results in the expected order of magnitude:

$$\frac{(k_{21})_{H_2O}}{(k_{21})_{D_2O}} = 3.42$$

The idea of an tyrosine–imidazole hydrogen bridge does not contradict the presented results. To explain the mechanism of aminopeptidase attack at the substrate amide bond, the following picture can be drawn (fig. 4):

The pi-bond in the carbonyl group is composed of p-orbitals and it is assumed that a nucleophile attacking this pi-bond must approach carbon in such a stereoselective way as to provide overlap between its own orbitals and that of the carbon atom. In addition, coulombic repulsion exists between the entering group and the high electron density at the oxygen atom. The role played by the histidine residue consists of accepting the proton from the hydroxyl group of the tyrosine side chain via a hydrogen bridge and then lending it in a pre-equilibrium step to the leaving moiety of the substrate. A favorable collision between the carbonyl atom of the substrate and oxygen atom of the tyrosine then triggers the catalytic process.

References

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