ON THE MECHANISM OF AMIDE BOND CLEAVAGE CATALYZED BY AMINOPEPTIDASE M. ENZYMATIC PROPERTIES OF NITROAMINOPEPTIDASE M

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1. Introduction

The presence of tyrosine residues at or near the active site of aminopeptidase M has been demonstrated [1]. This extend study was initiated to obtain further knowledge about the function of the reactive tyrosyl residues in aminopeptidase M as well as the cause of the loss of enzymatic activity upon nitration.

2. Materials

Aminopeptidase M, obtained from Röhm GmbH (Darmstadt) with a specific activity of 14.18 IU was further purified by repeated gel filtrations up to a specific activity of 60 IU. Tetranitromethane (TNM) was a product of Fluka (Switzerland), as was tributylphosphate used for solvent extraction processes which was redistilled immediately before use (Kp_{10} 159°).

The substrates for kinetic measurements, glycine-4-nitroanilide, L-alanine-4-nitroanilide, L-leucine-4-nitroanilide and L-phenylalanine-4-nitroanilide were generous gifts of Dr. Lang (Merck, Darmstadt).

The pseudosubstrate L-alanine-2,6-diethylanilide was prepared by the phosphazo-method [2].

3. Methods

Protein concentrations were determined at 280 nm ($A^{1\%}_{1\,cm}$ =17.0). The amount of nitrotyrosine present in the protein was obtained by measuring the difference in absorbance between the nitrotyrosyl (pH 3.0) and the nitrotyrosylate (pH 11.0) chromophores at 436 nm ($\epsilon = 4.6 \times 10^3$ mole$^{-1} \times$ cm^{-1}).

Kinetic measurements were performed as previously described [3]. Substrate was L-alanine-4-nitroanilide unless otherwise stated. The molecular weight of aminopeptidase M used in the calculations is 2.8×10^5.

Solutions of nitrated protein used for spectrophotometric titration of nitrotyrosine were adjusted to the desired pH by means of Knick pH-meter type 350/34. Constant ionic strength was maintained throughout the investigated pH range.

Nitration was carried out at room temperature by adding aliquots of an 1 M TNM solution in ethanol to a buffered, 4×10^{-6} M, solution of aminopeptidase M. Because, at the usual concentrations, the reagent largely exists as a separate phase, the reaction was allowed to proceed with vigorous stirring. In order to remove excess reagent and nitroformate produced, a solvent extraction procedure was used [4].

4. Results and discussion

4.1. *pH-dependence of the inactivation of aminopeptidase M by tetranitromethane*

The loss of activity of aminopeptidase M by TNM treatment was studied at various pH values between pH 6.0 and pH 9.0 with a 15-fold molar excess of reagent (fig. 1).

The inactivation rates increased with the pH of the reaction mixture. A half maximal rate of inactivation is reached at pH 7.6. Since only the ionized form of the tyrosine reacts with TNM [5] it appears that the tyrosine residues involved in the reaction have an apparent p*K* value of 7.6 which differs markedly from the value of surface tyrosines in proteins [6].

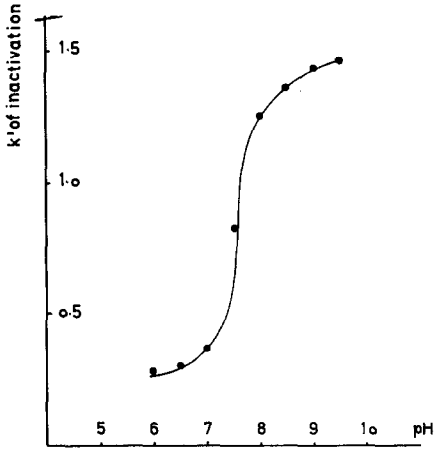

Fig. 1. Kinetics of the enzyme inactivation as a function of the pH of the solution. The apparent first order reaction constants of the inactivation process were determined graphically from semilogarithmic plots.

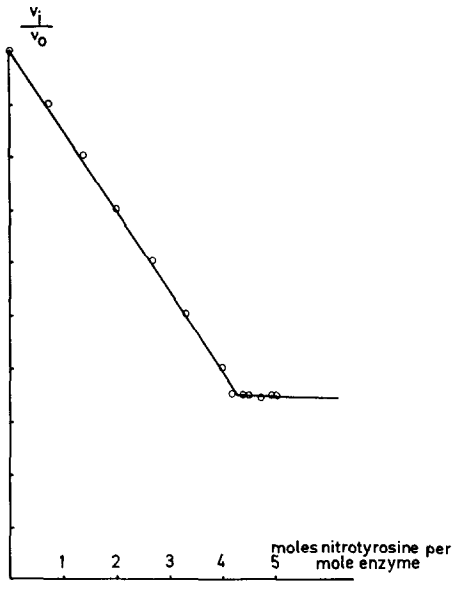

Fig. 2. Correlation between enzyme inactivation and tyrosine nitration. The number of the nitrotyrosines per mole of enzyme was determined spectrophotometrically.

Further nitration studies were performed at pH 7.5 with a 15-fold molar excess of reagent.

4.2. *Quantitation of the nitration reaction*

When measured spectrophotometrically the 3-nitrotyrosine content increases proportionally to the decrease of enzymatic activity (fig. 2). Maximum inactivation (65%) is obtained when 4.2 moles of nitrotyrosine per mole of enzyme are introduced. The presence of the pseudosubstrate L-alanine-2,6-diethylanilide modifies the nitration kinetics of the enzyme (fig. 3): the nitration of three of the accessible tyrosine residues is considerably retarded.

4.3. *Enzymatic properties of nitroaminopeptidase M*

Nitration of tyrosyl side chains increases their bulks and decreases the pK of the phenolic function.

TNM treatment of aminopeptidase M involves a first order loss of activity and both, K_m and V_{max} are altered considerably but the pH-dependence of V_{max} and K_m as well as the pH-optimum of the reaction remain unaltered compared to the native enzyme. Spectrophotometric titration of the nitro-

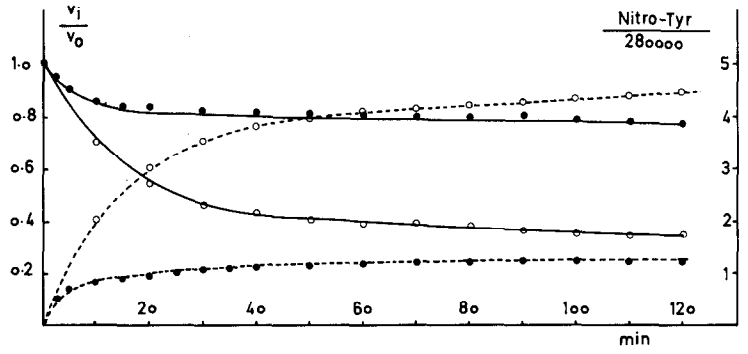

Fig. 3. Time course of the nitration of aminopeptidase M (dashed lines) without (o—o—o) and with (•—•—•) the pseudosubstrate L-alanine-2,6-diethylanilide and the concomitant loss of enzymatic activity (solid lines).

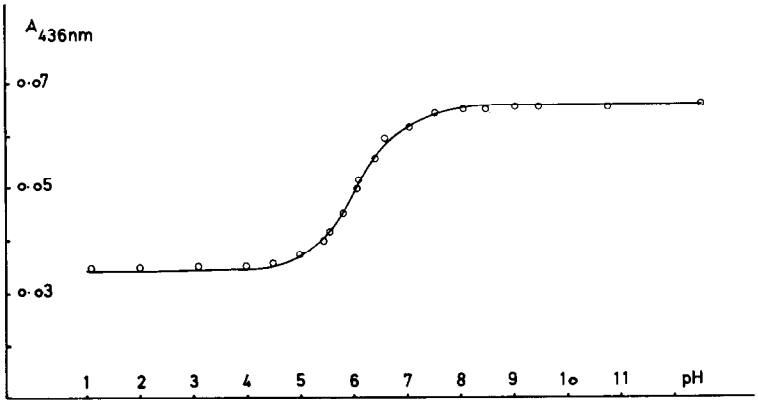

Fig. 4. Spectrophotometric titration of the nitrotyrosine ionization in nitrated aminopeptidase M. 4.2 moles nitrotyrosine per mole of enzyme.

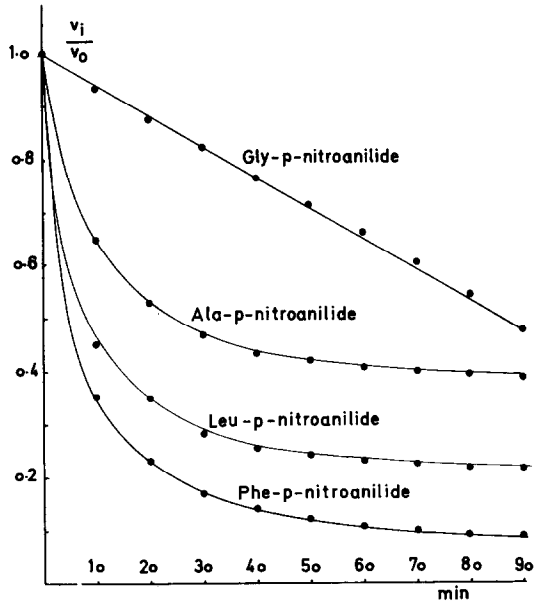

Fig. 5. Time course of decrease in enzymatic activity against different sized substrates after TNM treatment of aminopeptidase M.

tyrosines in nitroaminopeptidase M reveals a pK of 7.25 (fig. 4).

The unusual low apparent pK-value of the reactive tyrosines in native aminopeptidase M (pK = 7.6) further supports our idea about a hydrogen bond existing between histidine and tyrosine at the active center which enhances the nucleophilic character of the tyrosine residue.

Histidine is thought to act as an acid–base catalyst, which assists in the removal of a proton from the hydroxyl group of crucial tyrosine residues. Similar to the case of the serine proteases there is obviously no parallelism between the unusual nucleophility of the reactive tyrosines and their basicity.

The cooperative histidine–tyrosine system will ionize as a set and the pK value of 7.2, formerly obtained by plotting pK_m against pH [3] which was attributed to a reactive histidine residue is actually the value of the histidine–tyrosine system. Apparently the method of Dixon [7] will always give such combined values if there is any neighboring group cooperativity. After nitration a small pK shift (0.35 units) of the individual tyrosine causes a corresponding increase in the nucleophilic character but the enzymatic activity is actually diminished. This inhibition therefore must be due to the bulkiness of the ortho positioned nitro groups.

In order to decide whether this bulky substituent destroys the geometry of the active site or merely prevents binding of the substrate we compared the time dependent loss of activity upon nitration against several substrates (fig. 5). It is obvious that the degree of inhibition depends on the size of the substrate and we are in favour of the conclusion that nitration of aminopeptidase M causes inactivation by reducing the bulk tolerance of the active site.

References

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