THE EFFECT OF CATIONS AND COMPLEXING AGENTS ON THE HYDROLYSIS OF L-ALANINE-4-NITROANILIDE BY AMINOPEPTIDASE M

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1. Materials

The aminopeptidase M preparation was the same as described previously [1]. L-Alanine-4-nitroanilide-hydrochloride was purchased from Merck (Darmstadt, Germany). Cadmium acetate dihydrate, calcium acetate monohydrate, cobalt-II-acetate tetrahydrate, ferrous ammonium sulfate hexahydrate, ferric ammonium sulfate dodecahydrate, magnesium acetate tetrahydrate, manganese acetate tetrahydrate, nickel acetate tetrahydrate, ammonium molybdate tetrahydrate and mercurous acetate were obtained from the same source. Ethylenediamine tetraacetic acid, *p*-chloromercuribenzoate sodium salt (PCMB) and *N*-2-hydroxyethylpiperazine-*N*-ethane-sulfonic acid (Hepes) were commercial products of Serva (Heidelberg, Germany). The purity of all substances was reagent grade unless stated otherwise.

2. Methods

The involvement of metallic cations in the hydrolytic cleavage of L-alanine-4-nitroanilide catalyzed by aminopeptidase M was investigated in 0.2 M Hepes-buffer (pH 7.0, 25°).

Stock solutions (0.1 M metal salt in 0.2 M Hepes buffer pH 7.0) were diluted with the same buffer to obtain the required end concentration. The enzyme was added in appropriate volumes to give a final concentration of 4×10^{-8} M (molecular weight assumed to be 2.8×10^5).

In a typical run, 0.1 ml of an 1.2×10^{-6} M enzyme stock solution was mixed with 2.8 ml of metal salt solution and incubated for 30 min at 25°. After preincubation the reaction was started by addition of 0.1 ml substrate solution.

Evaluation of the kinetic parameters was performed as described previously [1].

3. Results and discussion

Particle-bound aminopeptidase (aminopeptidase M) was reported to be activated considerably by divalent cobalt [2] while other authors found none [3] or only slight activation [4]. We have reinvestigated the possible influence of metallic cations on hydrolysis of L-alanine-4-nitroanilide by aminopeptidase in connection with our studies concerning the mechanism of the enzymatic reaction.

Crude preparations of aminopeptidase M, as used in previous investigations (specific activity 16–17 IU), are indeed modestly activated by cobalt-II ions.

Preincubation of highly purified enzyme with cobalt-II-acetate (4×10^{-8} M aminopeptidase M in 0.2 M Hepes-buffer pH 7.0, 25°) for one hour causes no significant alteration of maximum rate of hydrolysis, at cobalt-II concentrations below 10^{-4} M. Higher concentrations cause no activation but, on the contrary, progressive inhibition (fig. 1) which can be reversed almost completely by 24 hr dialysis against large volumes of double distilled water (4°). The extent of the concentration-dependent inhibition also depends on length of preincubation (fig. 2). Similarly, other metal ions also exhibit a concentration-dependent inhibition (fig. 3).

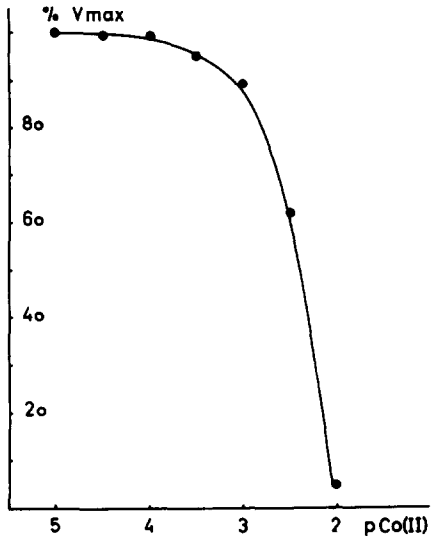

Fig. 1. Cobalt-II-dependent decrease of maximum rate. 0.2 M Hepes buffer, pH 7.0, 25°. Enzyme concentration 4×10^{-8} M (molecular weight assumed to be 2.8×10^5).

There is no obvious connection between the degree of inhibition and the characterization of the cations as hard or soft acids. Concentrations greater than 10^{-3} M EDTA also show an increasing inhibitory effect, as does *o*-phenanthroline (fig. 4). Both inhibitions can be reversed totally by dialysis against large volumes of double distilled water (24 hr, 4°). Extended dialysis of highly purified aminopeptidase M against 10^{-4} M EDTA (120 hr, 4°, batch changing every 12 hr) causes no loss of activity. After gel filtration of this preparation (Sephadex G 25, 120 × 10 mm, equilibration and elution with double distilled water) a small but significant alteration in the UV spectrum but no change in specific activity is observed.

The inhibition of an enzyme catalyzed reaction after the absence of a metal ion was for a long time viewed to be the classical proof of essential metal ion participation [5]; but this is a necessary but not sufficient condition, for the metal might stabilize active conformations of the enzyme without ever participating in the catalytic action. If the inactivating transconformations caused by removal of metal ions are totally reversible, it is rather difficult to distinguish between a stabilization effect and essential participation in the reaction pathway.

Since in our investigations, the inactivating cations form sufficiently stable complexes with phenols and imidazoles, it is feasible that, at cation concentrations greater than optimum, inhibition may be caused by cation binding to the active center.

No definite conclusions on inhibition by *o*-phenanthroline can yet be drawn; the presence of a metallic cation at the enzymes' active site, preventing hydrophobic binding or ion pair interaction with the active center, cannot be excluded. Phenanthrene shows a small but significant inhibition compared to *o*-phenanthroline (as does the sterically hindered 3,6-dimethylphenanthroline). The inhibiting action of the non-

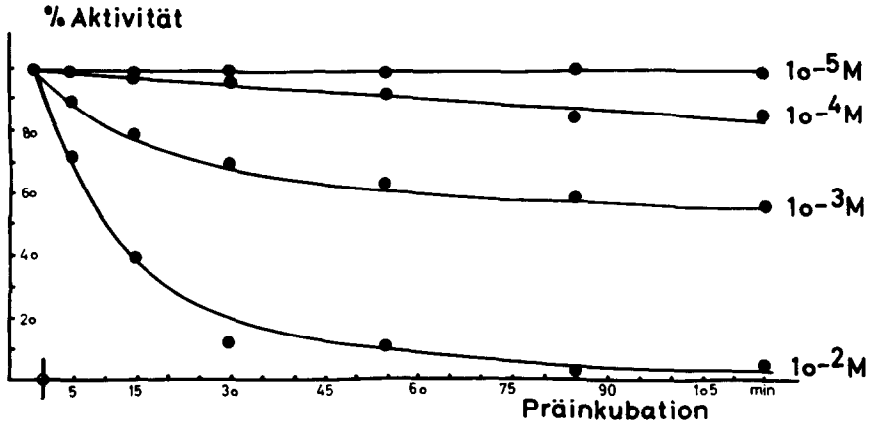

Fig. 2. Decrease in rate of hydrolysis effected by cobalt-II-ions under varied concentration and incubation conditions 0.2 M Hepes buffer, pH 7.0, 25°. Enzyme concentration 4×10^{-8} M.

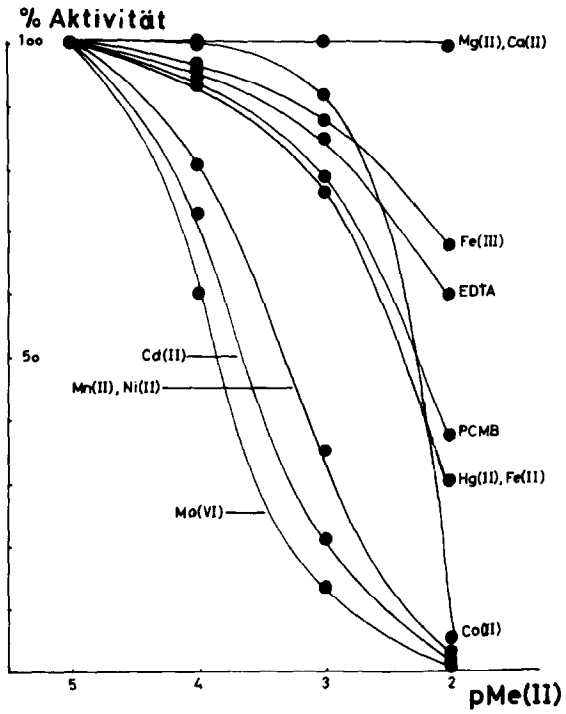

Fig. 3. Inhibition of aminopeptidatic solvolysis of L-alanine-4-nitroanilide by various cations. Enzyme concentrations 4×10^{-8} M, 120 min preincubation in 0.2 M Hepes buffer, pH 7.0, 25°.

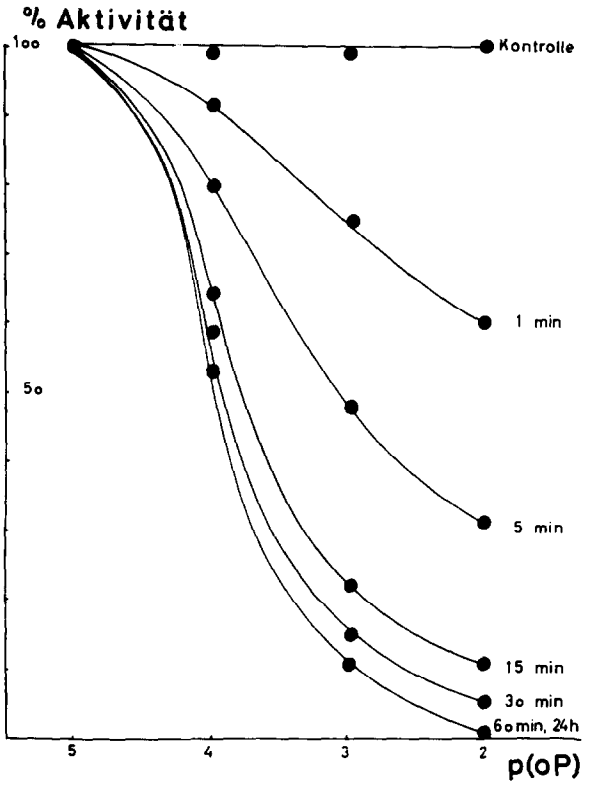

Fig. 4. Inhibition of aminopeptidase M by *o*-phenanthroline 0.2 M Hepes buffer, pH 7.0, 25°. Enzyme concentration 4×10^{-8} M.

chelating nitrogen containing compounds benz(a)acridine and benz(b)acridine, is of the same order of magnitude as that exhibited by phenanthroline. Because there is also no obvious connection between the inhibitory effect of the reagents and their hydrophobic character (expressed by the water–1-octanol partition coefficients), one might conclude with caution that the inhibitory action of phenanthroline and dipyridyl is due mostly to their basic character [6]. As the inhibition of aminopeptidase M by aliphatic carboxylic acids is a well known phenomenon [7], the inhibition by EDTA also cannot be explained unequivocally by the assumption that an essential metal ion has been captured. Further studies concerning the metal ion inhibition and the action of complexing and hydrophobic agents are in progress.

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