THE TYROSYL RESIDUES AT THE ACTIVE SITE OF AMINOPEPTIDASE M
Modifications by tetranitromethane

U. FEMFERT and G. PFLEIDERER
Abteilung für Chemie an der Ruhr-Universität Bochum, Bochum, Germany

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1. Materials and methods

Aminopeptidase M (commercial product of Röhm and Haas, Darmstadt) was purified by repeated gel filtration (Sephadex G 75 coarse, equilibrated with 0.1 M ammonium bicarbonate buffer pH 7) up to a specific activity of 20,000 I.U.

Tetranitromethane was purchased from Fluka (Buchs, Switzerland). The substrate L-alanine-4-nitranilide was made by the DCCI-procedure (mp. 97°C). Protein concentration was determined by the biuret method [4].

2. Nitration

Nitration of the enzyme was carried out at room temperature in 0.2 M phosphate buffer (pH 8.0). 1 ml protein solutions (3.2×10^{-5} M, the molecular weight of the enzyme assumed to be 280,000 [5]) were treated with aliquots of tetranitromethane in absolute ethanol, the end concentration of tetranitromethane varying from 10^{-5} M up to 10^{-2}M. One hour after incubation the mixture was passed through a column of fine grade Sephadex G 25 equilibrated with 0.1 M ammonium acetate buffer (pH 7.0).

Quantitative determination of the nitrotyrosine content was made by measuring the absorption at 428 nm in a Zeiss spectrophotometer PM Q II, to molar extinction coefficient assumed to be 4,100 [1].

The absorbance of the biuret complex is not affected by nitration of some tyrosyl residues and the protein content of the eluate can be obtained using this technique with sufficient accuracy to allow exact calculations.

3. Tryptic digests

The eluate from the gel filtration was lyophilized. 10 mg of the nitroenzyme were solved in 5 ml 50% trichloracetic acid and heated to boiling for 5 min. The precipitate was washed with absolute diethylether, dried, collected and suspended in 1 ml 0.1 M ammonium bicarbonate solution. After centrifugation the precipitate was suspended in 1 ml 0.1 M ammonium bicarbonate buffer (pH 8.0) and incubated with 1 mg trypsin for 6 hr at 37°C. After this period a repeated digestion under the same conditions was performed with another freshly added 1 mg trypsin.

4. Peptide maps

Peptide mapping was performed on the tryptic digests of nitroenzyme with the use of silica gel thin layer sheets. These were subjected to chromatography in 2-butanol-formic acid water (15:3:2, all volumes) followed by electrophoresis in pyridine-acetic acid buffer pH 6.5 (445 ml water, 50 ml pyridine, 5 ml acetic acid). Phenol red served as a marker in the chromatographic dimension [6], lysine released during tryptic digestion was used as standard in the electrophoretic rim [7]. Nitrotyrosine-containing peptides can be detected by their weak yellow color, which is considerably strengthened by exposure to ammonia vapors. The yellow spots were eluted with 50% aqueous pyridine and subjected to acid hydrolysis (6 N NCl, 105°C, 24 hr).

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5. Activity measurements

The cleavage of the substrate was measured by automatic registration of the increasing absorption at 405 nm in an Eppendorf photometer (Netheler and Hinz, Hamburg) at pH 7.5. The kinetic parameters resulted from a weighted statistical fit [8].

6. Results and discussion

Reaction of native enzyme with 60-fold molar excess of the reagent causes remarkable loss in maximum velocity which fades to about 35% of the uninhibited value. Surprisingly high molar excesses of reagent (up to 600-fold!) were not able to squeeze the maximum velocity below 25% of the native enzyme.

These findings confirm suggestions from kinetic investigations supposing the necessity of tyrosyl residues in catalytic action [9] and also in regard of quality of the earlier published results about iodination of reactive tyrosine in aminopeptidase M [10]. Concerning the quantitative aspects the results gained by the use of tetranitromethane differ markedly from those of iodination. During iodination 10 atoms of iodine are described to be incorporated rather unspecifically, while another 10 iodine atoms per mole are reported to inactivate the enzyme. Fig. 1 shows the inactivation of aminopeptidase M corresponding to the spectrophotometrically determined nitrotyrosine content. If the initial slope as shown is extrapolated towards total inactivation this would be due to almost 5 tyrosyl residues per mole enzyme. The specificity of the reagents seems to be quite inverse: with tetranitromethane selective modification of a few residues causes considerable loss in activity. Further nitration does not show additional effects. The possibility, that nitration might force a dissociation of native protein into enzymatically inactive subunits, could be ruled out by the fact, that the elution volumes in gel filtration of native enzyme and nitroenzyme are identical.

If preparations containing 3 to 5 nitrated tyrosine residues per mole enzyme were subjected to tryptic digestion and peptide mapping, there was only one single yellow spot detectable on exposure to ammonia vapors (fig. 2).

Acid hydrolysis of the eluted yellow spot yielded a product in which only 3-nitrotyrosine could be detected by reference chromatography.

The fact that only about five modified tyrosine residues per mole enzyme in exactly the same position

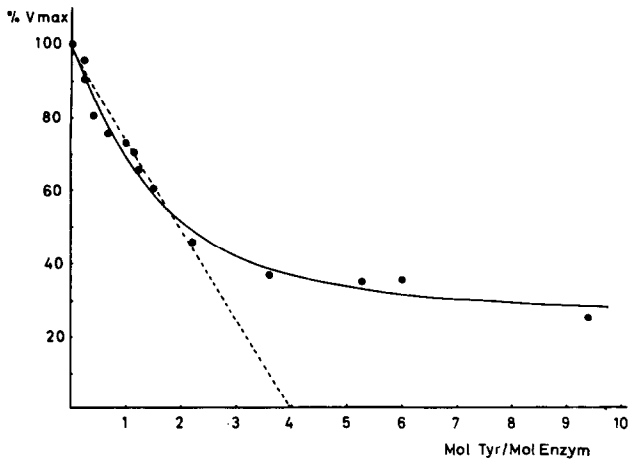

Fig. 1. Dependence of the decrease in maximum velocity of aminopeptidase M catalyzed hydrolysis of L-alanine-4-nitranilide on the nitrotyrosine content of the enzyme.

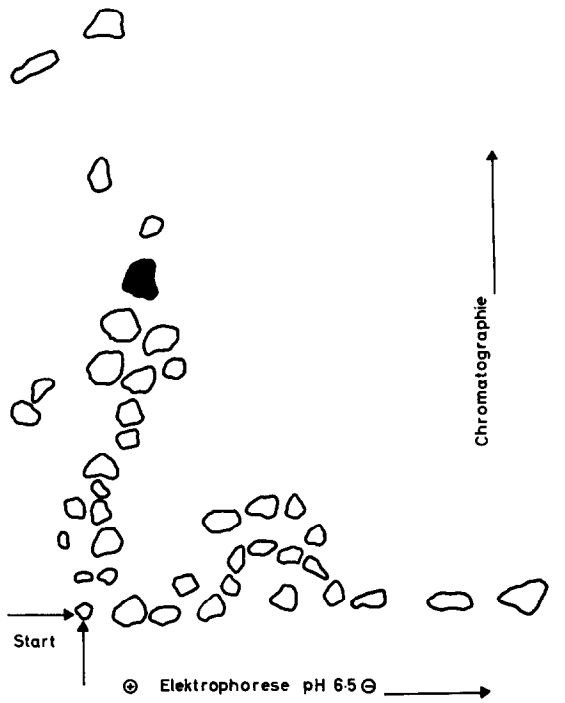

Fig. 2. Peptide map of nitrated aminopeptidase M containing 4 nitrotyrosine residues per mole enzyme.

correspond to inactivation, seems at the first glance to exclude the suggestion that the native enzyme might be composed of 10 subunits [10].

In connection with histidine modifying studies on aminopeptidase M using the diazonium-1-H-tetrazole reagent in which we find also a complete inactivation after reaction with about 5 histidine residues per mole enzyme [11] there are still remaining two possibilities if the reaction mechanism is assumed to consist of cooperative imidazole-tyrosine-catalysis [12]: (1) 10 nonidentical subunits with the active site on two different but corresponding chains, and (2) 5 identical subunits, the active site located on each single chain.

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