HISTIDINE RESIDUES AT THE ACTIVE SITE OF AMINOPEPTIDASE M
Modifications by Diazonium-1-H-tetrazole (DHT)

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1. Materials and methods

Aminopeptidase M, commercial product (Röhm and Haas, Darmstadt) was further purified by repeated gel filtration (Sephadex G 75 coarse equilibrated with 0.1 M ammonium bicarbonate buffer pH 7.0) up to a specific activity of 20,000 I.U.

The substrate for kinetic measurements L-alanine-4-nitranilide was synthetized by the DCCI-procedure (m.p. 97°C).

5-Amino-1-H-tetrazole was purchased from Schuchard (München). Protein concentration was determined by measuring the absorbance at 280 nm with the use of the value 16.2 for $E^{1\%}_{1cm}$ in the Zeiss spectrophotometer PM Q II. The same equipment was used for spectrophotometric titration of histidine-bisazo-1-H-tetrazole at 478 nm assuming a molar extinction coefficient of 21,000 [2].

The molecular weight of the enzyme used in the calculations was 280,000 [3].

2. Activity measurements

Kinetic measurements were performed by automatic registration of the increase of absorbance at 405 nm in an Eppendorf photometer (Netheler and Hinz, Hamburg) at pH 7.5 in 0.2 M phosphate buffer. The evaluation of the kinetic parameters resulted from a weighted statistical fit [4].

3. Diazotation

5-Diazonium-1H-tetrazole was prepared by diazotation of 0.1 g (0.95 mM) 5-amino-1-H-tetrazole-monohydrate dissolved in 2.3 ml 1.6 M hydrochloric acid with 0.07 g sodium nitrite solved in 1.5 ml water at 0°C. The nitrate solution was added slowly under vigorous stirring. Under continuous stirring in an ice bath the reagent is stable for at least 1 hr. The DHT content was determined by measuring the formation of N-carbobenzoxy-L-tyrosine-monoazo-1-H-tetrazole at 478 nm. Synthetic N-Cbo-L-tyrosine-monoazo-1-H-tetrazole (m.p. 193°C) was used as a standard, the molar extinction assumed to be 5,000 [2].

4. Coupling reaction

1 ml enzyme solution (3.10 M) in 0.2 M pyrophosphate buffer (pH 9.0) was mixed with increasing concentrations of DHT-reagent. After a reaction time of 5 min the pH was adjusted to 5.0 and the mixture was passed through a Sephadex column (G 25 equilibrated with 0.1 M ammonium acetate buffer pH 5.0).

With the eluate kinetic measurements and spectrophotometric titrations were performed after spectrophotometric determination of the protein content.

5. Tryptic digests

The eluate from gel filtration was freeze-dried, solved in 1 ml 8 M urea solution and heated to boiling for 5 min. The precipitate was collected, washed with

ethanol-acetone-water (1:1:1, all volumes), dried suspended in 0.5 ml 0.1 M ammonium bicarbonate buffer (pH 7.9) and incubated with 0.5 mg trypsin for 6 hr at 37°C. After this period the digestion was repeated for the same time with addition of another 0.5 mg trypsin.

6. Peptide maps

Peptide mapping was performed on the tryptic digest of the azoprotein. Thin layer chromatography in 2-butanol-formic acid-water (15:3:2, all volumes) was followed by electrophoresis in pyridine acetate at pH 6.5 (445 ml water, 50 ml pyridine, 5 ml glacial acetic acid). Azopeptides can easily be detected by their red-brown color.

7. Isolation of azopeptide

100 mg native aminopeptidase M in 4.5 ml 0.2 M pyrophosphate buffer (pH 9.0) were mixed with 0.5 ml of an ice cooled 5×10^{-3} M DHT-solution (pH 6.0). The pH was immediately readjusted to 9.0.

After 5 min the reaction was stopped by addition of resorcine. The azoprotein was precipitated by ice-cooled 60% perchloric acid. The collected precipitate was carefully washed several times with ethyl ether-acetone-absolute ethanol (1:1:1, all volumes).

The disulfide bridges of the dried azoprotein were reduced by mercaptoethanol [5] or sodium boranate [6] and carboxymethylated with iodoacetic acid [7].

After gel filtration (Sephadex G 25 equilibrated with 0.1 M phosphate buffer, pH 8.0) the freeze-dried eluate was again passed through a Sephadex G 25 column (equilibrated with 0.1 M ammonium bicarbonate), lyophilized, solved in 0.1 M ammonium bicarbonate buffer (pH 8.0) and subjected to a combined digestion with a mixture of 10 mg trypsin: chymotrypsin (1:1) for 6 hr at 37°C. The hydrolytic enzymes were precipitated by heating and the supernatant was passed through a Sephadex G 15 column (equilibrated with 0.1 M ammoniumbicarbonate). In the azopeptide-containing colored fraction the pH was adjusted to 4.0 and the azopeptide became adsorbed at a talcum column [8]. After washing with water (500 ml) elution was performed with 0.1% aqueous ammonia containing 1% pyridine. For further purification the eluate was adsorbed on an alumina column (Aluminium oxid, aktiv sauer, Merck), washed with 50 ml methanol and eluted with methanol-1N ammonia (2:1). After lyophilization the azopeptide shows one single band in electrophoresis and one spot in chromatography.

8. Digestion with aminopeptidase M

0.5 M purified azopeptide was incubated with the same amount of native aminopeptidase M in 0.1 M pyrophosphate buffer (pH 7.5). After 60 hr the mixture was dried *in vacuo* extracted with pyridine-methanol (1:1) and the colored product separated by preparative TLC on silica (development with pyridine-methanol 6:4).

9. Identification of the colored spot

The colored product proved identical in TLC on Silufol plates (Serva, Heidelberg) to standard histidine-bisazo-1-H-tetrazole in the solvent systems *n*-butanol-acetic acid-water (4:1:1) and pyridine-ethylacetate-acetic acid-water (5:5:1:3).

10. Results and discussion

Reaction of native aminopeptidase M with DHT causes profound loss in enzymatic activity. The inhibition strongly correlates to increasing absorbance at 478 nm due to the formation of histidine-bisazo-1-H-tetrazole [1]. Later it was found, that the absorption maximum of tyrosine-monoazo-1-H-tetrazole is identical with that of histidine-bisazo-1-H-tetrazole, its molar extinction coefficient being just one fourth of that of the histidine compound [2]. As fig. 1 indicates, total inhibition corresponds to bisazocoupling of five histidine residues, but this could just be due to monoazocoupling of maximally one tyrosine residue and in the peptide map gained from tryptic digests there is in fact only one colored spot (fig. 2). We had to find out if this was due to the existence of at least 5 identical subunits or if there was only one super-reactive tyrosine in the whole enzyme molecule. We coupled a greater amount of native enzyme with

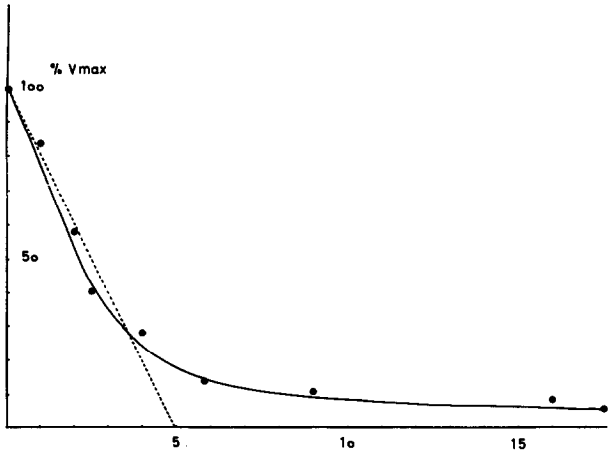

Fig. 1. Loss in maximum velocity corresponding to formation of histidine-bisazo-1-H-tetrazole.

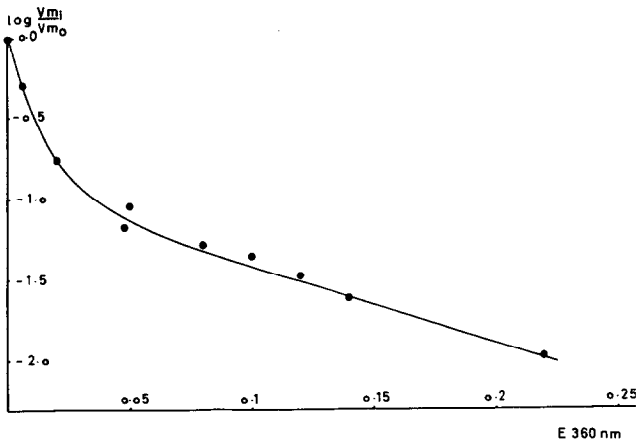

Fig. 3. Inhibition of aminopeptidase M by formation of histidine-monoazo-1-H-tetrazole.

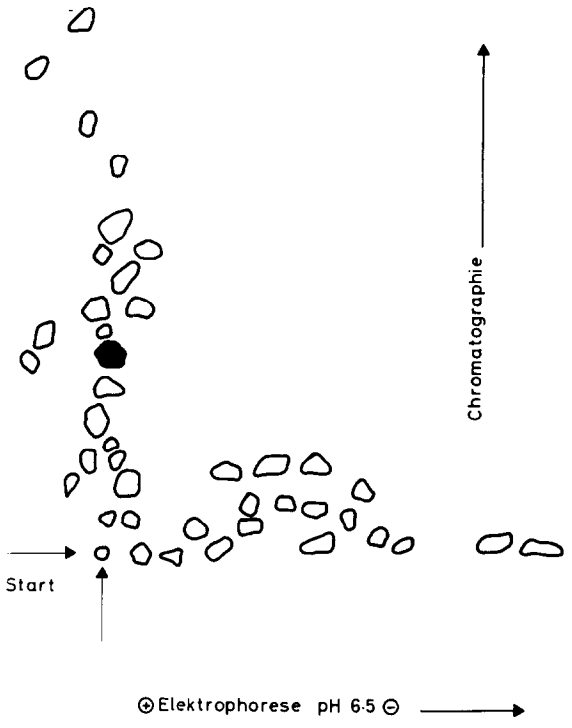

Fig. 2. Peptide map from tryptic digest of azocoupled aminopeptidase M containing four modified histidine residues per mole.

amounts of DHT-reagent which in previous experiments proved to be sufficient to modify just 3–4 "histidine" residues per mole enzyme and isolated one single azopeptide after the azopeptide was subjected to a combined tryptic/chymotryptic digestion.

After the isolated azopeptide was further digested by aminopeptidase M, we were able to identify histidine-bisazo-1-H-tetrazole by tlc and to exclude by the same means the formation of tyrosine-monoazo-1-H-tetrazole which moves much further. The tyrosine-bisazo-compounds could easily be excluded by spectrophotometry.

We find a surprising close similarity between the behavior of aminopeptidase M when reacted with DHT or with tetranitromethane [9].

Nitration of about five tyrosine residues would cause a total inhibition by extrapolating the initial alteration slope just as does bis-azocoupling of five histidine residues. Mono-azocoupling of histidine is already sufficient to inactivate the enzyme.

Very small molar excess of reagent (5–10-fold) and limited reaction times (1–2 min) cause partial inactivation without any increase in absorbance at 478 nm. Corresponding to the loss in activity however there was an increase in absorbance at 360 nm (fig. 3).

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