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Hydrolysis of Some Cystine Aminopeptidase and Aminopeptidase Substrates by Human Placental Enzymes after Isoelectric Focusing

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Summary: Several cystine aminopeptidase- and aminopeptidaselike activities of mature human placenta were demonstrated by chromatography on CM-Sephadex and subsequent preparative flat bed isoelectric focusing. Enzyme tests were carried out by using a micro modification of the *Bratton-Marshall* reaction with seven amino acid-*p*-nitroanilides and one amino acid β -naphthylamide as substrates.

By this method three main activities were detected; each was nonhomogeneous and capable of hydrolyzing cystine aminopeptidase- as well as aminopeptidase substrates. The substrates benzyl-cystine-*p*-nitroanilide, cystine di-*p*-nitroanilide and cystine di- β -naphthylamide were split by different enzymes. By studying the effects of several effectors or of heating, no enzyme with the properties described in the literature for serum cystine aminopeptidase could be unequivocally demonstrated.

The results reported here suggest that serum cystine aminopeptidase — in contrast to placenta cystine aminopeptidase — is either altered severely in its structure or that it represents a multiplicity of enzymes, which attack "specific" substrates and are therefore jointly manifested as cystine aminopeptidase.

Hydrolyse einiger Substrate für Cystinaminopeptidase und Aminopeptidase durch Enzyme aus der Placenta vom Menschen nach isoelektrischer Fokussierung

Zusammenfassung: Durch Chromatographie an CM-Sephadex und nachfolgende präparative isoelektrische Fokussierung lassen sich in der reifen menschlichen Placenta unterschiedliche Cystinaminopeptidase- und Aminopeptidase-Aktivitäten nachweisen. Die Enzymtests wurden mit einer Mikro-Modifikation der *Bratton-Marshall*-Reaktion und sieben Aminosäure-*p*-nitroaniliden sowie einem Aminosäure- β -naphthylamid als Substrate durchgeführt.

Durch diese Methodik wurden drei Hauptaktivitäten nachgewiesen, die selbst wiederum inhomogen sind und sowohl Cystinaminopeptidase- als auch Aminopeptidase-Substrate hydrolysieren können. Die Substrate Benzyl-cystin-*p*-nitroanilid, Cystin-di-*p*-nitroanilid und Cystin-di- β -naphthylamid wurden von unterschiedlichen Enzymen gespalten. Auch nach Zugabe verschiedener Effektoren oder Hitzeexposition konnte keine Enzymaktivität entdeckt werden, für die die in der Literatur beschriebenen Eigenschaften der Serum-Cystinaminopeptidase ohne Einschränkungen zutreffen.

Die hier berichteten Ergebnisse legen die Annahme nahe, daß die Serum-Cystinaminopeptidase gegenüber der Placenta-Cystinaminopeptidase entweder stark in ihrer Struktur verändert ist oder daß mit den meisten „spezifischen“ Substraten auch im Serum eine Vielzahl von Enzymen gemessen wird, die insgesamt als Cystinaminopeptidase imponiert.

Introduction

The placenta is said to be the source of serum enzymes which inactivate the labour-inducing posterior pituitary hormone oxytocin by splitting an amino terminal cysteinyl tyrosine bond (1). Following the introduction of amino acid β -naphthylamides and amino acid *p*-nitroanilides as ostensibly specific substrates, the properties of this serum oxytocinase or cystine aminopeptidase (EC 3.4.11.3) were extensively analyzed and are now well known (for review see l.c. (2)). It was also shown that the assay of serum cystine aminopeptidase activity with these substrates reveals the presence of other aminopeptidases of so far unknown origin and properties, in addition to true oxytocinase (3). Enzyme activities which split substrates specific for oxytocinase are very low in normal serum (4). When such unspecific activities are measured during pregnancy, they might therefore also originate from the placenta. Hydrolysis of cystine aminopeptidase substrates by enzymes of human placenta has — in contrast to the hydrolysis of this substrate by serum activities — not yet been systematically investigated. In the course of our ongoing studies on proteolytic enzymes of this organ (5, 6), we therefore partly enriched such activities from mature human placenta, and measured their action upon several amino acid *p*-nitroanilides and cystine di- β -naphthylamide, with and without addition of effectors and with exposure to heat.

We also hope to improve our understanding of serum cystine aminopeptidase.

Experimental

Materials

The substrates used are listed in table 1.

Alanine *p*-nitroanilide, cystine di-*p*-nitroanilide, glycine *p*-nitroanilide and phenylalanine *p*-nitroanilide were from Merck, Darmstadt; benzyl-cysteine *p*-nitroanilide and leucine *p*-nitroanilide were purchased from Boehringer, Mannheim; cystine di- β -naphthylamide was a preparation from Serva, Heidelberg.

Tab. 1. Relative hydrolysis rates of several cystine aminopeptidase- and aminopeptidase substrates after focusing (hydrolysis of alanine *p*-nitroanilide in peak I taken as 1.00. For further details see text.

Substrate	Peak I	Peak II	Peak III
Alanine <i>p</i> -nitroanilide	1.00	0.46	0.28
Leucine <i>p</i> -nitroanilide	0.98	0.54	0.43
Lysine <i>p</i> -nitroanilide	0.41	0.35	0.17
Benzyl-cysteine <i>p</i> -nitroanilide	0.23	0.26	0.05
Phenylalanine <i>p</i> -nitroanilide	0.20	0.16	0.07
Glycine <i>p</i> -nitroanilide	0.10	0.04	0.02
Cystine di- <i>p</i> -nitroanilide	0.08	0.06	0.03
Cystine di- β -naphthylamide	0.01	0.07	0.00

Tab. 2. Relative hydrolysis rates after addition of effectors (leucine *p*-nitroanilide as general substrate). Remaining activities are expressed as fractions of the activities of controls without addition of effectors.

Effector	Peak I	Peak II	Peak III
Control	1.00	1.00	1.00
Ca ⁺⁺	1.05	1.10	1.05
Co ⁺⁺	1.10	1.15	1.00
Mg ⁺⁺	1.00	1.10	1.05
Pb ⁺⁺	0.05	0.10	0.05
Zn ⁺⁺	1.00	1.30	1.05
ϵ -aminocaproic acid	1.05	1.20	1.25
Cysteine	1.05	1.10	1.10
Dithioerythritol	1.10	1.25	1.05
Ethylene diaminetetraacetate	0.95	0.90	0.90
<i>p</i> -hydroxymercuribenzoate	0.95	0.75	0.30
Iodacetamide	1.10	1.15	1.00
<i>L</i> -methionine	0.70	0.90	1.00
<i>D</i> -penicillamine	0.90	1.00	1.00
Puromycin	0.15	0.45	0.70
Aprotinin	1.10	1.25	1.10

The effectors used are listed in table 2.

ϵ -aminocaproic acid, 4-hydroxymercuribenzoate, ethylene diamine tetraacetate, iodacetamide and *D*-penicillamine were from Serva, Heidelberg; puromycin was from Boehringer, Mannheim, aprotinin from Bayer, Leverkusen. All the other effectors were from Merck, Darmstadt.

Full term *placentae* without macroscopically visible pathological changes served as enzyme sources. They were freed from remaining blood, washed with tap water and frozen at -20°C until they were worked up.

Methods

The enzymes were enriched prior to isoelectric focusing by a procedure comprising elution in ammonium acetate solution 0.1 mol/l, ammonium sulfate precipitation up to 0.80 saturation, dialysis against ammonium acetate buffer 0.1 mol/l pH 6.0, chromatography on a CM Sephadex C 50 column, previously equilibrated and eluted with the same buffer, and freeze drying of the protein which was not bound on to the exchanger. The procedure is described in more detail in l.c. (6).

Subsequently, 500 mg of the dry material were mixed with 6 ml of Servalyt pH 4–6 (Serva 42904) and 94 ml of Ultradex 4 g/100 ml (LKB, Munich), swollen in double distilled water. A plate of 24.5×10.8 cm was cast and focused for 7 h at 20 W power under cooling with tap water in a double chamber (Desaga, Heidelberg).

After focusing the gel was cut into 30 fractions and transferred into plastic columns (LKB, Munich). Elution was carried out by addition of 2 ml double distilled water to each fraction; pH was measured in these eluates. Then a second elution of the fractions was performed by adding of 3 ml Tris-HCl buffer 0.2 mol/l pH 7.5. Protein concentrations in the eluates were determined by measurement of absorbance at 280 nm after ten-fold dilution.

The enzyme assay was based on our micro modification of the Bratton-Marshall reaction as described elsewhere (7).

Enzyme samples were incubated in Tris-HCl buffer 0.20 mol/l (total incubation volume 800 μl) pH 7.5; tests were started by adding of 10 μl substrate 0.1 mol/l in dimethyl sulfoxide.

In order to check the action of some effectors, 200 μl samples from the enzymatically most active fractions of the focusing experiments were pre-incubated for 5 min at 37°C with 100 μl each of the effector stock solutions listed in table 2. The concentrations of the stock solutions were uniformly 10 mmol/l; aprotinin was used undiluted. For these effector experiments enzyme tests were started by adding 10 μl leucine *p*-nitroanilide solution 0.1 mol/l in dimethylsulfoxide as a general substrate.

After stopping the test incubation, the remaining activities were measured and expressed as fractions of the controls without addition of effectors.

Heating experiments: Samples from the enzymatically most active peak fractions were pre-incubated for 30 min at 56 °C prior to the enzyme assays and followed by the tests with leucine *p*-nitroanilide as substrate as described above. Remaining activities were expressed again as fractions of the controls without heating.

Results

1. Focusing

The results of the preparative isoelectric focusing experiments are presented in figure 1–3. All data are arithmetic means taken from double determinations.

In the main the focusing experiments yielded three peaks with the isoelectric points 3.8–4.3 (peak I), 4.8–5.1 (peak II) and 5.4–5.6 (peak III). The highest hydrolysis rates were measured with alanine *p*-nitroanilide and leucine *p*-nitroanilide as substrates, followed by lysine *p*-nitroanilide, benzyl-cysteine *p*-nitroanilide, phenylalanine *p*-nitroanilide, glycine *p*-nitroanilide, cystine di-*p*-nitroanilide and cystine di- β -naphthylamide.

It is clearly evident from the figures that the activities in the actual peaks do not represent homogeneous enzymes; in a future paper we will deal with the further differentiation of these activities by gel chromatography. If the measured hydrolysis rates are expressed for an uniform enzyme quantity of 200 μ l the resulting relative activities can be compared as shown in table 1.

Fig. 1. Flat bed isoelectric focusing of human placental enzymes (500 mg protein: ampholytes pH 4–6; 20 W; 7 h) after chromatography on CM Sephadex C 50.

Abcissa: fraction numbers; left ordinate: absorption at 280 nm (—) after ten-fold dilution; right ordinate: pH values (---). For further details see text.

Fig. 2. Hydrolysis of cystine aminopeptidase substrates by placental enzymes after isoelectric focusing. Enzyme (200 μ l) tests were carried out uniformly at 30 min/37 °C. Abcissa: fraction numbers; ordinate: absorptions at 550 nm.

● Benzyl-cysteine *p*-nitroanilide
▲ Cystine di-*p*-nitroanilide
○ Cystine di- β -naphthylamide

Fig. 3. Hydrolysis of aminopeptidase- and arylamidase substrates by placental enzymes (50 μ l) after isoelectric focusing. Test conditions as mentioned for fig. 2.

○ Glycine *p*-nitroanilide
● Alanine *p*-nitroanilide
■ Lysine *p*-nitroanilide
○ Leucine *p*-nitroanilide
▲ Phenylalanine *p*-nitroanilide

2. Substrate specificity

The distribution of hydrolysis rates shows that most of the substrates used here are of relative poor specificity; there are only small differences between classic cystine aminopeptidase substrates on the one hand and simple aminopeptidase substrates on the other. Peak I contains considerable activities towards typical arylamidase- and aminopeptidase substrates alanine *p*-nitroanilide, leucine *p*-nitroanilide, phenylalanine *p*-nitroanilide as well as cystine aminopeptidase substrates (benzyl-cysteine *p*-nitroanilide and cystine di-*p*-nitroanilide), but there is practically no activity towards cystine di- β -naphthylamide. Peak II contains the strongest benzyl-cysteine *p*-nitroanilide and cystine di- β -naphthylamide hydrolyzing activities, but it is clear that the cystine di- β -naphthylamide cleaving enzyme is by no means identical with the former one. By using a more advanced focusing technique they indeed are quite well separated (paper in preparation). In peak III, mainly leucine *p*-nitroanilide and alanine *p*-nitroanilide are hydrolyzed, but there are still activities towards benzyl-cysteine *p*-nitroanilide and cystine di-*p*-nitroanilide, whereas the hydrolysis of lysine *p*-nitroanilide shows an irregular pattern, which is distributed over a wide pH range.

To summarize, one can say that the substrates benzyl-cysteine *p*-nitroanilide, cystine di-*p*-nitroanilide and cystine di- β -naphthylamide are split to a great extent by different enzymes.

3. Effectors

The results of the effector experiments are given in table 2.

Of the tested divalent cations, only Pb^{++} inhibits all activities, whereas Zn^{++} shows a clear activating capacity upon the enzymes in peak II. Cysteine or ethylenediamine tetraacetic acid are more or less inefficient: *p*-hydroxymercuribenzoate inhibits the enzymes of the peaks II and III, which leads to the conclusion that SH-enzymes are incorporated there. These, however, can only be activated to a small extent by addition of cysteine or dithioerythritol. Methionine inhibits the enzymes of peak I weakly, whereas puromycin represents the most efficient inhibitor after Pb^{++} , especially for the activities of the peaks I and II.

4. Heating experiments and addition of methionine

After pre-incubation of the test mixture at 56 °C (see "Materials and Methods") remaining activities of 0.83 (peak I), 0.37 (peak II) and 0.16 (peak III) were measured, whereas values of 0.70 (I) and 1.00 (III) were found (leucine *p*-nitroanilide as substrate) when heating was performed in the presence of *L*-methionine.

Discussion

Serum cystine aminopeptidase (EC 3.4.11.3) is not inhibited by *L*-methionine (8, 9), but strongly inhibited by ethylenediamine tetraacetic acid (10), *p*-chloromercuribenzoate (11) and Pb^{++} (12); it is activated by Co^{++} and Zn^{++} (13), is sensitive to heat (8, 9) and hydrolyzes cystine di-*p*-nitroanilide (14), benzyl-cysteine *p*-nitroanilide (15,16) and cystine di- β -naphthylamide (1) ostensibly in a specific way, in addition to leucine *p*-nitroanilide and glycine *p*-nitroanilide (14). According to Yman (17) its isoelectric point lies at pH 3.7, but can be raised to pH 4.5 by desialiation. In polyacrylamide electrophoresis one can detect — subject to the chosen test conditions — one or two bands which are named cystine aminopeptidase 1 and cystine aminopeptidase 2 (18, 19).

In contrast to our expectations that one or two cystine aminopeptidase would also be found in placenta, focusing led to three main peaks, which were nonhomogeneous. This means that there is a multiplicity of enzymes which can hydrolyze classic cystine aminopeptidase substrates as well as aminopeptidase substrates, moreover, there is no distinct relationship between the hydrolysis rates of various "specific" cystine aminopeptidase substrates. An enzyme contained in peak II, however, resembles the slowly migrating sialic acid-free cystine aminopeptidase 2 of serum, since it shows — in contrast to other activities — a certain specificity for the first synthetic cystine aminopeptidase substrate cystine di- β -naphthylamide (1).

It is also possible to differentiate between arylamidases and cystine aminopeptidase of placental origin, according to Oya *et al.* (8), by studying the divergent reactions of these enzymes after addition of *L*-methionine and heating; but even on addition of *L*-methionine, ethylenediamine tetraacetic acid, hydroxymercuribenzoate and Pb^{++} or exposure to heat, an enzyme corresponding directly to serum cystine aminopeptidase could not be unequivocally detected.

Hence, in the present work on placental hydrolytic enzymes, the substrates were not hydrolyzed by oxytocinase-cystine aminopeptidase. This especially applies to benzyl-cysteine *p*-nitroanilide, which was claimed to be specific for serum cystine aminopeptidase (16); moreover, it cannot be hydrolyzed "ex definitione" by aminopeptidases, because of the substitution at the amino terminal of cysteine.

The results reported here lead to the conclusion that serum cystine aminopeptidase is either altered severely in its structure, or that it represents a multiplicity of enzymes, which attack "specific" substrates and are therefore jointly manifested as cystine aminopeptidase. At any rate one can suppose that serum enzymes obtained from placenta would show activity towards the mentioned substrates as well as glycine *p*-nitroanilide and lysine *p*-nitroanilide, which are hardly split by arylamidases of normal serum (4). Until the problem as to which enzymes split oxytocin and which split cystine

aminopeptidase substrates is solved, it might be useful — for reasons of specificity and comparability with older investigations — to go back to the primary substrate cystine di- β -naphthylamide for assaying serum cystine aminopeptidase. Investigations on this problem are under way.

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Senior Award
der National Academy of Clinical Biochemistry
für *Johannes Büttner*

Anlässlich des Fourth Annual Meeting der National Academy of Clinical Biochemistry in Boston, Mass., hielt Herr Professor Dr. Dr. *Johannes Büttner*, Geschäftsführender Direktor des Instituts für Klinische Chemie der Medizinischen Hochschule Hannover und einer der Herausgeber dieser Zeitschrift, am 19. Juli 1980 den Festvortrag „From Chemistry of Life to Chemistry of Disease: The Rise of Clinical Biochemistry“ und wurde in Anerkennung seiner wissenschaftlichen Originalität, Initiative und seiner Beiträge zum Fortschritt der Klinischen Biochemie mit dem Senior Award ausgezeichnet sowie zum Ehrenmitglied ernannt.