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Enzymic properties of intestinal aminopeptidase P: a new continuous assay

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A continuous photometric assay of aminopeptidase P activity was developed which is based on a coupled enzymic assay with the substrate Gly-Pro-Pro-pNA and DPP IV as auxiliary enzyme. This assay was used to evaluate the kinetic parameters and inhibitory profile of intestinal brush border aminopeptidase P.

Aminopeptidase P; Coupled enzymic assay; Azaproline substrate; Enzyme assay

1. INTRODUCTION

Because of their unique specificity, imido bond hydrolysing proteases of the intestinal microvillar membrane hold a key position in degradation of food proteins. Aminopeptidase P, recently demonstrated to be an integral membrane enzyme of the intestinal brush border [1], is highly specific in cleaving N-terminal imido bonds in peptides of the type Xaa-Pro-..., standard substrates being Gly-Pro-Pro or Gly-Pro-Hyp. This enzyme was first isolated and characterised as a soluble cytosolic enzyme from *E. coli* by Yaron and Mlynar [2]. Later, APP activity has been found in a number of organs [3,4] and in human serum [4]. Mammalian membrane-associated aminopeptidase P has been enriched from pig kidney microsomes [5] and microvillous membranes [6], from rat intestinal brush border membranes [1] and from bovine lung [3]. The physiological function of the enzyme is not known, though a number of suggestions have been put forward [1,3,4]. Studies of enzymic properties of APP have been hampered for a long time by the time-consuming discontinuous assay for glycine cleaved off from the standard substrates [5].

In 1982, Yaron's group [7] introduced an elegant fluorometric assay based on dequenching of intramolecularly quenched fluorophore-containing substrates upon splitting, the sensitivity of which was recently [4] very much improved. The fluorometric analysis required, however, reduction of the absorbance by dilution with a stopping solution.

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Abbreviations: APP, aminopeptidase P (EC 3.4.11.9); DPP IV, dipeptidyl peptidase IV (EC 3.4.14.5); -pNA, *p*-nitroanilide; DFP, diisopropyl fluorophosphate; E-64, L-*trans*-epoxysuccinylleucylamido(4-guanidino)butane; THF, tetrahydrofurane; DCC, dicyclohexyl carbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, dimethyl formamide; BBMV, brush border membrane vesicle; bestatin, (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine; DTE, dithioerythritol

2. MATERIALS AND METHODS

Buffer A: 50 mM Tris-HCl, pH 7.4, 3 mM $MnCl_2$, 0.15 M NaCl; buffer B: 50 mM Tricine, pH 7.4, 50 μM $MnCl_2$, 200 μM Na-citrate, 0.15 M NaCl. Phosphoramidon, E-64 and bestatin were gifts from Dr H. Kirschke (Institute of Biochemistry, Martin-Luther-University). DFP was purchased from Serva (Heidelberg, FRG). DPP IV, prepared from pig kidney according to [8], had a specific activity of 0.46 μkat/mg

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(27.8 U/mg) against Gly-Pro-pNA. APP was either used in situ, i.e. as chromatographed brush border vesicles [1] or enriched by butanol extraction. Papain-treated brush border vesicles [1] were mixed with *n*-butanol (25%, v/v) and stirred at room temperature for 1 h. The two phases were dialysed separately at 4°C against buffer A containing 0.1% Triton X-100. Enzyme activity remained in the aqueous phase. Photometric measurements were performed with an Eppendorf photometer M 1100 or Zeiss UV/VIS Spektralphotometer M40.

2.1. *Synthesis of substrates*

2.1.1. Gly-Pro-Pro

Gly-Pro-Pro was synthesised by conventional methods described in [1].

2.1.2. Ala-AzaPro-pNA

Ala-AzaPro-pNA was synthesised as described in [9].

2.1.3. Z-Pro-pNA

Z-Pro-pNA was prepared from Z-ProOH and 4-nitroaniline by the mixed anhydride method with isobutyl chloroformate in THF in the presence of *N*-ethylmorpholine and recrystallised several times from ethyl acetate. The yield-limiting step in further synthesis was the introduction of the Pro-Pro bond. After some trials, the DCC/HOBt method [10] was found to give the best results.

2.1.4. Boc-Pro-Pro-pNA

To a solution of Boc-ProOH (1.5 nmol) in 10 ml DMF cooled to −20°C were added 1.5 mmol HBr·Pro-pNA, 1.5 mmol triethylamine and 1.65 mmol HOBt under stirring. Then, 1.5 mmol DCC in 10 ml precooled DMF were added and the reaction mixture stirred for 1 h at −20°C. The agitation was continued overnight at room temperature. The mixture was left at 4°C for 24 h and separated from precipitated dicyclohexyl urea by filtration. The solvent was evaporated and the solid residue taken up in ethyl acetate. The organic phase was extracted with 5% $KHSO_4$, saturated $NaHCO_3$, saturated NaCl and dried over $MgSO_4$. As the solution was still contaminated with urea it was put into the refrigerator several times and the urea filtered off (with loss of yield). The product precipitated as a white syrup from ethyl acetate/petrol ether which was triturated with hexane. The yield of the amorphous product was 69.3%. Anal.: f.p. 79–83°C; $[\alpha]_D^{20} = -120.2°$ ($c = 1$ in THF); TLC, $R_f = 0.52$ (chloroform/methanol, 9:1).

2.1.5. Boc-Gly-Pro-Pro-pNA

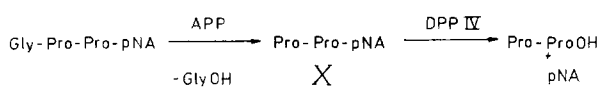
A solution of 4 mmol Boc-GlyOH in 15 ml dry THF and 4 mmol *N*-ethylmorpholine was cooled to −20°C and isobutyl chloroformate (4 mmol) was added to it. The reaction mixture was stirred at −20°C and 4 mmol HCl·Pro-Pro-pNA and 4 mmol *N*-ethylmorpholine added. After the usual purification steps the product was taken up in a small volume of ethyl acetate and precipitated with diisopropyl ether. Yield: 79.2%; Anal.: f.p. 100–106°C, $[\alpha]_D^{20} = -130.9°$ ($c = 1$ in THF); TLC, $R_f = 0.5$ (ethyl acetate/pyridine/HAc/H_2O, 90:15:4.5:2.3). Blocked amino groups were deprotected by either HBr/HAc (Z-groups) or HCl/HAc (Boc-groups).

2.1.6. HCl·Gly-Pro-Pro-pNA

HPLC of the final product (Merck Hitachi 655/A, column 250/4, LiChrospher RP 8, eluent 0.01 M KH_2PO_4, pH 3.3/acetonitrile (70:30), pumping speed 1 ml/min) revealed a single peak at 9.57 min comprising 99.5% of the integrated area when monitored at 220 nm.

2.2. *Kinetic assays*

A continuous coupled enzyme assay was developed for routine APP activity measurements. It takes advantage of the reaction sequence given in scheme 1. The necessary amount of



Scheme 1. Reaction sequence of the coupled enzymic aminopeptidase P assay.

auxiliary enzyme was calculated from the relationship (3.25) in [11]. Accordingly, the standard assay mixture was composed of 100 μl enzyme sample (preincubated for 1 h in buffer A or B), 100 μl buffer A or B and 5 μl DPP IV with a specific activity of 47.3 nkat/ml against Pro-Pro-pNA, corresponding to 0.79 μkat/l in the assay mixture. The reaction was started with 100 μl substrate solution and the nitroaniline absorbance recorded at 405 nm. Under these conditions, the absorbance change measures the velocity of glycine release, the first step of the reaction sequence being rate limiting (i.e., adding more DPP IV in portions of 5 μl did not change the slope of the progress curve). Gly-Pro-Pro hydrolysis was determined essentially as in [5]. Cleavage of Ala-AzaPro-pNA by APP was ascertained qualitatively by TLC in *n*-propanol/conc. NH_3 (7:3). Its binding constant was extracted from substrate competition experiments with Gly-Pro-Pro. All kinetic parameters were evaluated by non-linear regression using a modified Sinclair Basic version of the program KINFIT [12].

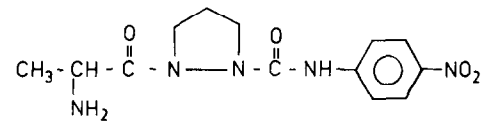
3. RESULTS AND DISCUSSION

Kinetic parameters of DPP IV for the hydrolysis of Pro-Pro-pNA under APP assay conditions were found to be: $K_m = 13.82 \pm 1.22$ μM and $k_{cat} = 47.24 \pm 1.33$ s^{-1}. The kinetics of Gly-Pro-Pro-pNA hydrolysis was found to obey Michaelis behavior. K_m values are given in table 1. When the auxiliary enzyme was omitted and residual endogeneous DPP IV inhibited by DFP, no change in absorbance was recorded indicating that the Pro-pNA bond is not attacked by any other enzyme in purified BBMs.

Preincubation with the Mn^{2+}-containing buffers A and B for 1 h at 25°C increased initial velocities 2-fold without change of K_m. When increasing amounts of mercaptoethanol were added to the Mn^{2+}-activated enzyme a corresponding drop in

V_m was seen without any change in K_m. This corroborates Mn^{2+} being a pure V_m effector. As shown in table 2, the aminopeptidase inhibitor bestatin and metal chelating agents were inhibitory to intestinal APP. Inhibition by bestatin was competitive. Phosphoramidon, DFP and E-64 do not influence the activity at the concentrations given. Taken together, these findings indicate that the enzyme like several other aminopeptidases is a metalloenzyme.

More insight into the electronic and structural prerequisites of a peptide or peptide derivative in order to become susceptible to attack by APP was gained from studies with Ala-AzaPro-pNA (scheme 2). TLC analysis of the incubation mixture at different times clearly revealed that only alanine is split off from the substrate analog. Thus, the activity of traces of contaminating DPP IV which would cleave off Ala-AzaPro [9] is negligible. The Aza-peptide as an alternative substrate for APP competitively inhibits the Gly-Pro-Pro hydrolysis. From substrate competition studies a K_m value of 0.37 mM was derived for the Aza analog. This value is comparable to that for Gly-Pro-Hyp (K_m = 0.34 mM [3]) and one order of magnitude larger than for the tripeptide nitroanilide (cf. table 1).



Scheme 2. Structure of the Aza-substrate analog Ala-AzaPro-pNA.

Table 1

Michaelis constants of aminopeptidase P with the substrate Gly-Pro-Pro-pNA at pH 7.4 and 25°C under various conditions

Condition	K_m (± s) (μM)	Substrate concentration range (μM)
Buffer A without Mn^{2+}	33.0 ± 3.9	12.5– 200
Buffer A	37.3 ± 3.7	50 –2220
Buffer A (BuOH)	31.1 ± 3.2	12.5– 200
Buffer B without Mn^{2+}	80.2 ± 1.0	50 –2220
Buffer B	69.9 ± 1.8	12.5– 200

In all cases the enzyme source was in situ APP (i.e. proteolytically shaved and chromatographed BBMVs) with one exception (3rd row) which was the butanol extract of BBMVs

Table 2

Inhibitory profile of intestinal aminopeptidase P

Inhibitor	Concentration of inhibitor (mM)	Inhibition (%)
Phosphoramidon	0.16	0
E-64	0.16	0
DFP[a]	14.50	0
Bestatin	0.20	55 (K_i=0.13 mM)
EDTA	1.00	80
1,10-Phenanthroline	6.70	100
2-Mercaptoethanol	1.60	91
DTE	1.00	84

[a] Substrate: Gly-Pro-Pro

All inhibition studies were carried out without Mn^{2+} activation. Samples were preincubated for 1 h at 25°C with the respective inhibitor. Substrate: Gly-Pro-Pro-pNA (200 μM)

The carbonyl carbon of AzaPro in the Aza-peptide is less electrophilic than that of proline due to resonance delocalisation of electrons on the neighbouring nitrogen. Furthermore, the Aza-proline ring is more planar than the proline ring. Thus, we may conclude that the active site of APP has no strict requirements for ring conformation and electronic structure around the carbonyl and α-carbons of the proline moiety.

The behavior toward inhibitors of the in situ intestinal aminopeptidase P resembles that of crude extracts [4] and enriched preparations [3] from lung, though we never found inhibition by DFP as reported in [3]. Of the microbial peptidase inhibitors only bestatin seems to have an effect but had a rather high K_i (0.13 mM). Surprisingly, the K_m values for Gly-Pro-Pro-pNA and Yaron's fluorogenic substrate Lys(ϵ-Dnp)-Pro-Pro-NH-CH_2-CH_2-NH-CO-C_6H_4-NH_2 are nearly identical, 33 μM and 38 μM, respectively.

The continuous assay for aminopeptidase P described in this paper should greatly facilitate further purification and characterisation of the enzyme and help to clarify its physiological significance.

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