Biochimica et Biophysica Acta, 742 (1983) 452–462
Elsevier Biomedical Press

BBA 31462

THE CONFORMATION AROUND THE PEPTIDE BOND BETWEEN THE P_1- AND P_2-POSITIONS IS IMPORTANT FOR CATALYTIC ACTIVITY OF SOME PROLINE-SPECIFIC PROTEASES

GUNTER FISCHER, JOCHEN HEINS and ALFRED BARTH

Martin-Luther-Universitat Halle, WB Biochemie (Abteilungen fur Enzymologie und Wirkstoffbiochemie), Domplatz 1, 402 Halle (G D R)

(Received June 29th, 1982)
(Revised manuscript received October 25th, 1982)

Key words *Conformational specificity, Proline peptidase, Substrate conformation, Dipeptidyl peptidase IV*

Proline-containing dipeptidyl-4-nitroanilides have been synthesised and subjected to dipeptidyl peptidase IV-catalysed hydrolysis at high enzyme concentrations to collect information on the conformational specificity of the enzyme active site for a nonscissile bond. Descriptions of the biphasic kinetics were carried out in terms of *cis/trans* interconversion of the substrates. The results show that the enzyme can cleave only the *trans*-conformation of the substrate. The competitive inhibition by Gly-Pro-OH and Ala-Pro-OH is also specific for the *trans* form of the dipeptides. The interpretation of the results obtained from these kinetic studies has led to proposals for the stepwise cleavage of biologically active peptides like substance P and β-casomorphine by dipeptidyl peptidase IV.

Introduction

Proline-containing peptides are widely distributed among biological active sequences The proteolytic cleavage of peptide bonds in this type of compound is catalysed by various types of proline-specific endo- or exopeptidases [1]. The specificity of these enzymes can be described, in part, by the position of the proline residue with respect to the susceptible peptide bond.

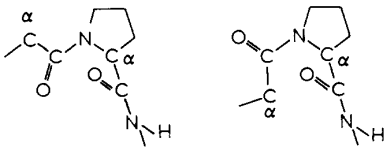
If the proline is located in a peptide chain, as in.

P_3-P_2-Pro-P'_1-P'_2-

the peptide bond C-terminal to proline is hydrolysed by proline-specific endopeptidases (EC 3.4.21 26) like postproline-cleaving enzyme [2] and the proline-specific endopeptidase from *Flavobacterium meningosepticum* [3]. Some of these enzymes have further structural requirements. For example dipeptidyl peptidase IV (dipeptidyl peptidase IV, EC 3 4 14.-) [4,5] requires a protonated α-amino group in the P_2- position for successful cleavage.

H_3^+ N-P_2 Pro-P'_1-P'_2

When an imidic group is present in the P'_1- position, neither proline-specific endopeptidase nor dipeptidyl peptidase IV is active. It is, however, a fact that amino acids like proline, hydroxyproline or sarcosine within a peptide chain might introduce structural heterogeneity because of the two possible conformations about the P_x-Pro- bond Although the -CO-NH- peptide unit generally shows a large preference for the *trans* conformation, imino acids are known to introduce consider-



Scheme I

0167-4838/83/0000–0000/$03 00

able amounts of *cis*-conformation in solution [6–8]. In contrast to the situation with the -P_x-Pro unit, the *cis* population of the Pro-P_x- unit is surely lower than 1% [9].

In fact, investigations of the conformational specificity of aminopeptidase P (EC 3 4.11.4) [10,11] and prolidase (EC 3.4.13.9) [12] have demonstrated a requirement for the *trans* conformation about the scissile bond. For this type of proteolytic enzyme, the secondary amide bond itself is the center of catalytic action:

$$-P_1^{\downarrow}\text{-}Pro\text{-}P_2'$$

On the other hand, the cytosolic leucine aminopeptidase (EC 3 4 11 1) shows no preference for a specific conformation about a nonscissile bond. When Leu-Phe-Pro-OH is used as a substrate, it is possible to find hydrolysis of the Leu–Phe bond in the conformation with a *trans* Phe-Pro unit as well as with the *cis* Phe-Pro peptide bond [11].

In accord with these results, it is clear that the interpretation of kinetic constants of proteolytic enzymes working on proline-containing peptides will require in every case a knowledge of conformational specificity of the enzyme and, if necessary, the *cis / trans* isomer ratio for the substrate. In fact, because the free energy, $\Delta G°$, for the *cis / trans* isomerisation of oligopeptides containing imino acids is intrinsically rather small [13,14], minor changes of external conditions for enzyme catalysis (state of protonation, solvent composition) might produce large differences in the amount of enzymatically hydrolysable isomer in solution

The present paper reports the conformational specificity of dipeptidyl peptidase IV and, to a smaller extent, that of proline-specific endopeptidase.

For this purpose, the reaction of Gly-Pro-4-nitroanilide (Gly-Pro-NHNp) and Ala-Pro-4-nitroanilide (Ala-Pro-NHNp) was investigated at very high enzyme concentrations. Conformational specificity in catalysis could be also important in relation to product inhibition by the product P_2-Pro-OH. The proline-specific-endopeptidase-substrate Ala-Pro-Pro-4-nitroanilide (Ala-Pro-Pro-NHNp) has four possible isomers [6], any or all of which might serve as a substrate for proline-specific endopeptidase. This could be detected by the observation of different kinetic phases at high proline-specific endopeptidase concentrations.

Materials and Methods

Dipeptidyl peptidase IV was purified from pig kidney as described in a previous paper [4] Proline-specific endopeptidase was gift from T. Yoshimoto, Faculty of Pharmaceutical Sciences, Nagasaki, Japan. The lyophylised enzyme was dissolved in buffer and centrifuged before the kinetic runs. Ala-Pro-4-nitroanilide and Gly-Pro-4-nitroanilide were synthesised by using the mixed-anhydride technique and were characterised as described previously [18]. The chemical purity of the substrates was checked additionally by following the alkaline hydrolysis to the endpoint. The optical purity was assayed using dipeptidyl peptidase IV in long incubation (4–8 h) experiments. Dipeptidyl peptidase IV can hydrolyse only the L-isomer in both positions of the peptide chain. The concentrations given in the results were calculated from these determinations The purity of the substrates was approx. 93% Lys-ε-(4-NO_2Z)-Pro-OH and Gly-Pro-OH HCl were gifts from K. Neubert

Enzyme and protein assay

Dipeptidyl peptidase IV was assayed by active-site titration using Pro-Pro-4-nitroanilide as titrant. The molecular weight of dipeptidyl peptidase IV was assumed to be 115000 per catalytic subunit [2,4,20] The fraction of active enzyme was determined by measuring the 'burst' region in the pre-steady state phase of the enzyme kinetics at various substrate concentrations. A typical purity of the enzyme used in this work was 60–70% of total protein concentration.

Proline-specific endopeptidase activity was assayed by a spectrophotometric method using Ala-Pro-Pro-NHNp as substrate. Protein was measured by the method of Lowry et al. [22] using bovine serum albumin as standard.

Estimation of pK_a values

pK_a values of substrates were determined by potentiometric titration with Radiometer titration equipment (PHM 26, TTT 1, ABU 13, G 2222 C, K 4112) at 30.0°C and 0.1 μ KCl In order to

avoid incorrect results from the hydrolysis of the nitroanilides in more basic solutions, only the first part of the titration curve was used to calculate pK_a values

^{13}C NMR spectra

The ^{13}C NMR spectra were recorded by pulsed Fourier transform methods on a Bruker WP-200 spectrometer Carbon-13 chemical shifts are ppm downfield from an external reference of TMS.

The p^2H of the peptide samples was adjusted with ^2HCl and was measured by a combination electrode (Radiometer) at 25°C. The p^2H was calculated from $p^2H = pH_{reading} + 0.4$ [19]

Kinetic experiments

Fast kinetic experiments were performed on an Dionex model 110 stopped-flow spectrophotometer with a dead time of 3.5 ms One syringe contained enzyme in 0.1 M phosphate buffer and the other contained the substrate in the same buffer solution. Reaction progress was monitored at 390 and 450 nm with a 20 mm cell The spectrophotometer was used only in the transmittance mode. The time-dependent transmittance was digitised and stored by a transient recorder (Biomation, model 2805). The stored 2048 data points were transmitted on-line to a 9825 desk-top computer (Hewlett-Packard) and converted to the time-dependent absorbances Different sets of data points were taken for calculation of the first order kinetic parameters and for time-course analysis Slow kinetic measurements were done on a Perkin-Elmer 356 spectrophotometer and Specord 40 M (VEB Carl Zeiss, Jena).

Results

a) The substrate hydrolysis

The enzymatic rate of hydrolysis of Gly-Pro-NHNp was determined at a dipeptidyl peptidase IV concentration of $2.1 \cdot 10^{-7}$–$4.7 \cdot 10^{-7}$ mol · l^{-1} Since crystal structure data for the N-terminal-protonated substrate show exclusively the *trans* conformation [23], the dissolved compound was preequilibrated in the final incubation buffer (pH 7.2; 0.1 mol l^{-1} phosphate) for at least 30 min

After the enzymatic reaction was initiated by adding dipeptidyl peptidase IV, two kinetically different processes were monitored by means of the increase of absorbance at 390 nm or 450 nm, produced by the reaction product 4-nitroaniline (Fig 1a and b)

The kinetic pattern of the proline-specific endopeptidase (0 184–0 329 mg/ml) catalysed hydrolysis of Ala-Pro-Pro-NHNp looks very similar to that of the dipeptidyl peptidase IV experiments (Fig. 1c and d) In all three cases the rate of the slower kinetic phase, as measured by k_{slow}, is nearly independent of the enzyme concentration (Table I).

In contrast to this, the rate of the fast kinetic process increases with increasing enzyme concentration. For Gly-Pro-NHNp, the initial concentration of the substrate S_o is much smaller than the K_m value, so that the relationship $v = (k_{cat}/K_m)\,[E_o]\,[S_o]$ is approximately valid. This led to a nearly first order decrease of the substrate concentration for the fast, enzyme-catalysed process This allows a rough comparision of k_{slow} and the apparent rate constant for the enzymatically catalysed reaction The slow kinetic phase itself follows first-order kinetics for more than six $t_{1/2}$ values

In order better to estimate the amplitude ΔS_{slow}, related to the substrate concentration involved in the slow phase, and the rate constant k_{slow}, the time-dependent absorbance signal was digitised as 100 data points at equal intervals. By non-linear regression analysis [24], the first order rate con-

TABLE I

INFLUENCE OF ENZYME CONCENTRATION ON RATE CONSTANTS k_{slow} AND AMPLITUDE RATIOS $\Delta S_{fast}/\Delta S_{slow}$

Substrate	E_o (mol l^{-1})	k_{slow} (s^{-1}) ($\times 10^{-2}$)	$\Delta S_{fast}/\Delta S_{slow}$
Gly-Pro-NHNp [b]	1 55 10^{-6}	6 26	9 0
	2 37 10^{-6}	6 45	9 06
Ala-Pro-NHNp [c]	1 55 10^{-6}	7 41	15 9
	2 37 10^{-6}	6 64	16 9
Ala-Pro-Pro-NHNp	0 329 [a]	1 66	10 6
	0 185 [a]	2 03	9 3

[a] g l^{-1}, substrate concentration 2 58 10^{-5} mol l^{-1}
[b] 6 45 10^{-5} mol l^{-1} substrate concentration
[c] 4 6 10^{-5} mol l^{-1} substrate concentration

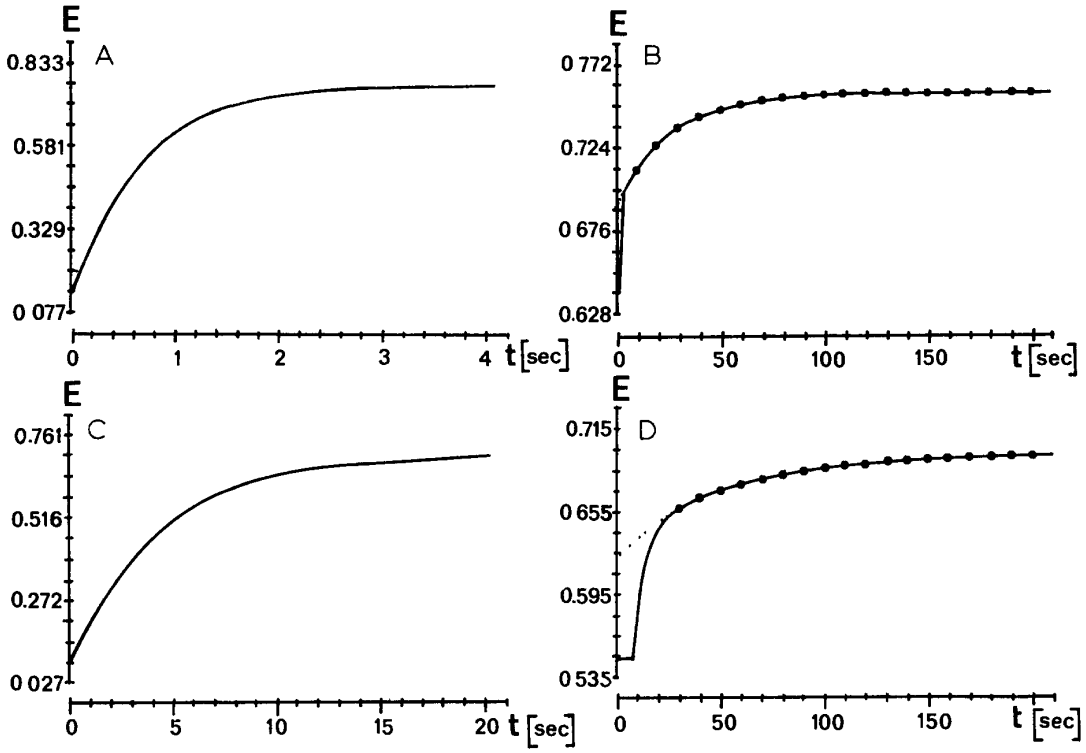


Fig 1 A Rate of dipeptidyl peptidase IV-catalysed hydrolysis of Gly-Pro-NHNp at 25 0°C and pH 7 2 in phosphate buffer (0 1 mol l^{-1}) The concentration of Gly-Pro-NHNp was 3 09 10^{-5} mol l^{-1}, the concentration of enzyme was 1 08 10^{-6} mol l^{-1} B Same reaction monitored on a slower time base The first order rate constant was calculated from the data points collected after termination of the rapid change in absorbance The calculation gives k_{slow} = 3 82 10^{-2} s^{-1} and ΔS_{slow} = 0 065 The theoretical curve is drawn as a line, with some of the experimental values shown as points C Rate of proline-specific endopeptidase-catalysed hydrolysis of Ala-Pro-NHNp at 30°C and pH 7 5 in phosphate buffer The concentration of the substrate was 2 58 10^{-5} mol l^{-1}, the concentration of the enzyme was 0 185 g l^{-1} D Conditions as in b, k_{slow} = 2 03 10^{-2} s^{-1} and ΔS_{slow} = 0 073

stant k_{slow} and the amplitude ΔS_{slow} were obtained. The theoretical curve and some of the experimental data points used in the regression analysis are shown in Fig 1b and c to permit a check of the quality of curve fitting.

TABLE II
INFLUENCE OF SUBSTRATE CONCENTRATION ON THE KINETIC CONSTANTS OF DIPEPTIDYL PEPTIDASE IV CATALYSIS

Ala-Pro-NHNp (mol l^{-1}) [a]	k_{slow} (s^{-1})($\times 10^{-2}$)	% *cis*	$\Delta S_{fast}/\Delta S_{slow}$
2 83 10^{-4}	5 98	5 88	16 0
6 90 10^{-5}	4 36	7 15	13 0
1 38 10^{-5}	6 64	5 58	16 9
2 75 10^{-6}	2 55	8 46	10 8

[a] Enzyme concentration 2 37 10^{-6} mol l^{-1}

Both fast and slow kinetic phases are different in their rates by a factor of 31, when the experimental conditions of Fig 1a and b are used. The amplitude ΔS_{fast} of the fast reaction was obtained from the knowledge of the slow phase value ΔS_{slow} and the total change of absorbance after long incubation experiments ($\Delta S_{slow} + \Delta S_{fast}$). For some cases, especially at low temperatures, the rate constants are different enough ($k_{fast}/k_{slow} \gg 50$) to permit direct measurements of absorbance of the fast process as a plateau region.

The ratio of amplitudes $\Delta S_{fast}/\Delta S_{slow}$ is independent of the enzyme concentration and of the substrate concentration as well (Table II)

For the experimental conditions of Fig 1a and b, for Gly-Pro-4-NHNp the ratio 9 1 is measured. The sum of both amplitudes $\Delta S_{fast} + \Delta S_{slow}$ is directly proportional to the 4-nitroaniline concentration, which can be calculated from the substrate concentration, after correction for optical and chemical purity of the dipeptidyl-4-nitroanilides The rate constant k_{slow} is also independent of the substrate concentration (Table II)

It has been demonstrated that the activity of dipeptidyl peptidase IV can be competitively inhibited by Lys-ε-(4-NO_2Z)-Pro-OH in a powerful manner If the slow phase of the kinetics pattern reflected any property of dipeptidyl peptidase IV, this process would be influenced by the inhibitor. In the presence of 3.24 10^{-5} mol · l^{-1} Lys-ε-(4-NO_2Z)-Pro-OH and 4.24 · 10^{-5} mol · l^{-1} Gly-Pro-NHNp k_{slow} is unaffected, while the fast kinetic process is slowed down to one third of this original velocity The apparent small influence of the inhibitor on ΔS_{slow} (Table IV) seems to be an artefact It appears from the difficulty of evaluating this quantity, that the rates of the two kinetic phases approach each other. The limits of error in the nonlinear regression analysis also become greater, which is consistent with this explanation In summary, these results suggest that the slow phase is an intrinsic property of the substrate, while the rapid phase results from enzyme catalysis

Biphasic kinetics were found not only at 30°C, but throughout the temperature range from 9 to 30°C. For Gly-Pro-NHNp, the dependency of the fractional concentration of the slow phase and the rate constants of the slow phase from the temperature are summarised in Table III.

The activation enthalpy $\Delta H\ddagger$ for the slow phase

TABLE III

TEMPERATURE DEPENDENCE OF *CIS-TRANS* INTERCONVERSION OF Gly-Pro-NHNp

t (°C)	k_{slow} (s^{-1})	% *cis*
9 2	8 15 10^{-3}	7 84
15 0	1 43 10^{-2}	8 39
19 9	2 37 10^{-2}	8 52
25 0	3 82 10^{-2}	9 09
30 4	6 45 10^{-2}	9 94

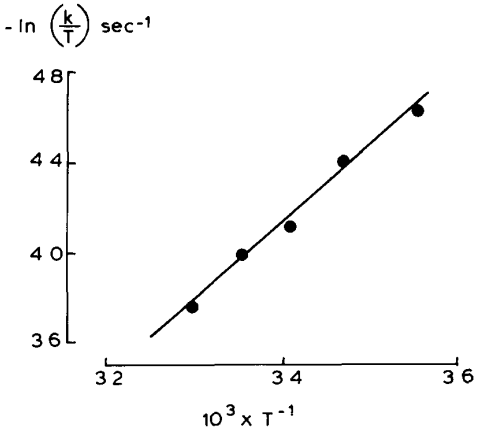


Fig 2 Eyring-plot for k_{slow} of Gly-Pro-NHNp For experimental conditions, see Fig 1a The linear regression analysis gives $y = -3\,50\ 10^3\ x + 7\,87$ ($r^2 = 0\,99997$, $n = 5$)

of Gly-Pro-NHNp obtained from the Eyring plot given in Fig. 2, is +66.9 kJ mol^{-1}. For the reaction enthalpy $\Delta H°$ of the same process (K_{eq} = [fast]/[slow]) a value of −8.2 kJ · mol^{-1} (r^2 = 0.952; $n = 5$ for linear regression) was estimated from a van't-Hoff plot. The population and the rate constant of the slow-fast interconversion do not depend markedly on the pH in the range 5.5–7.5 (Table IV).

TABLE IV

pH DEPENDENCE OF RATE CONSTANTS AND FRACTIONAL CONCENTRATION OF *CIS* ISOMER, AT 30°C IN PHOSPHATE BUFFER

k_{slow} values are in s^{-1}

pH [b]	Gly-Pro-NHNp		Ala-Pro-NHNp	
	k_{slow} ($\times 10^{-2}$)	% *cis*	k_{slow} ($\times 10^{-2}$)	% *cis*
5 5			3 88	6 4
6 8	5 82	10 8	7 64	6 5
7 2	6 45	9 9	7 41	5 9
7 5	5 81	10 5	7 34	6 6
7 5 [a]	6 64	15 3		

[a] In presence of 3 24 10^{-5} mol l^{-1} Lys-(ε-4NO_2-Z)-Pro-OH HCl as a competitive inhibitor (K_i = 2 8 10^{-6} mol l^{-1}) The initial rate of the hydrolysis in 4 25 10^{-5} mol l^{-1} Gly-Pro-NHNp is reduced to 38%

[b] Enzyme concentration 3 10 10^{-6} mol l^{-1}

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.