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DIPEPTIDYLPEPTIDASE IV – INACTIVATION WITH *N*-PEPTIDYL-*O*-AROYL HYDROXYLAMINES

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Eleven *N*-peptidyl-*O*-aroyl hydroxylamines have been synthesized and their hydrolytic stability, acidity and properties during reaction with dipeptidyl peptidase IV (E.C. 3.4.14.5) investigated. *N*-peptidyl-*O*-(4-nitrobenzoyl) hydroxylamines act as irreversible inhibitors of serine proteases[1]. The serine enzyme, dipeptidyl peptidase IV (DP IV), is inactivated by substrate analog derivatives of this class by a suicide inactivation mechanism. During the enzymic reaction of DP IV with the suicide substrates most molecules are hydrolyzed but some irreversibly inactivate the target enzyme. In contrast to porcine pancreatic elastase and thermitase, DP IV exhibits a high ratio for hydrolysis of the compounds versus inhibition during their interaction with the enzyme. Variation of the leaving aroyl residue lowers this ratio. Variation of the substrate analog peptide moieties of the DP IV-inhibitors increases their ability to inhibit the enzyme to a remarkable extent. Possible reaction pathways are discussed.

KEY WORDS: Dipeptidyl peptidase IV, diacyl hydroxylamines, nitrenes, mechanism-based inactivation, Lossen reaction.

INTRODUCTION

Dipeptidyl peptidase IV, a serine peptidase with pronounced specificity for proline residues, is believed to be involved in modulation of proline containing peptide hormones and activation of proteins by limited proteolysis[2–4].

The enzyme is a membrane-bound aminopeptidase anchored by a hydrophobic peptide chain into the bilayer and is widely distributed in organs of mammals. Dipeptidyl peptidase IV has two identical but catalytically independent subunits and removes dipeptide units of the structure Xaa-Pro from the N-terminal end of polypeptides and proteins (Xaa-Pro-Yaa). Substrates containing amino-acids other than proline in the P_1-position are also accepted by the enzyme but the turnover rate is reduced. The unprotected and protonated aminofunction of the P_2-amino acid is essential for catalysis (for a review see Walter[5]).

The enzyme activity in plasma is useful for diagnostic purposes since it deviates significantly from normal values in several pathological circumstances, e.g. blood diseases and different cancers[6–8]. However, the enzyme's physiological role and the cause of the deviation of DP IV-activity in pathological processes are not yet fully understood.

Enzyme inhibitors can be useful tools to help clarify the biological functions of

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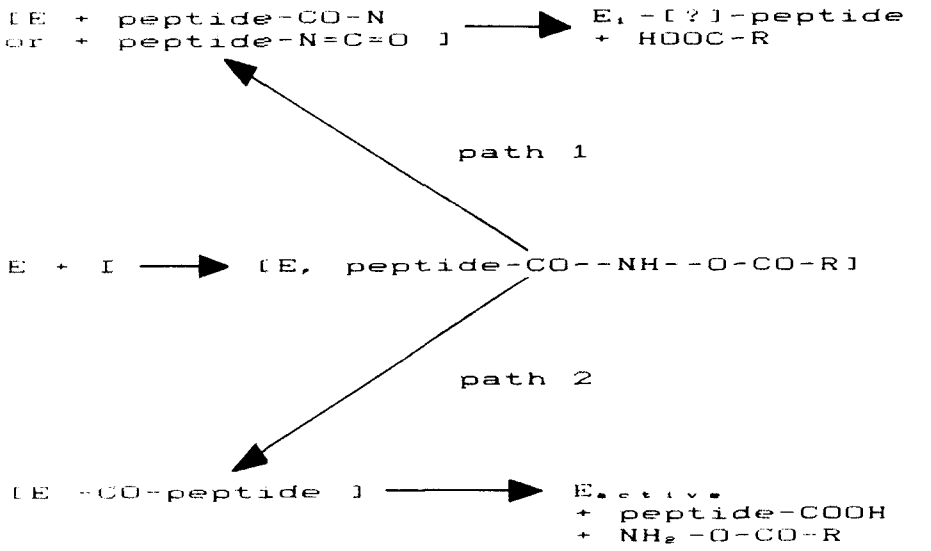
enzymes and accordingly we have studied *N*-peptidyl-*O*-aroyl hydroxylamines as mechanism-based inactivators of serine proteases[1]. *N*-alanyl-prolyl-*O*-(4-nitrobenzoyl) hydroxylamine has been used successfully as a specific inhibitor in the investigation of DP IV in human lymphocytes[9].

Two reaction pathways between serine peptidases and the substrate analog diacyl hydroxylamines were proposed (see Scheme 1).



E represents the free enzyme, R may be alkyl or phenyl, E_1-[?] means the nature of the inactive complex is unknown

SCHEME 1 Pathways of reaction of peptidases with *N*-peptidyl-*O*-aroyl hydroxylamines.

Pathway 1: During the formation of noncovalent or covalent complexes between suicide-substrate and target enzyme, N–O bond fission occurs leading to reactive intermediates (carbonyl nitrenes or isocyanates) which irreversibly modify the protein. Final products are the inactivated enzyme and the *O*-acyl residue.

Pathway 2: The target enzyme hydrolyzes the compound as a substrate. The release of *O*-acyl hydroxylamine during the catalytic process prevents N–O bond fission leading to a simple acyl enzyme. Final products are active enzyme, peptide and the *O*-acyl hydroxylamine. The partition ratio (r) between both processes (substrate hydrolysis/enzyme inactivation) is a characteristic measure of mechanism-based inactivation[10].

In this paper work is reported on the mechanism of reaction of *N*-peptidyl-*O*-aroyl hydroxylamines with porcine kidney DP IV.

MATERIALS AND METHODS

Synthesis of Diacyl Hydroxylamines

All *N*-peptidyl-*O*-benzoyl hydroxylamines used in this work were synthesized as

described previously[1] by hydroxylaminolysis of the corresponding Boc-dipeptidyl-methylesters[11]. The resulting peptide hydroxamic acids (Boc-dipeptide-NHOH) were acylated to give diacyl hydroxylamines by treatment with the appropriate benzoyl chlorides in Schotten-Baumann-reactions.

The Boc-dipeptidyl-methyl esters were synthesized according to standard methods[12] starting with C-terminal amino acid methyl esters.

Amino acids were purchased from Reanal, Budapest. Di-tert. butylcarbonate was from Serva, Heidelberg and tert. butylchloroformate, 3-chlorobenzoyl chloride, 4-nitrobenzoyl chloride and 3,5 dinitrobenzoyl chloride were obtained from Merck, Darmstadt. All other chemicals were research grade from Laborchemie, Apolda. Organic solvents were dried before use by standard procedures.

Melting points are uncorrected. TLC in product anlysis was performed on silicagel plates (Silufol, Kavalier, Czechoslovakia). Intermediate and final products were further characterized by ^{1}H-NMR, elemental analysis and uv-spectrometry.

Peptide hydroxamic acids were obtained amorphous in all cases with yields between 90 and 95%. Their acylation to *N*-Boc-dipeptidyl-*O*-benzoyl hydroxylamines using substituted benzoyl chlorides gave yields of 60–80% after crystallization from ethyl acetate/petrol ether. Finally, the Boc-groups were removed by HCl/CH_3COOH to give the hydrochlorides of *N*-dipeptidyl-*O*-benzoyl hydroxylamines listed in Table I.

All N-terminal deprotected compounds were highly hygroscopic, thus results of elemental analysis were influenced by moisture and these are not shown for compounds 1–5. Their purity was checked by TLC, by comparison of the free dipeptides (Xaa-Pro) with the products of DP IV-catalyzed total hydrolysis of compounds 1–11 to the appropriate dipeptides and *O*-benzoyl hydroxylamines[14].

Comparison of the absorption spectra of compounds 1–11 after complete degradation to dipeptidyl hydroxamic acids and substituted benzoic acids with the spectra of solutions of the corresponding recrystallized commercial benzoic acids gave purity higher than 95.2% in all cases.

TABLE I

Hydrochlorides of *N*-peptidyl-*O*-benzoyl hydroxylamines; analytical parameters

Compound*	M_2	Formula	Mp. [°C]		C%	H%	N%
1 Gly-Pro-NHO-Bz(4-OCH_3)	357.81	$C_{15}H_{20}N_3O_5Cl$	141–142				
2 Gly-Pro-NHO-Bz(4-CH_3)	341.81	$C_{15}H_{20}N_3O_4Cl$	133–134				
3 Gly-Pro-NHO-Bz	327.78	$C_{14}H_{18}N_3O_4Cl$	157–158				
4 Gly-Pro-NHO-Bz(3-Cl)	362.23	$C_{14}H_{17}N_3O_4Cl_2$	146–148				
5 Gly-Pro-NHO-Bz(3,5-$(NO_2)_2$	417.79	$C_{14}H_{16}N_5O_6Cl$	see text				
6 Gly-Pro-NHO-Bz(4-NO_2)	372.80	$C_{14}H_{17}N_4O_4Cl$	148–150	found:	44.20	4.58	14.22
				requ.:	45.10	4.60	15.01
7 Ala-Pro-NHO-Bz(4-NO_2)	386.77	$C_{15}H_{19}N_4O_6Cl$	148–149	found:	46.70	4.73	14.49
				requ.:	46.53	4.91	14.48
8 Leu-Pro-NHO-Bz(4-NO_2)	428.87	$C_{18}H_{25}N_4O_6Cl$	114–117	found:	48.52	6.21	11.72
				requ.:	50.41	5.88	13.07
9 Phe-Pro-NHO-Bz(4-NO_2)	462.92	$C_{21}H_{23}N_4O_6Cl$	100–105	found:	52.81	5.07	11.90
				requ.:	54.48	5.01	12.10
10 Lys(Z)-Pro-NHO-Bz(4-NO_2)	578.01	$C_{26}H_{32}N_5O_8Cl$	93– 95	found:	51.55	5.59	10.68
				requ.:	54.02	5.58	12.12
11 Lys(Z-4-NO_2)-Pro-NHO-Bz(4-NO_2)	623.01	$C_{26}H_{31}N_6O_{10}Cl$	101–103	found:	49.20	4.97	13.70
				requ.:	50.12	5.02	15.74

*Nomenclature of peptide residues according to Schechter and Berger[13], -NHO- is the hydroxylamine function, *N*-acylated by petidyl residues, *O*-acylated by various benzoic acids (Bz), substituent in brackets, Bz(X).

No melting point is reported for 5 but the analytical data of the corresponding Boc-protected compound is as follows:

N-Boc-Gly-Pro-NHO-Bz(3,5-$(NO_2)_2$). M.p.: 131–132°C, (Found: C, 47.69; H, 5.06; N, 13.99. $C_{19}H_{23}N_5O_{10}$ requires C, 47.40; H, 4.82; N, 14.54%).

Kinetic Methods and Inactivation Experiments

All enzyme activity assays and inactivation experiments were performed and all spectra were recorded using a Unicam SP 800 and the microprocessor controlled Specord M 40.

Estimation of pK-values was performed by measurement of spectra between 250 and 450 nm for 0.1 mM solutions of diacyl hydroxylamines in silica cells containing 2.0 ml McIlvain-buffer (pH 2.2–pH 8.0) and 0.1 ml acetonitrile or dimethyl formamide. Graphic analysis of differences in absorption at 300 nm was used for pK-calculation.

Spontaneous degradation of diacyl hydroxylamines was followed spectrometrically in the range of 225 to 400 nm at 30°C.

Solutions contained 40 mM sodium phosphate buffer, ionic strength 0.125, pH 7.6 and 0.1 mM concentrations of diacyl hydroxylamines.

Data at several wavelengths were collected as functions of time and the pseudo-first-order rate constants calculated using nonlinear regression programs provided with the Specord M 40 by Carl-Zeiss-Jena and using a Hewlett-Packard desktop computer HP 9825 A.

The activity of DP IV was determined with Gly-Pro-4-nitroanilide and Ala-Pro 4-nitroanilide in 40 mM sodium phosphate buffer with an ionic strength of 0.125, maintained by potassium chloride as described previously[1].

Specific activity of DP IV was in the range 35–45 U/mg. The k_{cat}-values given in Table 3 have been standardized and calculated assuming a maximal activity of 55 U/mg and a molecular weight of 115 000 per subunit of the enzyme.

DP IV-catalyzed hydrolysis of substrate analog diacyl hydroxylamines has been analyzed following the absorption change due to the release of *O*-benzoyl hydroxylamines between 260 and 360 nm (for wavelengths and absorption coefficients see Table 3).

Activity was estimated in 10 mm silica cells containing 2.5 ml of 40 mM sodium phosphate buffer, pH 7.6, ionic strength 0.125 at 30°C. The pseudosubstrate concentration was varied between 1.0 μM and 0.1 mM. Final DP IV concentration was in all cases 50 nM after addition of 50 μl aliquots to initiate reaction.

Initial rates were analyzed using the software cassette "reaction kinetics" built into the spectrophotometer M 40. The parameters k_{cat} and K_m were calculated from the initial rates using nonlinear regression programs to fit a hyperbola using a Sinclair Spectrum Plus computer.

Residual activity of DP IV after preincubation with several substrate analog diacyl hydroxylamines was estimated as follows: DP IV was incubated with suicide substrates in concentrations of 20 μM to 1.04 mM in 2.5 ml (40 mM) sodium phosphate buffer, pH 7.6, ionic strength 0.125 at 30°C. The reaction was initiated by adding enzyme to give 0.15 nM DP IV in the mixture. Decrease of activity was followed by withdrawing 0.1 ml aliquots of the incubation mixture and estimating its residual DP IV activity against 1.23 mM alanyl-prolyl-4-nitroanilide under the same conditions described above. The inactivation reaction was monitored until a completion but usually not longer than two hours.

The effectiveness[10] of suicide inactivation is characterized by the partition ratio $r = k_{cat}/k_{inact}$. Deviations from linearity in semilogarithmic plots (log residual activity versus time) occur caused by enzyme catalyzed hydrolysis ((Figure 3A) and an exact estimation of k_{inact} is not possible. Using the following approximation here a value representing "r" could be obtained:

Since the partition ratio, r, is equal to the number of molecules processed as "substrate" divided by the number of molecules processed as "inhibitor" (giving inactivated enzyme), r can be set equal to $[S]/[E_i]$ in our case. The final concentration of processed substratemolecules is approximately equal to its initial concentration, if after the reaction has finished all suicide substrate was consumed and only catalytic quantities of enzyme were used.

If in the experiment an enzyme concentration was chosen that after the reaction is complete still gives an active enzyme then the molar concentration of inactivated enzyme and therefore the concentration of molecules leading to inactivation can be calculated.

Dipeptidyl peptidase IV was purified according to reference 24 using a slightly modified procedure.

RESULTS

The structure of diacyl hydroxylamines (R_1-CO-NH-O-CO-R_2) ideally permits variation of affinity and reactivity of the molecule towards a target enzyme simply by selection of appropriate *N*-acyl- and *O*-acyl residues.

Bearing in mind the substrate specificity of DP IV two sets of modified diacyl hydroxylamines were synthesized and used in this study: (1) Compounds with the same substrate analog peptide moiety but different substituted benzoyl residues as leaving groups (R_1-CO- = Gly-Pro, R_2-CO- = substituted benzoic acid) and, (2) compounds with same leaving groups but different amino acids in the P_2-position of the N-peptidyl residue of the molecule (R_1-CO- = Xaa-Pro, R_2-CO- = 4-nitrobenzoyl) (see Table II).

Stability and Ionization of Diacyl Hydroxylamines in Aqueous Solution

Degradation studies with diacyl hydroxylamines in buffer solutions using TLC in silica gel plates showed peptide hydroxamic acids and benzoic acids as products of the spontaneous degradation of *N*-peptidyl-*O*-benzoyl hydroxylamines[14].

Taking advantage of the difference in absorption between solutions of free substituted benzoic acids and solutions of *N*-peptidyl-*O*-benzoyl hydroxylamines the pseudo-first-order rate constants of this degradation have been estimated spectrometrically (Table II).

The uv-spectra of 0.1 mM buffered solutions (pH 2.2–8.0) of *N*-peptidyl-*O*-(4-nitrobenzoyl) hydroxylamines exhibited a bathochromic shift of absorption maxima (263 nm to 268 nm) due to the known acidity of diacyl hydroxylamines[15]. The pH-dependent absorption change at 300 nm was used for estimation of pK-values of some N-peptidyl-*O*-(4-nitrobenzoyl) hydroxylamines (Figure 1, Table II).

With typical pK-values of 4.8 the compounds exist at neutral pH as anions as a result of the acidity of the -CO-NH-O- linkage. Investigation of the stability of Xaa-Pro-NHO-Bz(4-NO_2) derivatives (Xaa = Gly, Ala, Leu, Phe, Lys(Z)) in the

TABLE II

Rate of spontaneous degradation and pK value for *N*-peptidyl-*O*-benzoyl hydroxylamines in buffer solutions

Suicide substrate	Pseudo-first order rate constant[+], k_{obs} [min^{-1}]	pK value[#]
Gly-Pro-NHO-Bz(4-CH_3)	$0.82 \cdot 10^{-3}$	n.e.
Gly-Pro-NHO-Bz(3-Cl)	$1.13 \cdot 10^{-3}$	n.e.
Gly-Pro-NHO-Bz(4-NO_2)	$2.20 \cdot 10^{-3}$	4.77
Gly-Pro-NHO-Bz(3,5-(NO_2)$_2$)	$6.30 \cdot 10^{-3}$	n.e.
Ala-Pro-Bz(4-NO_2)	$1.30 \cdot 10^{-3}$	4.9
Leu-Pro-NHO-Bz(4-NO_2)	$2.20 \cdot 10^{-3}$	4.8
Phe-Pro-NHO-Bz(4-NO_2)	$2.00 \cdot 10^{-3}$	4.5
Lys(Z)-Pro-NHO-Bz(4-NO_2)	$2.80 \cdot 10^{-3}$	4.85
Lys(Z-4-NO_2)-Pro-NHO-Bz(4-NO_2)	$2.30 \cdot 10^{-3}$	4.80

[+] Release of benzoic acid followed uv-spectrometrically (see MATERIAL AND METHODS) in 0.04 M sodium phosphate buffer, pH 7.6, ionic strength was adjusted with potassium chloride to 0.125, temperature 30°C.

[#] pK-values of ionisation of the -CO-NH-O- linkage were estimated in McIlvaine-buffer (pH 2.2–8.0) at 25°C.

n.e. = not estimated.

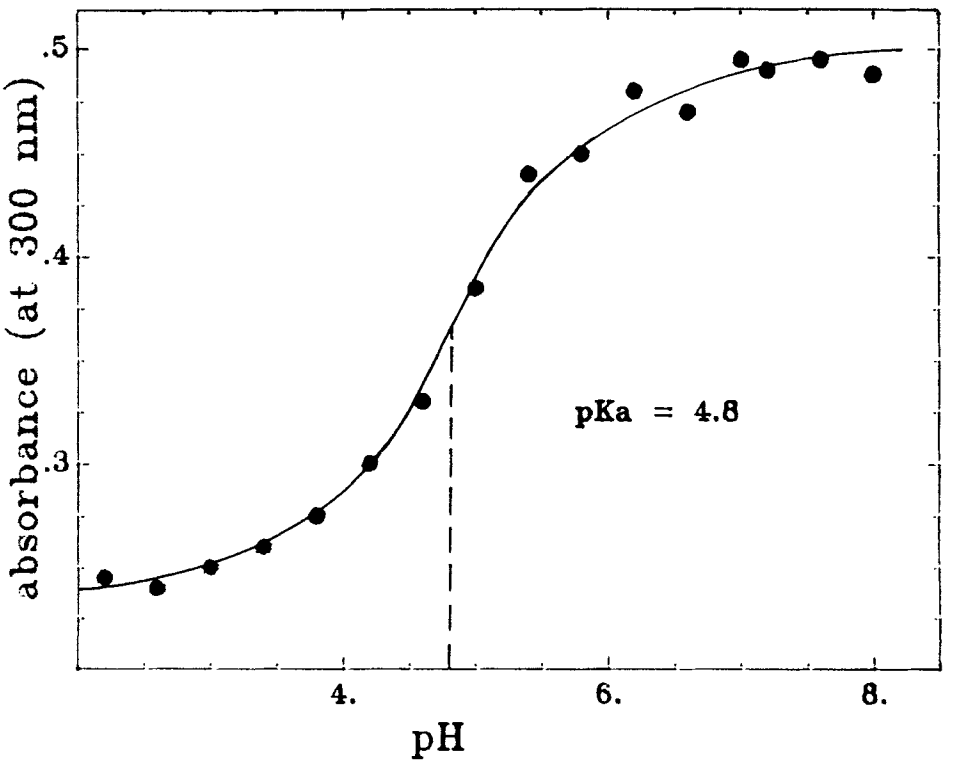
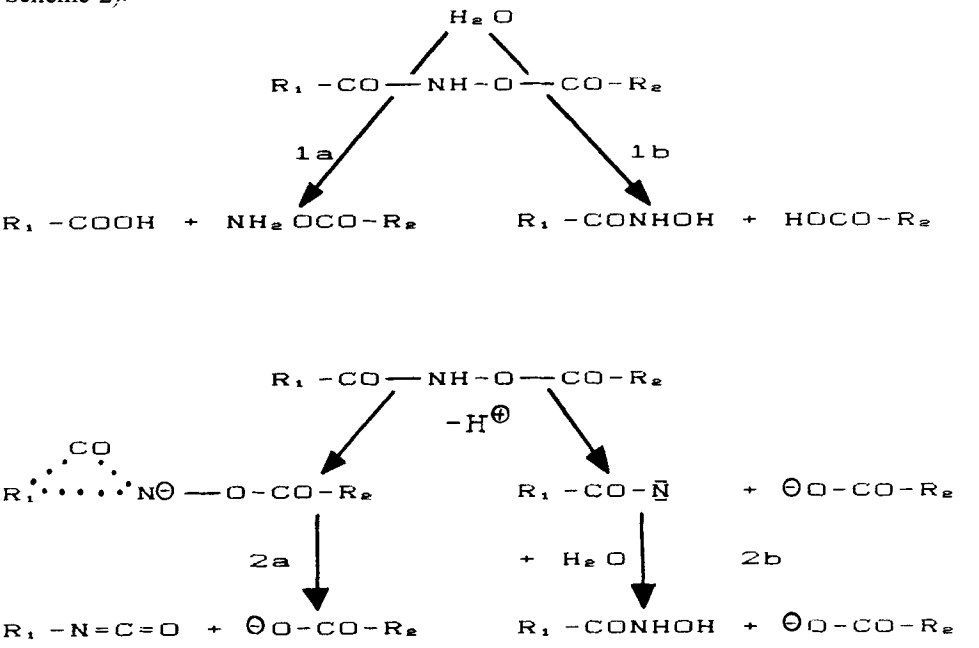


FIGURE 1 pH-dependence of absorption of Lys(Z)-Pro-NHO-Bz(4-NO_2) 0.1 mM in McIlvaine buffer, pH 2.2–8.0, at 25°C.

range pH 5–pH 8 showed that they did not exhibit significant pH-dependence of the degradation reaction. This result does not fit a model involving hydroxyl ion catalyzed hydrolysis of either amide or ester bonds of the compounds (pathway 1a, 1b in Scheme 2).



SCHEME 2 Degradation pathways for diacyl hydroxylamines.

Due to their relative stability i.e. half lives of 2–5 h under conditions used, investigation of their reactions with DP IV were possible.

DP IV-catalyzed Hydrolysis of Diacyl Hydroxylamines

By definition, mechanism-based inactivation of enzymes includes inactivation of the enzyme by the suicide substrate as well as the enzyme-catalyzed turnover of the suicide substrate (Scheme 1).

Indeed, incubation of *N*-Xaa-Pro-*O*-benzoyl hydroxylamines in buffered solutions with substantial amounts of DP IV results, in contrast to the nonenzymic degradation, in complete hydrolysis to *O*-benzoyl hydroxylamines and appropriate dipeptides as proved by TLC[14] (see pathway 2 in Scheme 1). The reaction leads to an absorption decrease in the uv-spectra between 250–400 nm due to the release of *O*-benzoyl hydroxylamines which permits estimation of the catalytic constants K_m and k_{cat} (Table III). Figure 2 illustrates the dependence of the rate of DP IV-catalyzed hydrolysis with different concentrations of Leu-Pro-NHO-Bz(4-NO_2).

Inactivation of DP IV by Substrate Analog Diacyl Hydroxylamines

Incubation of DP IV with suicide substrates (shown in Table IV) and measurement

TABLE III
Kinetic parameters for dipeptidyl peptidase IV-catalyzed hydrolysis of *N*-peptidyl-*O*-benzoyl hydroxylamines

Suicide substrate	Km × 10^5) [M]	k_{cat}* [sec^{-1}]	k_{cat}/K_m (× 10^7)	Wavelength⁺/ absorb. coeff.
Gly-Pro-NHO-Bz(4-OCH_3)	3.0	117	3.90	286 :3600
Gly-Pro-NHO-Bz(4-CH_3)	3.9	108	2.77	263.5:4800
Gly-Pro-NHO-Bz	3.7	101	2.73	263.5:3372
Gly-Pro-NHO-Bz(3-Cl)	3.7	101	2.73	263.5:3285
Gly-Pro-NHO-Bz(3,5-$(NO_2)_2$)	2.4	108	4.5	270 :1400
Gly-Pro-NHO-Bz(4-NO_2)	4.0	116	2.9	303.5:2000
Ala-Pro-NHO-Bz(4-NO_2)	1.8	74.5	4.1	325 :1230
Leu-Pro-NHO-Bz(4-NO_2)	4.0	55	1.38	300 :1847
Phe-Pro-NHO-Bz(4-NO_2)	6.8	43	0.63	300 :1864
Lys(Z)-Pro-NHO-Bz(4-NO_2)	3.8	28	0.74	300 :1540
Lys(Z-4-NO_2)-Pro-NHO-Bz(4-NO_2)	2.1	22	1.05	300 :1513

⁺DP IV-catalyzed release of *O*-(4-nitrobenzoyl) hydroxylamine followed spectrometrically in 0.04 M sodium phosphate buffer, pH 7.6, at 30°C with ionic strength adjusted with potassium chloride to 0.125. Wavelength in nm.

*The k_{cat}-values have been calculated here assuming a molecular weight of 115000 per subunit of DP IV and a specific activity of 55 U/mg for the enzyme used.

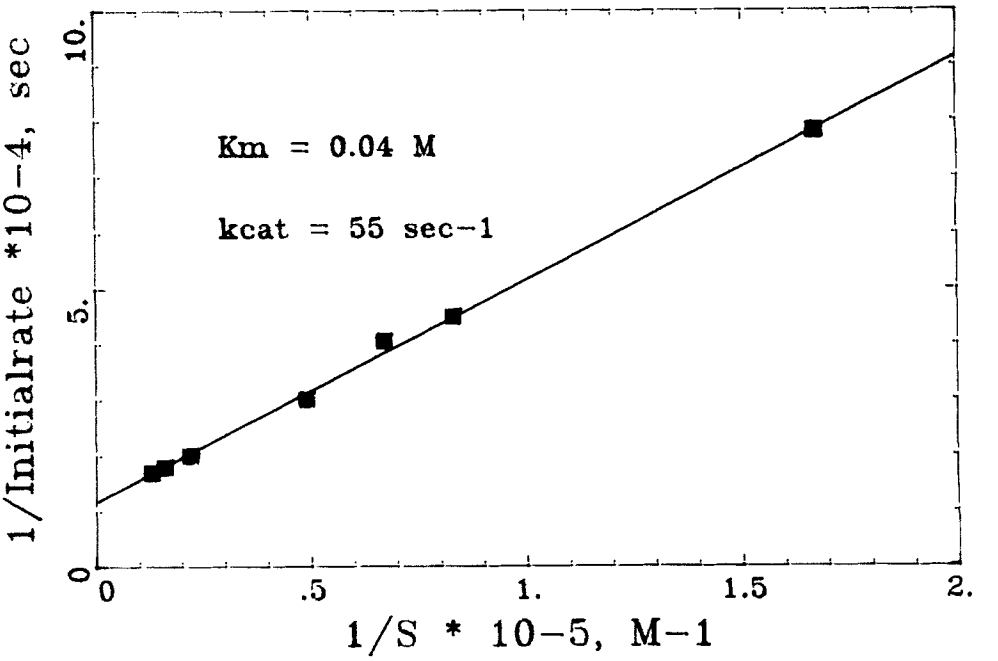


FIGURE 2 Lineweaver-Burk plot of kinetic results in the DP IV-catalyzed hydrolysis of *N*-Leu-Pro-*O*-(4-nitrobenzoyl) hydroxylamine at pH = 7.6 in 0.04 M sodium phosphate buffer at 30°C with ionic strength of 0.125 maintained with potassium chloride.

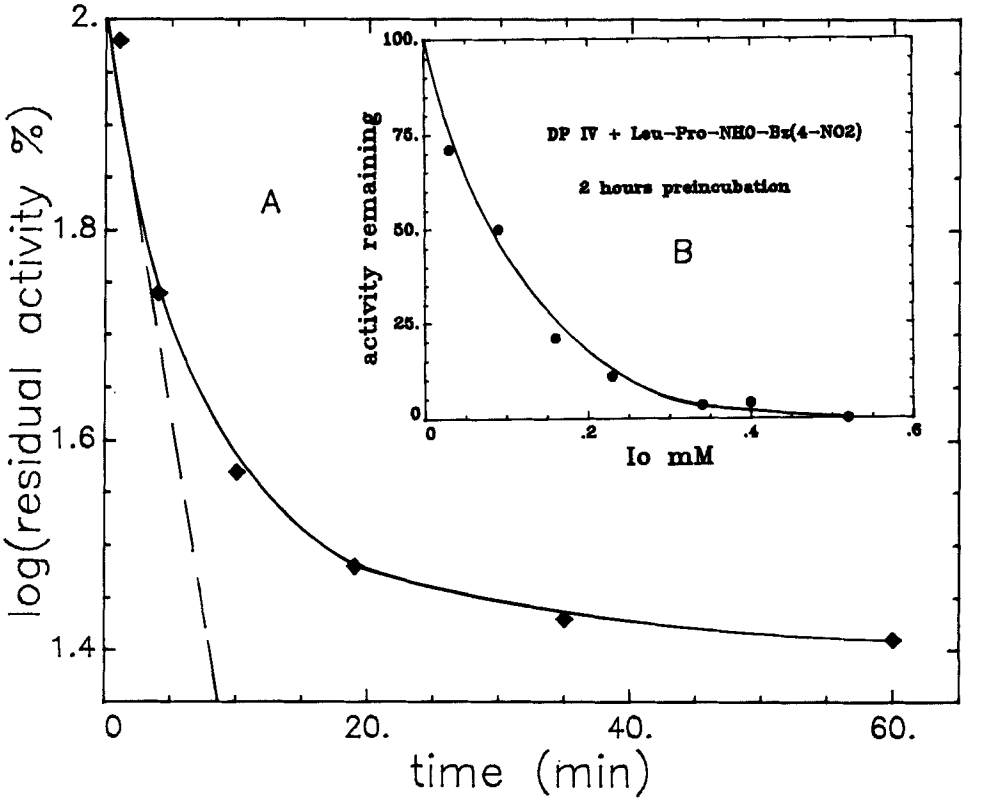


FIGURE 3 (A) – Inactivation of Dipeptidyl peptidase IV with *N*-Leu-Pro-*O*-(4-nitrobenzoyl) hydroxylamine. Measurements carried out at 30°C, pH 7.6, in 0.04 M sodium phosphate buffer with ionic strength 0.125, maintained with potassium chloride. DP IV = 0.165 nM, I_o = 0.26 mM. (B) Insert shows dependence of inactivation of DP IV with *N*-Leu-Pro-*O*-(4-nitrobenzoyl) hydroxylamine as function of inhibitor concentration at 2 hours incubation time. DP IV = 0.146 nM.

of the residual activity at different time intervals shows a time-dependent loss of enzyme activity (Figure 3). The irreversibility of the inactivation was proved by dilution experiments and by gel chromatographic separation of the inactivated protein from excess of inhibitor. Incubation of the enzyme with diacyl hydroxylamines having other *N*-acyl-residues as Xaa-Pro- does not affect DP IV activity[1].

Diminution or suppression of the inactivation process by adding "normal" substrate or competitive inhibitors to the incubation mixture may indicate that a specific reaction within the active site of a target enzyme is occurring. Thus, a change of the half life for inactivation in the presence of Lys(Z-4-NO_2)-Pro, a competitive inhibitor of DP IV[14], was expected:

To a preincubation mixture containing 2.8 mM *N*-Ala-Pro-NHO-Bz(4-NO_2) and 57 μM Lys(Z-4-NO_2)-Pro, DP IV was added to give a final concentration of 1.1 nM. The resulting inactivation rate constant (residual activity estimated as described in Material and Methods), $k_{obs} = 7.7 \times 10^{-3} min^{-1}$, is about one order of magnitude lower than the rate obtained in a control experiment in the absence of Lys(Z-4-NO_2)-Pro.

TABLE IV
Inactivation of dipeptidyl peptidase IV by *N*-peptidyl-*O*-benzoyl hydroxylamines

Suicide substrate	$I_0(\times 10^4)$ $[M]^+$	Turnover ratio "r"	$k_{inact}(\times 10^4)$ $[sec^{-1}]^\#$
Gly-Pro-NHO-Bz(4-OCH_3)	5.0	$2.03 \cdot 10^6$	0.58
Gly-Pro-NHO-Bz(4-CH_3)	5.0	$6.97 \cdot 10^5$	1.54
Gly-Pro-NHO-Bz	5.0	$6.67 \cdot 10^5$	1.52
Gly-Pro-NHO-Bz(3-Cl)	5.0	$3.47 \cdot 10^5$	2.92
Gly-Pro-NHO-Bz(3.5-$(NO_2)_2$)	5.0	$2.13 \cdot 10^5$	5.08
Gly-Pro-NHO-(4-NO_2)	5.0	$3.01 \cdot 10^5$	3.83
Ala-Pro-NHO-Bz(4-NO_2)	4.4	$6.73 \cdot 10^4$	11.07
Leu-Pro-NHO-Bz(4-NO_2)	2.6	$1.50 \cdot 10^4$	36.69
Phe-Pro-NHO-Bz(4-NO_2)	2.6	$1.40 \cdot 10^4$	30.07
Lys(Z)-Pro-NHO-Bz(4-NO_2)	2.6	$8.00 \cdot 10^3$	35.10
Lys(Z-4-NO_2)-Pro-NHO-Bz(4-NO_2)	2.6	$1.20 \cdot 10^4$	18.31

[+] Initial inhibitor concentration.

*DP IV incubation with inhibitors in 0.04 M sodium phosphate buffer, pH 7.6, at 30°C with ionic strength adjusted with potassium chloride to 0.125. Preincubation time: all Gly-Pro-derivatives one hour, all others two hours. Estimation of "r"-values see "Material and Methods".

[#] The k_{inact}-values in this paper have been calculated assuming $r = k_{cat}/k_{inact}$ and r = [hydrolyzed inhibitor]/[inactivated enzyme] using k_{cat}-values in Table 2.

The results described meet the typical criteria established for suicide inactivators[16] which also include enzyme-catalyzed turnover of inhibitor during the incubation period. In Figure 3 the deviation of the estimated residual activities from a straight line is due to this turnover. Although some suicide substrate molecules inhibit the target enzyme others are hydrolyzed by it simultaneously (compare Scheme 1).

The higher the enzyme concentration or the less effective the inactivation process a more curved dependence of residual activity from incubation time in a semilogarithmic presentation may be observed. Alternatively the lower the enzyme concentration or the more effective the inactivation compared to catalysis is, a closer approach to a semilogarithmic presentation of residual activity versus time will be obtained.

Ideally, if no inhibitor molecule is destroyed by the enzyme or the inhibitor concentration does not change significantly during incubation, kinetics follow pseudo-first order reactions. Accordingly suicide inactivation is best characterized by ratios of catalysis versus inactivation[10,17,18] i.e. values of "r" (ratio of k_{cat}/k_{inact}).

Values of "r" for the reaction of DP IV with the substrate analog diacyl hydroxylamines estimated as described previously[10], are summarized in Table IV.

DISCUSSION

The concept of introducing substrate analog diacyl hydroxylamines as specific inhibitors of DP IV was aimed at overcoming the undesirable features of N-terminal unprotected proline containing dipeptide derivatives which as amides or esters tend to form diketopiperazines during intramolecular reactions. Earlier attempts to use Xaa-Pro-chloromethylketones gave compounds with half lives less than ten minutes and complete inactivation of the target enzyme was achieved only with substantial amounts of inhibitor. Compounds were sought where the chemical reactivity is

masked and are only activated by the catalytic attack of the target enzyme which so generates its own inactivation.

Falling in this category of masked compounds that deserves special mention, are *N*-halogenamides, carbonylazides and diacyl hydroxylamines i.e. compounds prone to rearrangement (Hofmann-, Curtius- and Lossen reactions). It was considered that should the compounds generate reactive intermediates during degradation, the mechanism could be used to inactivate proteolytic enzymes.

Azides and diacyl hydroxylamines may share this feature since possible intermediates in the Curtius and Lossen reactions are carbonyl nitrenes and/or isocyanates[19]. As expected the peptide azides, Boc-Ala-Ala-N_3 and Ala-Ala-N_3, were found to be inhibitors of chymotrypsin and DP IV, respectively but there was still substantial hydrolytic degradation of these compounds in buffer solution due to the good leaving azide ion[20].

Since diacyl hydroxylamines exist as anions at neutral pH-values[15] a higher stability to hydrolysis of the scissile carbonyl linkage in *N*-peptidyl-*O*-benzoyl hydroxylamines was expected. Incubation of chymotrypsin, elastase, thermitase and dipeptidyl peptidase IV with specific substrate analog *N*-peptidyl-*O*-benzoyl hydroxylamines resulted in irreversible enzyme inhibition[1]. In most cases enzyme-catalyzed hydrolysis of the substrates was negligible. Only during reaction between substrate analog diacyl hydroxylamines with DP IV have we found substantial enzyme-catalyzed hydrolysis of the suicide substrates.

Therefore a systematic study of the factors influencing the effectiveness of inactivation of DP IV was perforemd. The compounds used are stable and suitable in the enzymological experiments under the standard conditions used. Their degradation in solution may be caused by hydrolysis of the ester or the amide bonds of the hydroxylamine function or by N–O bond fission (see pathways 1a, 1b and 2 in Scheme 2).

Variation of the peptidyl residue of the compounds does not influence the degradation rate remarkably (Table III). Obviously, the distance between the P_2-amino acid and reactive center of the molecule excludes noticeable effects of side chain structure in the reaction. In contrast, rate constants increase proportionally to the electron withdrawing effects of substituents in the leaving benzoic acid in compounds 1–4 (Table II).

These kinetic findings are in good agreement with studies on the degradation of diacyl hydroxylamines in aqueous ammonia described as Lossen degradation of the substrates[21,22].

Hammett correlation of the pseudo-first-order rate constants of the degradation give for *N*-Gly-Pro-*O*-benzoyl hydroxylamine derivatives a correlation coefficient $\varrho = 0.62$ (X = 4-CH_3-, 3-Cl-, 4-NO_2-, 3,5-$(NO_2)_2$, r = 0.97). The value resembles the value $\varrho = 0.86$ found for the degradation of dibenzoyl hydroxylamines, with variation of the *O*-benzoyl substituents, in 0.1 M ammonia[21].

However, here no rearrangement products of a complete Lossen reaction are detectable (2a, Scheme 2), thus excluding the occurrence of isocyanates as intermediates[19]. The decomposition products are peptide hydroxamic acids and *O*-benzoyl hydroxylamines, so that only distinction between hydrolysis of the ester bond (pathway 1a), and insertion of carbonyl nitrene in water (pathway 2b), is necessary.

Recently we obtained evidence of a nitrene-generating α-elimination step followed by insertion in H–O bond of a water molecule (2b, Scheme 2)[22].

In contrast to the nonenzymic degradation of the compounds, the electronic nature

of the leaving group does not influence the k_{cat} values for DP IV-catalyzed hydrolysis of Glycyl-Prolyl- derivatives (Table III). Departure of *O*-benzoyl hydroxylamine as the first product of catalysis occurs prior to the step analyzed as k_{cat} so that k_{cat} must reflect the rate determining deacylation of Glycyl-Prolyl-DP IV (see items 1–6, Table III).

This observation is in complete agreement with similar results obtained using a series of substituted alanyl-prolyl-anilides as substrates[24]. In control measurements using Gly-Pro-4-nitroanilide as a substrate a k_{cat} value of 111 sec^{-1} has been estimated supporting the above view (Table III: mean k_{cat} value 109 sec^{-1} $\pm$ 6%).

Analysis of DP IV-catalyzed hydrolysis of suicide substrates with different peptide residues but a constant leaving group shows that a slight variation of the deacylation rate constant k_{cat} occurs (Table III, items 6–11). The rate constants obtained resemble similar values obtained[25] for DP IV substrate specificity, which was interpreted as being due to structural differences of Xaa-Pro-DP IV (acyl enzymes).

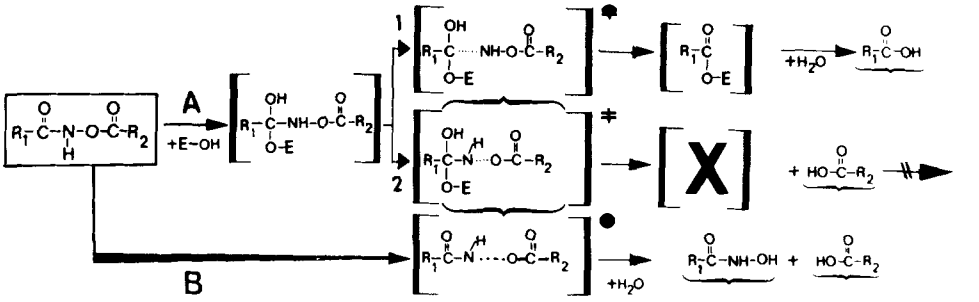
As in nonenzymic degradation, but in contrast to the independence of k_{cat} values for enzyme-catalyzed hydrolysis, *N*-Gly-Pro-*O*-benzoyl hydroxylamines exhibit a remarkable influence of the nature of benzoyl substituents on the inactivation of DP IV. A Hammett correlation of the k_{inact}-values for compounds 1–6, (Table IV) gives a ϱ-value of 0.49 ($r = 0.9$). This result indicates electronic control by the leaving *O*-benzoyl group in the transition state of the enzyme inactivation pathway. Electron withdrawing substituents promote rate determining N–O bond fission by stabilization of the leaving negative charged benzoyl residue.

A comparison of the turnover values, r, of suicide substrates with a constant leaving group but different Xaa-residues in the P_2-position demonstrates that the more hydrophobic or longer the side chains in substrate analog molecule are, the more successfully DP IV is inactivated. The enhancement of the rate of inactivation between the Gly-Pro- and the Lys(Z)-Pro-derivatives is two orders of magnitude.

With a turnover ratio of 8000 and an apparent pseudo-first-order inactivation rate constant of 0.21 min^{-1} *N*-Lys(Z)-Pro-*O*-(4-nitrobenzoyl) hydroxylamine is the most potent of the inhibitors tested and may be used in further biological studies. However reasons for this dramatic change in activity are unclear. Possibly, the mechanism of reaction of DP IV with substrate analog diacyl hydroxylamines includes a thermodynamic partitioning between processes leading to hydrolysis as substrate or enzyme inactivation (Scheme 3, A). If so, both processes, enzyme catalyzed hydrolysis and inactivation, are influenced by the nature of the peptide part of the molecules since k_{cat} values increase as the size of side chain decreases (Table III), consistent with decreasing k_{inact} values (Table IV).

During the intermediate steps of catalysis of suicide substrate molecule, tighter binding caused by bulky or hydrophobic P_2-side chains may increase the lifetime of noncovalent or covalent complexes formed prior to the acyl enzyme so that more time exists for N–O bond fission as the initial step of the inactivation; the ratio between catalysis and inactivation events would decrease. The lower k_{cat} values for DP IV catalyzed hydrolysis of the compounds with Ala, Leu, Phe, Lys(Z) and Lys(Z-4-NO_2) as P_2- amino acids compared with the Gly-derivative may support this hypothesis (Table III).

Possible reactions occurring during interaction of DP IV with substrate analog diacyl hydroxylamines are summarized in Scheme 3. It becomes clear that for both events occurring – hydrolysis and inactivation – two different steps within the catalytic process are rate determining. The nature of the product formed during modification of the enzyme remains unknown.



SCHEME 3

Recently, 3-benzyl-N-(methanesulfonyloxy) succinimide has been shown to be an inhibitor of serine proteases[26]. The authors propose, similar to us, that the mechanism of inhibition results from a Lossen reaction[27].

Studies on the structural modifications by diacyl hydroxylamines of proteases, such as porcine pancreatic elastase and proline specific protease, are in progress.

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