J. Enzyme Inhibition, 1988, Vol. 2, pp. 129–142
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DIPEPTIDYLPEPTIDASE IV – INACTIVATION WITH *N*-PEPTIDYL-*O*-AROYL HYDROXYLAMINES

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(Received 10 August 1987)

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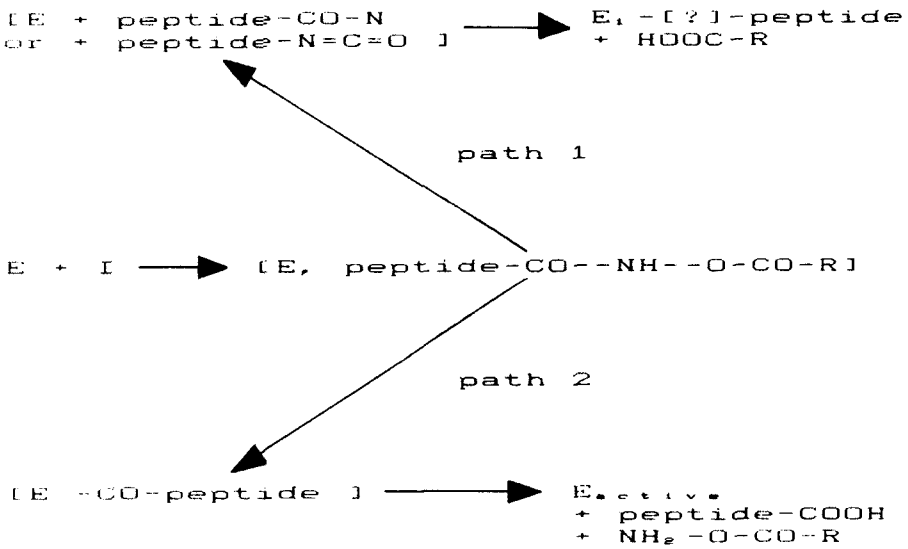
enzymes and accordingly we have studied *N*-peptidyl-*O*-aroyl hydroxylamines as mechanism-based inactivators of serine proteases[1]. *N*-alanyl-prolyl-*O*-(4-nitrobenzoyl) hydroxylamine has been used successfully as a specific inhibitor in the investigation of DP IV in human lymphocytes[9].

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

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