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REACTIONS BETWEEN DIPEPTIDYL PEPTIDASE IV AND DIACYL HYDROXYLAMINES: MECHANISTIC INVESTIGATIONS

HANS-ULRICH DEMUTH[1]*, ULF NEUMANN[2] and ALFRED BARTH[1]

[1] *Department of Biotechnology, Martin-Luther-University, Halle* [2] *Department of Pharmacology, Medical Academy, Erfurt German Democratic Republic*

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Kinetics of inactivation of dipeptidyl peptidase IV (DP IV, EC 3.4.14.5) by *N*-peptidyl-*O*-(4-nitrobenzoyl) hydroxylamines and their enzyme-catalyzed hydrolysis were followed using independent monitoring methods, all giving similar efficiency ratios of K_{cat}/K_{inact}.

Different temperature dependences of the DP IV-inactivation and enzyme-catalyzed hydrolysis provide evidence of independent rate determining steps for both reactions. Activation parameters of inactivation are similar to those of spontaneous decomposition of the compounds, suggesting a mechanistic relationship.

Investigation of DP IV-inactivation, DP IV-catalyzed hydrolysis of *N*-Ala-Pro-*O*-Bz(4-NO_2) and the decomposition of the suicide substrate in H_2O and D_2O gave solvent isotope effects of 4.65, 2.54 and 1.02, respectively. A proton inventory of the inactivation reaction indicates involvement of more than one proton in the formation or breakdown of its transition state. The linear proton inventory found for the hydrolytic reaction is consistent with one proton transition in the rate determining step and resembles the rate limiting deacylation of Ala-Pro-DP IV. The hypothetical reaction model now locates splitting in both reactions prior to formation of a covalent intermediate during the catalytic cycle.

KEY WORDS: Dipeptidyl peptidase IV, diacyl hydroxylamine, mechanism-based inhibitor, activation parameter, solvent isotope effect, proton inventory.

INTRODUCTION

Dipeptidyl peptidase IV has been studied intensively over the past two decades with respect to its biochemistry[1], pathobiochemistry[2,3] and molecular enzymology[4,5]. The unique specificity of the enzyme, which mainly releases Xaa-Pro dipeptide units from N-terminal parts of peptides, makes DP IV an ideal mediator within physiological pathways involving deactivation or activation of peptides by limited proteolysis. Thus, numerous authors have discussed the participation of DP IV in processing of peptide hormones and in the induction of biological effects[6,7]. Indeed, deviations from normal DP IV activity especially in blood diseases, cancers and AIDS has stimulated growing interest in DP IV-tests for diagnostic use[8-10]. However, opinions on the exact manner of participation of DP IV in biological processes remain obscure. Since naturally occurring inhibitors of DP IV are not known, the use of inhibitors as tools in biological investigations was restricted to Xaa-Pro dipeptides as product inhibitors and DFP and DEP as markers of seryl and histidyl residues, respectively[11]. After

*Correspondence to: Dr. H.-U. Demuth, Department of Biotechnology, Martin Luther University, Domplatz 1 Halle (Saale), DDR-4020.

unsuccessful attempts with substrate analog chloromethyl ketones, which decompose with half lives of less than 10 minutes[12], we introduced substrate analog diacyl hydroxylamines[13] as mechanism-based inhibitors for serine proteases (R_1-CO-NHO-CO-R_2). Using the substrate analog *N*-Ala-Pro-*O*-(4-nitrobenzoyl) hydroxylamine participation of DP IV in the proliferation of lymphocytes could be proved[14,15]. In a preceding paper we have described the characteristics of eleven *N*-peptidyl-*O*-benzoyl hydroxylamines as inhibitors and substrates for the enzyme[16].

Now, we present kinetic studies on the mechanisms of interaction of DP IV with *N*-peptidyl-*O*-(4-nitrobenzoyl) hydroxylamines to extend the mechanistic investigation of this reaction.

MATERIALS AND METHODS

Suicide substrates and substrates

N-peptidyl-*O*-(4-nitrobenzoyl) hydroxylamines were synthesized as described before[13,16].

Table I lists structural parameters of the Boc-derivatives. The Boc-protecting groups were removed using HCl/glacial acetic acid to give the appropriate hydrochlorides of *N*-Xaa-Pro-O-(4-nitrobenzoyl) hydroxylamines, whose structural parameters are given in reference 16. Melting points are uncorrected.

Gly-Pro-, Ala-Pro- and Phe-Pro-4-nitroanilide were provided by Dr. K. Neubert, Division of Drug Biochemistry, University of Halle, GDR.

Spectrophotometric measurements

Spectra and kinetic runs were recorded with a Carl-Zeiss-Jena microprocessor controlled spectrophotometer M 40 equipped with a jacketed cell compartment, containing heat and temperature control. Temperature readings were correct within ± 0.1°C. Data collected and stored to an internal buffer were analyzed using software packages provided on an application card "reaction kinetics" for the instrument allowing analysis of zero-order, first-order and reactions following more complex rate laws. Reactions were carried out in 1.0 cm teflon stoppered silica cells.

TABLE I

N-Boc-peptidyl-*O*-(4-nitrobenzoyl) hydroxylamines, analytical parameters

Boc-Xaa-Pro-NHO-Bz(4-NO_2)							
	Xaa	MG	Formula	Fp.[°C]	C%	H%	N%
1	Gly-	436.41	$C_{19}H_{24}N_4O_8$	134-137	found:52.06	5.51	11.57
					requ. :52.29	5.54	12.84
2	Ala-	450.45	$C_{20}H_{26}N_4O_8$	121-122	found:53.13	5.93	12.25
					requ. :53.33	5.82	12.44
3	Leu-	492.52	$C_{23}H_{32}N_4O_8$	79-81	found:56.07	6.54	10.95
					requ. :56.09	6.55	11.38
4	Phe-	526.53	$C_{26}H_{30}N_4O_8$	69-71	found:59.15	5.89	09.76
					requ. :59.31	5.74	10.64

D_2O used in estimation of solvent isotope effects and for proton inventories was distributed by Berlin Chemie, Berlin, and contained 99.8% deuterium (lot 010584). Sodium phosphate buffers were prepared with special care in H_2O or D_2O to give 0.1 M stock solutions and 40 mM concentrations within reaction tubes or cells. Solution were kept under a nitrogen atmosphere to avoid isotope dilution caused by atmospheric moisture. Ionic strength was maintained with potassium chloride to final I = 0.125, taking into consideration the isotope effect of phosphoric acid dissociation in D_2O and H_2O with a Δ pKa of 0.52. Preparation of solutions with different mole % deuterium oxide was performed according to reference 17.

Spontaneous decomposition of diacyl hydroxylamines has been followed uv-spectrometrically in the range of 225 to 400 nm at 30°C. Data at several wavelengths were collected as functions of time and the pseudo-first-order rate constants calculated using nonlinear regression programs on the Specord M 40[16].

DP IV catalyzed hydrolysis of substrate analog diacyl hydroxylamines was analyzed by monitoring absorption decrease due to the release of *O*-(4-nitrobenzoyl) hydroxylamine at 300 and 325 nm[16]. 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 the reactions. Parameters k_{cat} and k_m were calculated by fitting initial rates to a hyperbola using nonlinear regression programs running at a VICTOR PC-compatible computer.

Residual activity of DP IV after preincubation (methods 1 and 2, see results) 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 test tube.

Decrease in 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 below. The inactivation reaction was monitored until a complete halt but usually not longer than two hours.

For direct monitoring of progress curves of DP IV-inactivation (method 3, RESULTS) pseudosubstrate concentrations between 0.45 and 0.49 mM and enzyme concentrations of 0.5–2.0 nM were used. The decrease of absorption due to the DP IV catalyzed release of *O*-(4-nitrobenzoyl) hydroxylamine was recorded and the resulting progress curve analyzed by fitting data to a function $A = B \cdot t + C \cdot e^{-k \cdot t}$.

Enzyme concentration and activity

Dipeptidyl peptidase IV was purified according to Wolf *et al.* using a slightly modified procedure. The activity of DP IV has been determined with Gly-Pro-4-nitroanilide and Ala-Pro-4-nitroanilide in 40 mM sodium phosphate buffer with an ionic strength of 0.125. Protein concentration was estimated according to Lowry[18] and spectrophotometrically by use of $A^{1\%}_{280\,nM} = 19$. Specific activity of the DP IV was in the range of 35 to 45 U/mg. The k_{cat}-values given in the text have been standardized and calculated assuming a maximal activity of 55 U/mg against Gly-Pro-4-nitroanilide (at 30°C, pH 7.6, ionic strength of 0.125) and a molecular weight of 115000 per subunit of the enzyme.

RESULTS

Inactivation studies

Incubation of DP IV with diacyl hydroxylamines containing substrate analog peptide moities induces two parallel occurring reactions: DP IV catalyzed hydrolysis of the pseudosubstrate and inactivation of the enzyme[13,16]. This branched pathway of interaction between enzyme and ligand is characteristic for "suicide-" or mechanism-based inactivation, where latent chemical reactivity of a suicide substrate becomes activated by the target enzyme upon binding and/or catalytic action[19].

More complex kinetic analysis including estimation of activation parameters and examination of isotope effects to investigate the reaction mechanism seemed somewhat difficult since due to enzymic hydrolyis of the inhibitor the inactivation reaction does not follow simple pseudo-first-order kinetics and during the time course of inactivation cumbersome progress curves can be observed[20].

Considering the kinetic procedures proposed by several authors[20-25] we applied the following three methods to obtain k_{inact}-values:

1. Kinetic parameters of enzyme catalyzed hydrolysis k_{cat} and K_m were estimated as outlined in METHODS. Inactivation of the enzyme was monitored until the reaction ceased. Under conditions of complete consumption of pseudosubstrate but still residual enzyme activity k_{inact} can be calculated from $r = k_{cat}/k_{inact}$[20]. Ratio r is also

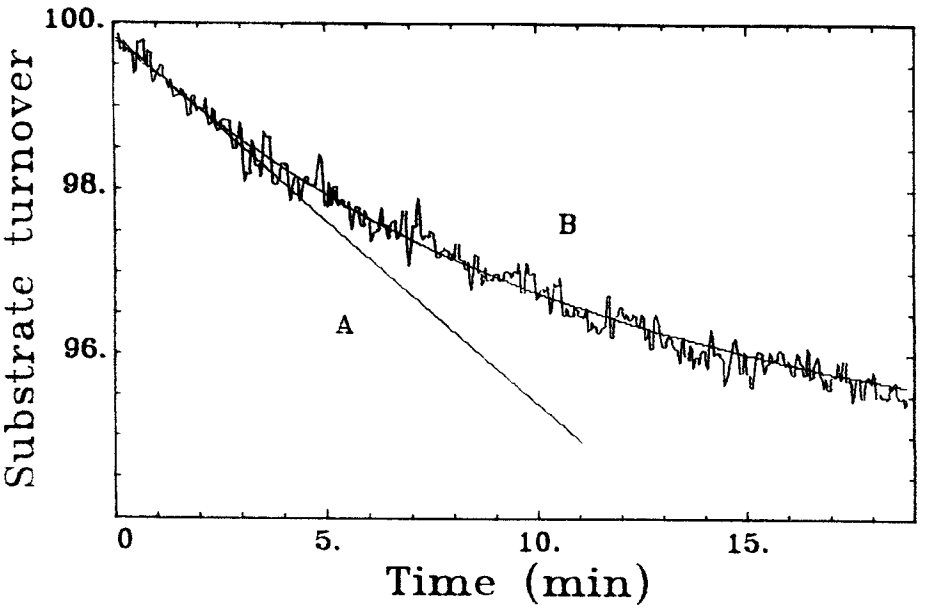


FIGURE 1 Progress curve of DP IV-catalyzed hydrolysis of Phe-Pro-NHO-Bz(4-NO_2), by measurement of absorbance decrease at 325 nm. Measurements carried out at 30°C, pH 7.6, in 40 mM sodium phosphate buffer with ionic strength 0.125, maintained with potassium chloride DP IV = 0.34 nM, pseudosubstrate = 0.5 mM.

Analysis of original data according to method 3. $S_o \gg K_m$.

(A) initial rate estimation, used to evaluate enzyme catalyzed hydrolysis

(B) approximation obtained by analysing data applying a first plus zero order rate equation

TABLE II
Investigation of suicide inactivation of DP IV with diacyl hydroxylamines using different monitoring methods

Compound	Hydrolysis		Method 1		Method 2		Method 3
	k_m	k_{cat}	I_o	k_{inact}	K_i	k_{inact}	k_{inact}
	μM	s^{-1}	μM	s^{-1}	μM	s^{-1}	s^{-1}
1	40	116	50	$3.8 \cdot 10^{-4}$	33	$7.4 \cdot 10^{-4}$	$4.8 \cdot 10^{-4}$
2	18	74.5	44	$11.1 \cdot 10^{-4}$	30	$6.2 \cdot 10^{-4}$	$10.7 \cdot 10^{-4}$
3	40	55	26	$36.7 \cdot 10^{-4}$	not estimated		$14.1 \cdot 10^{-4}$
4	68	43	26	$30.1 \cdot 10^{-4}$	160	$21.7 \cdot 10^{-4}$	$26.0 \cdot 10^{-4}$

represented by the ratio of the amount of pseudosubstrate converted to products to the amount acting as inactivator i.e. $r = [P]/[I]$. Assuming equimolar reaction between enzyme and inhibitor, [P] may be set equal to $[S_o]$-$[E_{inact}]$ and $[I] = [E_{inact}]$. With the resulting parameter r and the previously estimated k_{cat}-values, k_{inact} may be calculated.

2. Inactivation parameters were ascertained according to Kitz and Wilson[24] by estimation of k_{obs}-values at different pseudosubstrate concentrations. This is only possible using an enzyme concentration at a level where semilogarithmic plots of residual activity versus time appear linear over a suitable time interval. By fitting k_{obs}-values evaluated at different pseudosubstrate concentrations to a hyperbola, inactivation parameters K_i and k_{inact} could be obtained.

3. For different estimation of k_{inact} the method of Tian and Tsou[25] was applied. Pseudosubstrate concentrations were chosen sufficiently high to saturate the enzyme which was used in catalytic amounts to keep turnover low i.e. $[S_o] \gg K_m \gg [E_o]$. Then the absorption change caused by enzyme induced release of *O*-(4-nitrobenzoyl) hydroxylamine was recorded. Under these conditions the initial pseudosubstrate concentration remains practically constant in a certain time interval (with normal substrates zero-order-kinetics would be recorded). But due to the simultaneously occurring inactivation reaction, deviation from linearity was observed. Figure 1 illustrates a progress curve analyzed on-line as explained in methods to give the initial rate of pseudosubstrate hydrolysis as well as the pseudo-first-order rate constant of inactivation.

Table II lists the results obtained with compounds 1-4. K_m and k_{cat}-values are taken from Demuth *et al*[16]. Despite differences between some values, they are considered

TABLE III
Activation Parameters of spontaneous degradation of diacyl hydroxylamines, DP IV-catalyzed hydrolysis and enzyme inactivation

Compound	spontaneous degradation		hydrolysis by DP IV		inactivation of DP IV	
	ΔH^*	ΔS^*	ΔH^*	ΔS^*	ΔH^*	ΔS^*
	$kJ\,mol^{-1}$	$J\,mol^{-1}\,K^{-1}$	$kJ\,mol^{-1}$	$J\,mol^{-1}\,K^{-1}$	$kJ\,mol^{-1}$	$J\,mol^{-1}\,K^{-1}$
1	114.0	43.4	–	–	–	–
2	113.4	38.5	39.2	−83.6	98.1	13.8
3	114.8	41.1	–	–	–	–
4	113.5	44.2	38.6	−90.4	92.3	8.0

equivalent in view of the diversity of estimation methods used. Therefore in the experiments described below the time and material saving third method has been applied.

Activation parameters

It has been argued earlier[4] that during DP IV-catalyzed hydrolysis of Xaa-Pro-Yaa derivatives deacylation of Xaa-Pro-DP IV is rate determining and rates of hydrolysis are not influenced remarkably by the structural or electronic nature of the Yaa leaving group. Accordingly, investigation of the temperature dependence of the DP IV-catalyzed hydrolysis of *N*-Ala-Pro-and *N*-Phe-Pro-*O*-(4-nitrobenzoyl) hydroxylamine (Table III, column 2) gave almost the same values as obtained with the substrates Ala-Pro-4-nitroanilide ($\Delta H^* = 38.6$ kJ/mol, $\Delta S^* = -82.7$ J/mol K) and Phe-Pro-4-nitroanilide ($\Delta H^* = 36.1$ kJ/mol, $\Delta S^* = -92.6$ J/mol K) in reference experiments (see Figure 2a for the Eyring-plots of the Phe-Pro derivatives). These values are within the typical range of activation parameters to be expected for hydrolytic reactions.

The activation parameters for enzyme inactivation (Table II, column 3) are completely different and resemble more those of the spontaneous decomposition (Table III, column 1). Similar values are reported by Lwowski[26] for the Lossen degradation of several *N*-acyl-*O*-benzoyl hydroxylamines (e.g. *N*,*O*-dibenzoylhydroxylamine: $\Delta H^* = 111$ kJ/mol, $\Delta S^* = 25$ J/mol K), consistent with unimolecular fission of the -NH-O- bond of the compounds (see Figure 2b for Eyring-plots obtained with *N*-Phe-Pro-*O*-(4-nitrobenzoyl) hydroxylamine).

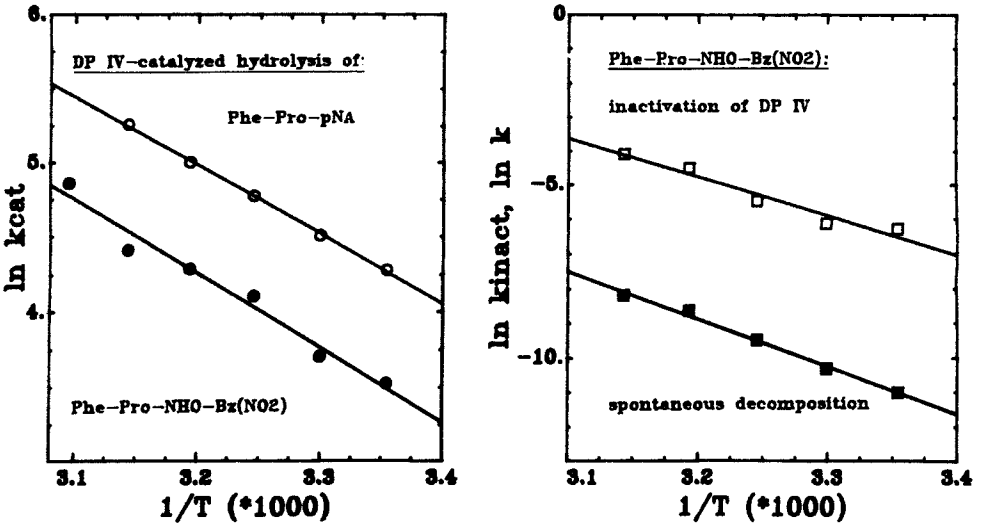


FIGURE 2 Eyring plots of temperature dependences of DP IV reactions with Phe-Pro derivatives
(a) hydrolysis of the Phe-Pro-4-nitroanilide
($r = 0.999$) and Phe-Pro-NHO-Bz(4-NO_2) ($r = 0.992$)
(b) inactivation of DP IV with Phe-Pro-NHO-Bz(4-NO_2) ($r = 0.975$) and nonenzymic decomposition of the compound ($r = 0.993$).
Measurements as in Figure 1 and under the conditions described in Methods, temperature range 25°C–50°C

Solvent isotope effects

Kinetic techniques of solvent isotope effect (SIE) analysis and "proton inventories" are used successfully to evaluate chemical and enzyme mechanisms in aqueous systems[17,28].

Whenever protons are transferred in rate-limiting steps of reaction pathways, solvent isotope effects (SIE) are expected to occur. The magnitude of the overall effect and the shape of the "proton inventory" (rate measurements in mixtures of protium and deuterium oxides) may give detailed information on the nature of the catalytic effects and the number of protonic sites involved, respectively[28,29]. Studies on solvent isotope effects and proton inventories of DP IV-catalyzed hydrolysis of 4-nitroanilide-substrates have been performed previously[5].

For the DP IV-catalyzed hydrolysis of *N*-Ala-Pro-*O*-(4-nitrobenzoyl) hydroxylamine at pL = 7.6 and 30°C we estimated a SIE of $k_{cat(H)}/k_{cat(D)} = 2.54 \pm 0.12$ (n = 5) which is equal to the value of 2.5 obtained for the DP IV-catalyzed hydrolysis of Ala-Pro-4-nitroanilide[5,29]. Additionally, the linear proton inventory (Figure 3a) corresponds to one proton catalysis consistent with rate-limiting hydrolysis of the acyl enzyme.

In accordance with a possible unimolecular fragmentation mechanism during decomposition of diacylhydroxylamines in aqueous solutions no solvent isotope effect was expected since rate-limiting transition state formation or breakdown occurs without appreciable proton transfer, bridging or change of solvation. The value we found for the decomposition of *N*-Ala-Pro-*O*-(4-nitrobenzoyl) hydroxylamine at pL = 7.6 and 30°C was $k_{(H)}/k_{(D)} = 1.015 \pm 0.02$ (n = 5).

Analysis of the inactivation of DP IV with pseudosubstrate gives a completely

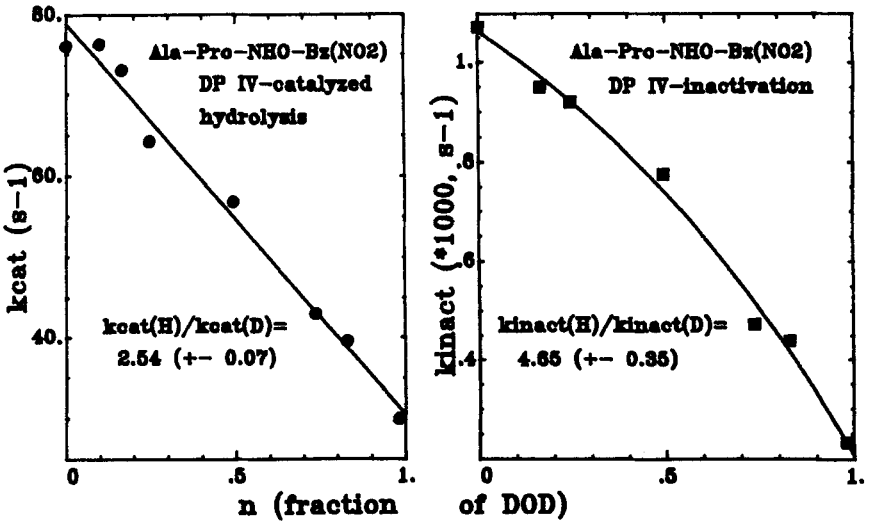


FIGURE 3 Proton inventory plots

(a) DP IV catalyzed hydrolysis of Ala-Pro-NHO-Bz(4-NO_2) in 40 mM sodium phosphate buffer, pL (lyonium concentration) = 7.6, 30°C, I = 0.125. For preparation of buffers see METHODS; adjustment of pL, buffer concentration according to Schowen[17]. Best fit of data to a straight line – one proton catalysis.

(b) DP IV inactivation by Ala-Pro-NHO-Bz(4-NO_2) under same conditions as 3 (a). Best fit of data to a third order degree polynomial – multiproton transition.

different situation where, in contrast to the decomposition, a high ratio of the pseudo-first-order rate constants in H_2O and D_2O could be detected: $k_{inact(H)}/k_{inact(D)} = 4.65 \pm 0.35$ (n = 5). Here, proton transfer reactions in the rate determining process are indicated. Also, the "dome-shaped" proton inventory (Figure 3b) is characteristic of contributions from more than one reaction step or from opposing normal and inverse isotope effects.

DISCUSSION

In the preceding paper[16] we compared inactivation rate constants and rates of hydrolysis estimated from reactions occurring between DP IV and a series of *N*-Gly-Pro-*O*-benzoyl hydroxylamines with various substituents of the benzoyl group[15]. Correlation between electronegativity of the substituent was found only in the inactivation rates, but not in the k_{cat}-values of the enzyme-catalyzed hydrolysis. We concluded both processes proceed with different rate determining steps.

The correspondence of activation parameters and solvent isotope effects of the hydrolysis of Xaa-Pro-4-nitroanalides to those reported here for the *N*-Phe-Pro- and *N*-Ala-Pro-*O*-(4-nitrobenzoyl) hydroxylamines provides further evidence for deacylation of Xaa-Pro-DP IV as the rate-limiting step in substrate hydrolysis.

Somewhat more complicated is the situation where spontaneous degradation of diacyl hydroxylamines and enzyme activation is compared. We believe that for both reactions the same reaction mechanism is responsible. The free energy of activation and the SIE of roughly 115kJ/mol and 1.0, respectively for the decomposition of the compounds are consistent with results characteristic for unimolecular reaction initiation in the Lossen reaction[26].

In a preliminary report[27] we have demonstrated that during decomposition of some diacyl hydroxylamines in the presence of ^{18}O containing water, no ^{18}O has been incorporated into the leaving nitrobenzoic acid ion which shows that nucleophilic attack of water at the ester carbonyl is not occurring. Accordingly, the decomposition of *N*-Boc-Ala-Pro-*O*-(4-nitrobenzoyl) hydroxylamine, labeled with ^{15}N at the N-O nitrogen, exhibits a kinetic isotope effect of $k_{14}/k_{15} = 1.09$, suggesting rate determining N-O bond fission in the degradation.

The deduced mechanism includes a nitrene-generating α-elimination of nitrobenzoate and subsequent nucleophilic attack of water at the nitrogen of the acyl nitrene producing hydroxamic acid as initial product.

If α-elimination of nitrobenzoic acid is also the crucial step of inactivation (releasing a fast reacting acyl nitrene as inactivating agent within the active site) the similarity of the activation parameters estimated is plausible. The fact that the inactivation of DP IV by diacylhydroxylamines takes place about 2 orders of magnitude faster than their spontaneous degradation may then be interpreted as "enzyme-catalyzed decomposition".

Certainly, the transition states are stabilized differently within a hydrophobic enzymic active site compared to the solvent cage in the aqueous solution. However, since diacyl hydroxylamines exist as monoanions at neutral pH[16], nucleophilic attack by the enzyme serine-hydroxyl function at the amide carbonyl carbon should be hindered initially.

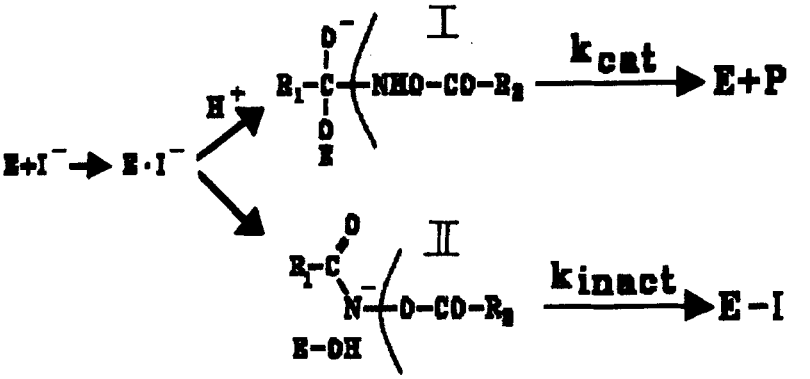
In contrast to uncharged "normal" substrate molecules forming an initial "non-charged" enzyme-substrate-complex during catalysis, the negatively charged –CO–

N^-–O–CO– linkage has to be held within the active site of the enzyme by proton bridges which possibly contribute to the isotope effect observed.

According to proton inventory theory, the overall SIE of 4.65 accompanied by a "dome-shaped" proton inventory may reflect single proton transfer in the transition state opposed by inverse contributions caused by conformational changes. Eventually, this behavior is connected to transition state stabilization by lowering the free energy of activation.

However, as soon as protonation of the scissile linkage has taken place, the substrate carbonyl carbon should be sufficiently electrophilic to form a covalent tetrahedral adduct with the attacking serine-hydroxyl residue of the enzyme.

Consequently, fast release of *O*-(4-nitrobenzoyl) hydroxylamine as first product during breakdown of the tetrahedral intermediate must prevent the -N-O-bond fission, which for its part hinders build up of the tetrahedral intermediate in the competing inactivation which releases nitrobenzoate.



Studying the inactivation of elastase and thermitase with diacyl hydroxylamines, in contrast to the DP IV-reactions discussed here, we can not detect enzyme-catalyzed release of substantial amounts of *O*-(4-nitrobenzoyl) hydroxylamine (paper in preparation).

Thus, we believe DP-IV possesses a catalytic apparatus able to protonate quickly the scissile hydroxylamine linkage within the noncovalent complex between enzyme and pseudosubstrate. This would explain its effectiveness in hydrolysing the inhibitors.

In conclusion we propose a mechanism locating the branching step for inactivation and hydrolysis prior to the formation of the first covalent intermediate within the catalytic sequence (Scheme).

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