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REACTION OF DIPEPTIDYLPEPTIDASE IV WITH SUBSTRATE-ANALOGOUS AZAPEPTIDES

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The reaction of dipeptidyl peptidase IV (EC 3.4.14.5.) with azapeptide substrates containing azaalanine or azaproline in the P_1-position was investigated. Accumulation of a fairly stable acyl-enzyme could be shown for ester substrates. Ala-AzaPro-pNA is a very poor substrate of DP IV and does not accumulate an acyl-enzyme. DP IV does not react with active-site titrants for trypsin-like serine proteases.

KEY WORDS: Dipeptidyl peptidase IV, azapeptides, azaproline, acyl-enzyme, deacylation.

ABBREVIATIONS: Ala, l-alanine; AzaAla, 1-methylcarbazate; Boc, tert.-butyloxy-carbonyl-; DP IV, dipeptidyl peptidase IV; DFP, diiso-propylfluorosphosphate; HEPES, *N*-(2-hydroxyethyl)piperazin-*N*-2-ethanesulfonic acid; LFE, linear free energy; MES, morpholino-ethanesulfonic acid; -OPh, phenylester; -ONp, 4-nitrophenylester; -pNA, 4-nitroanilide; Pro, l-proline; AzaPro, pyrazolidine-*N*-carboxylic acid; TLC, thin layer chromatography.

INTRODUCTION

Dipeptidyl peptidase IV (EC 3.4.14.5) is a dimeric membrane-bound exopeptidase with pronounced substrate specificity. The enzyme removes dipeptides from the *N*-terminus of polypeptides and proteins if the penultimate amino acid is proline or alanine. Because of this post-proline cleaving specificity DP IV is thought to be involved in a number of regulatory processes such as metabolism of neuropeptides,[1] immunoresponse[2,3] or cell proliferation.[4] Correlations have been found between plasma enzyme activity and several blood diseases and cancers.[5] Interestingly, no endogenous inhibitors have been found so far.[6] Many attempts have been made to clarify the catalytic properties of the enzyme and the molecular basis of its unusual substrate specificity.[7,8] It is generally accepted that DP IV is a serine protease because of its reactivity towards DFP. The results of LFE-studies with substrates containing alanine or proline in the P_1-position and differently substituted anilide leaving-groups support this view.[9] The independence of hydrolysis rate constants on the electronic nature of the leaving-group in the case of the proline substrates led to the assumption of the deacylation (k_3) being rate limiting, according to the general scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[P_1]{k_2} EA \xrightarrow{k_3} E + P_2$$

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where ES is the noncovalent recognition complex and EA represents the acylated enzyme. Formation of the acyl-enzyme EA (k_2) is thought to be rate-limiting with substrates containing alanine in the P_1-position.[9] A protonated N-terminus is also essential for catalysis.[10]

Several specific inhibitors for DP IV have been developed by derivation of known structures of simple dipeptidyl-amide substates such as *N*-dipeptidyl-*O*-aroyl hydroxylamines.[11,14] These compounds exhibit only moderate inhibitory potency, because most of the inhibitor was hydrolysed rapidly by the enzyme.[12] Enzyme inactivation occurs before a tetrahedral intermediate has formed.[1-3] Therefore it seems unlikely that more potent inhibitors for dipeptidyl peptidase IV will be obtained on the basis of diacyl hydroxylamines.

Powers and coworkers introduced azapeptides as substrate analogous reagents to generate stable, slowly hydrolyzing acyl-enzymes of some serine proteases.[15] Substitution of the α-carbon atom in an amino acid by nitrogen results in a hydrazine-like structure within the peptide backbone and gives compounds which retain the ability to acylate the target enzyme, but the rate of deacylation is decreased, as compared to normal peptide substrates. It has been shown that only activated azapeptide esters are reactive enough to yield acyl-enzymes.[17] If a chromogenic leaving group is used (nitrophenol), azapeptide esters are good activate-site titrants for a variety of serine proteases. Azapeptide esters are useful in preparation of stable and highly substrate-analog acyl-enzymes and have been used in NMR-investigations of active site properties of acyl-chymotrypsin.[18,19]

This paper is concerned with the synthesis, stability and inactivation properties of P_1-aza-substituted DP IV-substrates.

MATERIALS AND METHODS

Synthesis of Azapeptides

Amino acids were purchased from Reanal, Budapest, Hungary. Nitrophenyl-chloroformate, nitrophenylisocyanate, pyrazoline and methylhydrazine were obtained from Aldrich, Milwaukee, U.S.A., *N*-ethylmorpholine and isobutylchloroformate from Riedl de Haen, Hannover, FRG, and di-tert. butylcarbonate, HEPES and MES buffer substances were from Serva, Heidelberg, FRG. Sephadex LH 20 was supplied by Pharmacia, Uppsala, Sweden. All other chemicals were research grade from Laborchemie Apolda, GDR. Organic solvents were purified and dried by standard laboratory procedures.

Substrates glycyl-prolyl-4-nitroanilide and alanyl-prolyl-4-nitro-anilide were provided by Dr. K. Neubert, Halle.

Nitrophenyl guanidinobenzoate and amidinophenyl benzoate were gifts from Dr. J. Stürzebecher, Erfurt.

Melting points are uncorrected. Reaction control and product analysis were performed by TLC on silicia plates from Kavalier, Czechoslovakia. The following solvent systems were used: methanol/chloroform 9:1 (R_{f1}); benzene/acetone/acetic acid 27:10:0.5 (R_{f2}); *n*-butanol/acetic acid/ethylacetate/water 1:1:1:1 (R_{f3}); pyridine/n-butanol/ethylacetate/water 10:15:3:12 (R_{f4}). Spots were visualized by spraying with 0.2% ninhydrine or (for hydrazine compounds) $FeCl_3$/potassium hexacyanoferrate. Products were characterized either by ^{1}H-NMR-spectroscopy and elemental analysis or both.

Preparation of 1-benzyloxycarbonyl-1-methylhydrazine To a stirred solution of 1.38 g methylhydrazine in 22 ml chloroform were added 4.2 ml triethylamine and at −5°C, 4.25 ml benzyl-chloroformate. After stirring at room temperature for 3 h, the salt was filtered off and the organic layer was intensively washed with water. The solution was dried by addition of $MgSO_4$ and concentrated by rotary evaporation. The resulting oil was used in further synthesis. Yield: 3.2g = 62%. TLC: R_{f1} = 0.52, R_{f2} = 0.41, R_{f3} = 0.92, R_{f4} = 0.72. ^{1}H-NMR: δ, 3.07 ppm (s, 3 H), 5.107 ppm (s, 2 H), 7.34 ppm (m, 5 H)

Preparation of Boc-alanyl-azaalanyl-benzylester 3.31g Boc-alanine and 2.21 ml *N*-ethylmorphline were dissolved in 20 ml of tetrahydrofurane. The mixture was cooled to −15°C and 2.28 ml isobutylchloroformate were added slowly. After 5 min 3.13 g 1-benzyloxycarbonyl-1-methylhydrazine dissolved in tetrahydrofurane were added. After stirring overnight, water was added and tetrahydrofurane was removed *in vacuo*. The reaction mixture was redissolved in ethylacetate and washed with water, 5% $KHSO_4$ and saturated $NaHCO_3$ solution. The ethylacetate layer was dried and concentrated in a rotary evaporator. Addition of diisopropylether induced crystallization of the desired product. Yield: 2.9 *g* = 47%. Melting point: 100°C. TLC: R_{f1} = 0.55, R_{f2} = 0.44, R_{f3} = 0.92, R_{f4} = 0.81. Found: C 58.23 H 7.18 N 11.58, $C_{17}H_{25}O_5N_3$ requires: C 58.10 H 7.17 N 11.96%. ^{1}H-NMR: δ, 1.21 ppm (broad, 3 H), 1.42 ppm (s, 9 H), 3.14 ppm (s, 3 H) 4.05 ppm (broad, 1 H), 5.10 ppm (s, 2 H), 7.32 ppm (m, 5 H).

Preparation of Boc-alanyl-methylhydrazide 1 g of Boc-alanyl-azaalanyl-benzylester was disssolved in aqueous methanol and hydrogenated over 250 mg palladium-black for 3 h. The catalyst was removed and the filtrate was evaporated. After addition of hexane a solid product was obtained and could be recrystallized from ethylacetate/hexane. Yield: 0.4 *g* = 65%. Melting point: 83°C. TLC: R_{f1} = 0.27, R_{f2} = 0.11, R_{f3} = 0.60, R_{f4} = 0.67. ^{1}H-NMR: δ, 1.28 ppm (d, 3 H), 1.47 ppm (s, 9 H), 2.55 ppm (s, 3 H), 4.02 ppm (q, 1 H).

Preparation of alanyl-azaalanyl-nitrophenylesterhydrochloride 284 mg nitrophenylchloroformate were dissolved in 5 ml tetrahydrofurane and cooled to −10°C. A mixture of 300 mg Boc-alanyl-methylhydrazide and 0.18 ml triethylamine in 5 ml tetrahydrofurane was slowly dropped into the reaction vessel. After stirring in the cold for 1 h and at room temperature for 2 h the precipitated salt was filtered off and the solution was concentrated. The resulting solid was redissolved in a small amount of ether, filtered once more and crystallized by addition of hexane. The product was taken up in 2 ml HCl/acetic acid (1.1 mol/l), allowed to stand at room temperature for 20 min and precipitated by addition of dry ether. Yield: = 62%. Melting point: 70°C. TLC: R_{f1} = 0.00, R_{f2} = 0.00, R_{f3} = 0.53, R_{f4} = 0.74. Found: C 41.50, H 5.10, N 15.57. $C_{11}H_{15}O_5N_5N_4Cl$ requires: C 41.45, H 4.74, N 17.58%. ^{1}H-NMR: 1.56 ppm (d, 3 H), 3.24 ppm (s, 3 H), 4.06 ppm (q, 1 H), 7.39 ppm (d, 2 H), 8.26 ppm (d, 2 H).

Preparation of 1-butyloxycarbonyl-2-benzyloxcarbonylhydrazide 51 g benzylchloroformate were added to a intensively stirred solution of 39.6 g carbazinic acid tert.-butylester and 13.5 g NaOH in 500 ml chloroform and 150 ml water. After stirring overnight the chloroform layer was separated, washed with water and 20% solution of citric acid, dried and evaporated. The resulting oil was dissolved in ether

and precipitated by addition of hexane. Yield: 66.2 *g* = 82.9%. Melting point: 75°C. TLC: R_{f1} = 0.53, R_{f2} = 0.56, R_{f3} = 0.81. Found C 58.67, H 6.89, N 10.56. $C_{13}H_{18}N_2O_4$ requires: C 58.63, H 6.81, N 10.52. ^{1}H-NMR: δ, 1.40 ppm (s, 9 H), 5.09 ppm (s, 2 H), 7.29 ppm (m, 5 H).

Preparation of benzyloxycarbonylpyrazolidine hydrochloride Sodium hydride (4.8 g) was dissolved under argon in 100 ml dimethylformamide and a solution of 1-butyloxycarbonyl-2-benzyloxycarbonylhydrazide (26.6 g) in 250 ml dimethylformamide was slowly added with stirring. After 30 min, 20.2 g 1,3-dibrompropane were added and the solution was stirred overnight. The solvent was removed *in vacuo*, the residue was taken up in ethylacetate and washed with water, 20% solution of citric acid and saturated $NaHCO_3$. The ethyl acetate layer was dried with $MgSO_4$ and the solution was concentrated to an oil, which did not crystallize. 400 ml of HCl/acetic acid (1.1 mol/l), were added and after standing at room temperature for 20 min acetic acid was removed *in vacuo*. The product was precipitated with ether. The crude product was recrystallized several times from isopropanol/diiso-propylether. Yield: 8.9 g = 40%. Melting point: 142°C. TLC: R_{f1} = 0.07, R_{f2} = 0.0, R_{f3} = 0.63, R_{f4} = 0.66. Found: C 54.99, H 11.34, N 6.38. $C_{11}H_{15}N_2O_2Cl$ requires: C 54.43, N 11.54, H 6.23%. ^{1}H-NMR: δ, 2.37 ppm (m, 2 H), 3.62 ppm (t, 2 H), 3.76 ppm (t, 2 H), 5.27 ppm (s, 2 H), 7.35 ppm (m, 5 H).

Preparation of Boc-glycyl-azaprolylbenzylester 2.1 g Boc-glycine and 1.52 ml *N*-ethylmorpholine were dissolved in 25 ml tetrahydrofurane and cooled to − 15°C. 1.56 ml isobutyl-chloroformate were dropped into the solution and after stirring for 6 min, 2.91 g benzyloxycarbonylpyrazolidinehydrochloride and one equivalent *N*-ethylmorpholine were added. The solution was stirred overnight, tetrahydrofurane was removed after addition of 10 ml water and the residue was taken up in ethylacetate and worked up as usual. Yield: 1.33 *g* = 42%. Melting point: 77°C. TLC: R_{f1} = 0.75, R_{f2} = 0.42 R_{f3} = 0.85 R_{f4} = 0.76. Found: C 59.45, H 7.1, N 12.05. $C_{18}H_{28}O_5N_3$ requires: C 59.23, H 7.2, N 11.53%.

Preparation of Boc-glycyl-pyrazolidine The procedure was the same as that described for Boc-alanyl-methylhydrazide. Yield: 80%. Melting point: 78°C. TLC: R_{f1} = 0.40, R_{f2} = 0.21, R_{f3} = 0.68, R_{f4} = 0.64. Found: C 52.57, H 8.52, N 19.26. $C_{10}H_{20}N_3O_3$requires: C 52.15, H 8.75, N 18.24%. ^{1}H-NMR: δ, 1.41 ppm (s, 9 H), 2.01 ppm (m, 2 H), 2.96 ppm (t, 2 H), 3.51 ppm (t, 2 H), 4.07 ppm (d, 2 H).

Preparation of glycyl-azaprolylnitrophenylesterhydrochloride The synthesis was accomplished via Boc-glycyl-azaprolylnitrophenylester in the same manner as for alanyl-azaalanylnitrophenylester-hydrochloride. The resulting compound was extremely moisture-sensitive and could not be fully characterized. Yield: 52%. Melting point: 122°C. TLC: R_{f1} = 0.00, R_{f2} = 0.00, R_{f3} = 0.72, R_{f4} = 0.68.

Preparation of Boc-alanyl-azaalanyl-4-nitroanilide 450 mg of Boc-alanyl-methylhydrazide were dissolved in 30 ml dry benzene at 340 mg nitrophenylisocyanate were added. After stirring at 50°C for 24 h the benzene was removed. The residue was taken up in methanol, chromatographed over a LH-20 column with chloroform/methanol (1 : 1) as solvent. Product fractions were collected, evaporated and the product was crystallized from ethylacetate/hexane. Yield: 305 mg = 38.6%. Melting point: 213°C. TLC: R_{f1} = 0.55, R_{f2} = 0.45, R_{f3} = 0.81, R_{f4} = 0.75.

Preparation of alanyl-azaalanyl-4-nitroanilidehydrochloride The butyloxycarbonyl-protecting group was removed with 1.1 *N* HCl/acetic acid at room temperature. The product was precipitated by addition of diethylether and washed several times with ether. Yield: 87%. Melting point: 162°C. TLC: $R_{f1} = 0.00$, $R_{f2} = 0.00$, $R_{f3} = 0.48$, $R_{f4} = 0.61$. For analytical data compare reference 32.

Enzyme Preparation

Dipeptidyl peptidase IV was prepared from porcine kidney according to published methods.[18] The specific activities of DP IV preparations used were 30–35 U/mg determined with glycyl-prolyl-4-nitroanilide as substrate. A molecular weight of 115,000 per subunit was used for calculation.

Kinetic Methods

A uv-vis spectrophotometer M 40 (Carl Zeiss Jena, Jena) combined with temperature-controlled cuvette holder and application card "reaction kinetics" was used throughout the experiments. Standard conditions were 40 mM buffer, ionic strength 0.125 and 30°C in 10 mm cells.

Spontaneous degradation was followed spectrometrically from 220 to 450 nm. Data at several wave lengths were analyzed as functions of time, and pseudo-first-order rate constants were calculated by using nonlinear regression programs. Standard errors of individual rate constants were less than 1%.

Determination of deacylation rate constants Preincubation: acetate buffer 0.04 M, pH 5.2 or MES-buffer 0.04 M, pH 6.0 total volume 2.6 ml. Enzyme concentration was 32 nM, azapeptide concentration was 0.5 mM for azaprolyl compounds and 1 mM for alanyl-azaalanyl-phenylester. Enzyme and azapeptide were incubated for 10 min at 30°C, before 0.1 ml were transferred into a reactivation cuvette.

Measurement of reactivation: A cuvette containing 2.5 ml buffer and 0.2 mM alanyl-prolyl-4-nitroanilide was allowed to reach thermal equilibrium within the spectrophotometer. The reaction was started by addition of 0.1 ml of the preincubated enzyme and was followed by 390 nm until complete reactivation of the preincubated (acyl-)enzyme was reached. Under the conditions used only 5% of the total substrate concentration was hydrolyzed. 100–300 data points were used to calculate first order rate constant and steady state-velocity by fitting the curves to the general equation $P = A + Bt + Cexp(-kt)$. First order rate constants were obtained with errors less than 4% of the individual run.

Determination of acylation-rate-constants Buffer (MES 0.04 M, pH 6.0), substrate (glycyl-prolyl-4-nitroanilide, 0.067–0.2 mM) and azapeptide (0.1–0.5 mM) were incubated for 5 min to reach thermal equilibrium, then the reaction was started with enzyme (final concentration: 4 nM). The release of 4-nitroaniline was followed and data were again analyzed by nonlinear regression methods.

RESULTS AND DISCUSSION

Synthesis of Azadipeptide-derivatives

The goal of the present studies was to synthesize azaalanine and azaproline-contain-

ing dipeptide esters as acylating reagents for DP IV. Azaalanyl-dipeptide esters were synthesized from methylhydrazine;[20] temporary protection of the methylated hydrogen by a urethane-protecting group and further coupling to an acylamino acid gave the best results. NMR-analysis confirmed that acylation reactions led to the correct isomers. After deprotection, the *N*-acylaminoacidhydrazides were acylated by the appropriate aryl-chloroformates and the N-terminal blocking group was removed. Typical examples for preparation are given under Materials and Methods.

Dutta and Morley[20] proposed a route for synthesis of azaprolyl compounds. Following their strategy we synthesized the pyrazolidine derivatives from tert.-butyloxycarbonylhydrazide by acylating with benzylchloroformate to give a diacyl hydrazine. The product was cyclisized with 1,3-dibrompropane in the presence of sodium hydride. Purification and characterization of the product were done after removing the Boc-protecting group. Further coupling gave acylamino acid-azaprolyl-benzylester by conventional methods. The low yields indicate a decreased basicity of azaamino acid esters in comparison to amino acid esters. Catalytic hydrogenation and acylation with arylchloroformates or nitrophenylisocyanate resulted in the protected aza-dipeptide derivatives. Phenylesters could be obtained as oils, while nitrophenyl-esters and 4-nitroanilides could be crystallized. Finally, the N-terminal protecting group was removed yielding the substrate analogous azapeptides. Unprotected aza-dipeptides are highly hygroscopic compounds.

Degradation of Azadipeptide Esters in Solution

Before starting kinetic experiments with DP IV, the stability of the compounds in buffer solutions was investigated. Contrary to our expectations, unprotected aza-dipeptide esters showed extreme instability in solutions with pH-values in the range of 5.0 to 7.6. We determined pseudo-first order rate constants for degradation at different conditions (Table 1). Variations of buffer concentration or use of other buffer substances gave similar results (data not shown). Some important conclusions may be drawn from these results.

1) Unprotected azaprolyl-containing dipeptide nitrophenylesters are too unstable for enzymatic investigations at physiological pH. Even at pH 4 the half-life for decay of Ala-AzaPro-ONp is only 4.8 min (not shown in Table 1), whereas at neutral or

TABLE I

Pseudo-first order rate constants of non-enzymatic degradation of azapeptide esters and dipeptide esters in buffer solution

Compound	Rate constant k_{obs} [s^{-1}]	
	pH 6.0	pH 7.6
Ala-AzaAla-OPh	0.0001	0.0045
Ala-AzaAla-ONp	0.0015	0.022
Ala-Ala-ONp	0.0035	0.013
Ala-AzaPro-OPh	0.0009	0.028
Ala-AzaPro-ONp	0.082	> 0.7
Ala-Pro-ONp	0.0023	0.002

Reactions were followed in 0.04 M sodium phosphate buffer, ionic strength 0.125 adjusted with KCl, temperature 30°C.

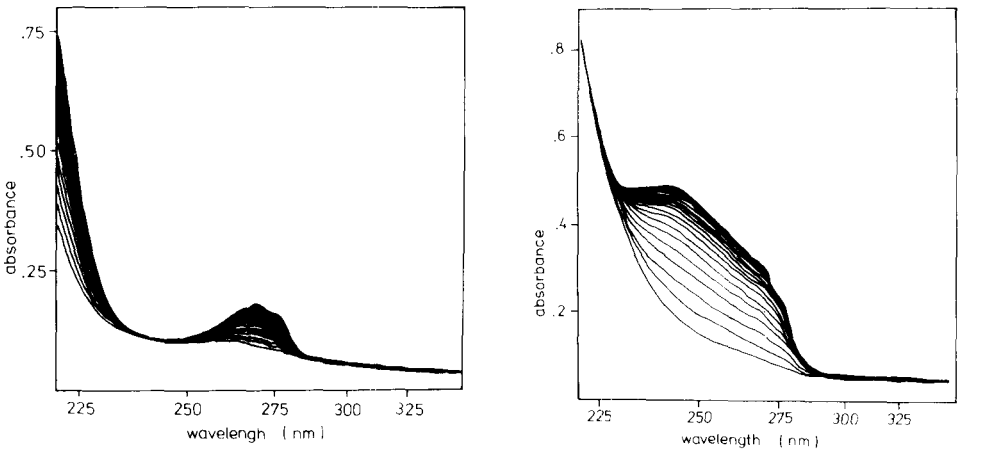

FIGURE 1 UV-spectra of non-enzymatic degradation of Ala-AzaAla-OPh, (left) followed to completeness, cycle time 120 s and UV-spectra of non-enzymatic degradation of Ala-AzaPro-OPh, (right) cycle time 80 s, pH 6.0.

slightly basic pH-values the half-life is in the range of 1s. The use of DP-IV specific azapeptides for active-site-titration is therefore impossible.

2) Substrate analogues containing the azaprolyl-residue degrade faster than the corresponding "normal" substrates (compare Ala-AzaPro-ONp and Ala-Pro-ONp in Table 1). This behaviour did not change during variations of pH or buffer conditions. The results of Gupton[21] with *N*-protected azaalanyl-nitrophenylesters indicate a decreased reactivity of the carbonyl group as the consequence of aza-substitution in P_1-position. Thus we assume intramolecular nucleophilic catalysis by the free N-terminal amino group to be responsible for the enhanced reactivity.

To gain further insight into the mechanism of degradation, the reaction was investigated by uv-spectroscopy. A characteristic difference between azaprolyl- and azaalanyl-containing compounds could be found (see spectra in Figure 1). Degradation of Ala-AzaAla-OPh exclusively yields phenol as absorbing product as detected at wave lengths greater than 220 nm. This is in agreement with the expected reaction mechanism being the hydrolysis of the ester bond leading to a linear dipeptide. In contrast, the decay of Ala-AzaPro-OPh in neutral or slightly acid solution yields an additional product adsorbing at 235–240 nm besides phenol (represented by shoulders at 269 and 278 nm). A similar spectrum is obtained after incubation of "normal", proline-containing dipeptide esters, but not at pH-values as low as 4.

It is known that unprotected proline-containing dipeptides give diketopiperazines as a result of intramolecular cyclization.[22] The absorbing product of azapeptide degradation should be the corresponding tetrahydrotriazin-3,6-dione.[20] Aza-substitution in the P_1-position seems to promote intramolecular cyclization, so that enhanced reactivity of the prolyl-derivatives in comparison to the results available from the literature can be explained by a different degradation mechanism.

Kinetic Analysis of Reactivation

Because of the high non-enzymatic reactivity of the azapeptides we selected the relatively stable phenylesters for further investigations. After addition of azapeptide

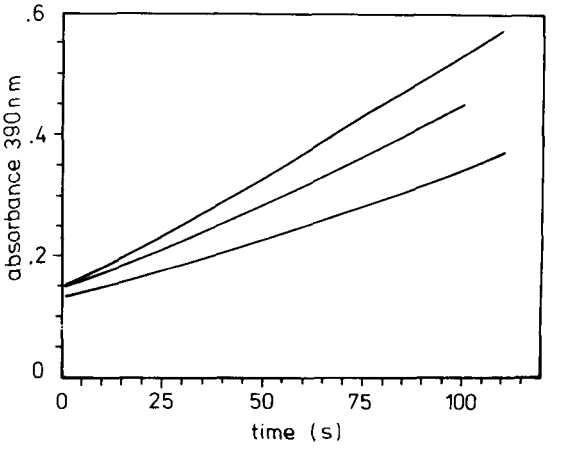

FIGURE 2 Reactivation of enzymatic activity after preincubation of DP IV with Ala-AzaPro-OPh in phosphate buffer pH 6.0 (bottom), pH 7.0 (middle) and pH 7.6 (top) containing 0.2 mM Ala-Pro-pNA at 30°C.

phenylesters to a DP IV-containing solution, its activity reaches zero within a short period of time, but reactivation is complete after a few minutes. The reactivation velocity was too fast to use the conventional kinetic technique of withdrawing aliquots from an incubation mixture and determining the concentration of the residual enzyme activity in a spectrophotometric test system with chromogenic substrate. Moreover, isolation of the inactiviated enzyme was impossible because of its instability.

Therefore a kinetic procedure had to be used, in which reactivation was continously followed in the presence of a substrate within the cuvette of a spectrophotometer. Since the amount of free (deacylated) enzyme is proportional to the slope of the progress-curve at each time, after complete reactivation the substrate turnover will reach the steady-state and correspond to the initial enzyme activity (see Figure 2). Substrate concentration was not allowed to change by more than 5% during the course of the experiment. The kinetic model for this procedure is:

$$S + EI \underset{l}{\overset{k}{\rightarrow}} S + E \overset{K_M}{\rightleftharpoons} ES \overset{k_{cat}}{\rightarrow} E + P$$

Where E is active enzyme, EI and ES are inactivated enzyme and enzyme-substrate complex, respectively. Generation of E from EI follows a pseudo-first order rate law:

$$[E] = [EI_o] \times (1 - \exp(-k \times t)) \quad (1)$$

Substitution into ordinary Michaelis-Menten Equation yields:

$$\frac{dP}{dt} = \frac{k_{cat} \times \{[EI_o] \times (1 - \exp(-k \times t))\} \times [S]}{K_M + [S]} \quad (2)$$

Integration of (2) yields:

$$[P] = v_s \times t + \frac{v_s \times (\exp(-k \times t))}{k} - \frac{v_s}{k} \quad (3)$$

TABLE II
First order rate constants of reactivation of DP IV, inactivated by Ala-AzaPro-OPh, in the presence of different inhibitor concentrations and substrate Gly-Pro-pNA (0.2 mM) at 30°C

Concentration of Ala-AzaPro-OPh, [M]	First order rate constant [s^{-1}]
9.6×10^{-6}	0.0212
1.9×10^{-5}	0.0128
3.8×10^{-5}	0.0094

For methods of determination see Materials And Methods. Reactivation was followed in 0.04 M sodium phosphate buffer, pH 7.6, ionic strength 0.125 (adjusted with KCl), at 30°C.

TABLE III
First order rate constant for reactivation of acyl-DP IV with Ala-AzaPro-OPh concentration 0.019 mM, in the presence of different substrate concentrations

Concentration of Ala-Pro-pNA. [M]	First order rate constant [s^{-1}]
2×10^{-5}	0.0148
5×10^{-5}	0.0215
1×10^{-4}	0.0315
2×10^{-4}	0.0367

Reactivation was followed in sodium phosphate buffer 0.04 M, ionic strength 0.125 (adjusted with KCl) at 30°C. Determination of rate constants is described under Materials And Methods.

where v_s = steady-state velocity =

$$\frac{k_{cat} \times [EI_o] \times [S]}{K_M + [S]}$$

Expressions like (3) can easily be solved with the help of non-linear fitting programs, developed for analysis of slow-binding inhibition or "burst"-kinetics.[23,24]

Since the inactivated enzyme is still contaminated by the inhibitor, substrate and inhibitor will compete for the free enzyme. Thus, the amount of free enzyme is lowered due to re-inhibition. Therefore the active-enzyme concentration is underestimated on each occasion and the calculated first-order rate constant will be too small. Table II shows the effect of increasing amounts of Ala-AzaPro-OPh in the reactivation mixture. Thus, the re-inhibition by a small amount of inhibitor may be depressed either by high substrate concentrations or the choice of substrates with a low Michaelis constant resulting in complete saturation of the enzyme with substrate. Table III shows that a concentration of the substrate Ala-Pro-pNA in the range of $20\,K_M$ (K_M = 0.01 mM) is high enough to avoid any re-inhibition. The experimental design described here allows determination of reactivation rate constants up to 0.05 s^{-1} (i.e. half-lives smaller than 0.5 min). Reactivation reactions can be followed without isolating the inactive species from excess inactivator.

Reaction of Dipeptidyl Peptidase IV with Azapeptide Substrates

The observed protection of the enzyme from azapeptide with substrate underlines the active-site directed mode of interaction of the substrate-analogous inhibitors. We

TABLE IV
First order rate constants for the reactivation of azapeptidyl-DP IV

Conditions	Azapeptide used for acylation		
	Ala-AzaPro-OPh	Gly-AzaPro-OPh	Ala-AzaAla-OPh
Phosphate buffer pH 7.6	0.0367	0.0397	0.0038
HEPES buffer pH 7.6	0.0395	0.0389	n.d.
Phosphate buffer pH 7.0	0.0311	0.0258	0.0019
Phosphate buffer pH 6.0	0.0236	0.0148	n.d.
MES buffer pH 6.0	0.0262	0.0207	n.d.

Reactions were followed as described under Materials and Methods at indicated buffer conditions and 30°C. Buffers were 0.04 M and ionic strength 0.125 (adjusted with KCl). n.d. = not determined. First-order rate constants are given in s^{-1}.

determined reactivation rate constants for different azapeptide derivatives under various pH and buffer conditions (Table IV). All compounds containing azaprolyl residues cause rapid reactivation, whereas the azaalanyl derivative reactivates one order of magnitude slower.

We attempted to gain additional information about the inactivation process. Unfortunately, the reaction products could not be quantitatively detected by uv-spectroscopy or thin layer chromatography because of the instability of compounds. Therefore we measured the inactivation rates indirectly by the addition of a chromogenic substrate into the mixture of enzyme and inhibitor. DP IV is inactivated in a time- and concentration-dependent manner. Progress curves of substrate turnover showed an exponentional decrease in the active enzyme concentration, followed by a linear release of 4-nitroaniline, indicating a low but constant concentration of active

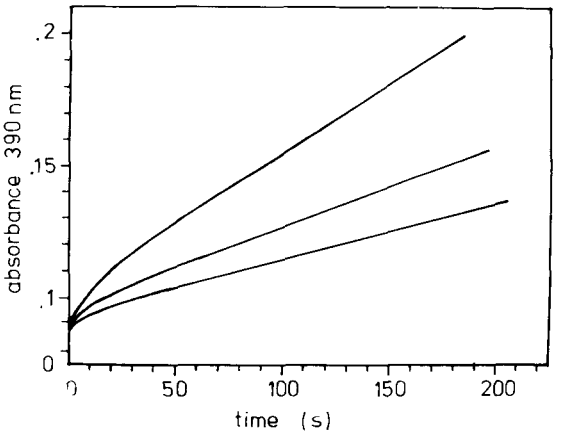

FIGURE 3 Inactivation of DP IV (4 nM) by Gly-AzaPro-OPh at 0.3 mM (bottom), 0.2mM (middle) and 0.1 mM (top) in 0.04 M MES-buffer, pH 6.0 containing 0.2 mM Gly-Pro-pNA as substrate at 30°C.

enzyme (see Figure 3). We determined first-order rate constants for inactivation at different substrate and inhibitor concentrations and were able to obtain second-order rate constants for inactivation at [S] = 0 by adopting the method of Tian and Tsou.[25] Linearity of plots of $1/k_{obs}$ vs. [S] justifies this approach. Rate constants for inactivation of DP IV by Gly-AzaPro-OPh and Ala-AzaAla-OPh are (609 ± 142) $M^{-1}s^{-1}$ and (154 ± 12) $M^{-1}s^{-1}$, respectively. Incubation of DP IV with decomposition products of the inhibitors did not result in any inhibition of the enzyme.

The results presented here indicate that dipeptidyl peptidase IV is in fact inactivated by formation of an acyl-enzyme intermediate during the reaction with substrate analogous azapeptides. Different reactivation properties of azaprolyl and azaalanyl compounds make an incorporation of the peptide portion into the acyl-enzyme very likely. The high deacylation rates of aminoacyl-azaprolyl-DP IV, as compared to results obtained with other serine proteases, can be discussed in steric terms of the pyrazolidine moiety (see below). In any case, these compounds will not lead to powerful inhibitors of dipeptidyl pepidase IV.

Reaction of Dipeptidyl Peptidase IV with Active-site-titrants

All DP IV substrates investigated so far, require a positively charged amino group at the N-terminus. In this respect, DP IV resembles the family of tryspin-like proteases, which do also need a protonated function for substrate recognition and catalysis. Therefore, we examined whether inhibitors developed for tryspin-like proteases will also inhibit with DP IV. We selected nitrophenyl guanidinobenzoate (NPGB) and amidinophenyl benzoate (APB) for acylation studies. Both substances are known to give stable acyl-enzymes with all trypsin-like proteases.[26,27] NPGB is also described as inactivating dipeptidyl peptidase II (EC 3.4.14.2.), an enzyme with properties quite similar to those of DP IV.[28,29] DP IV is neither inactivated by these compounds nor were any hydrolysis products detectable even at very high enzyme concentrations and at prolonged reaction times. Obviously, the compounds do not fulfil the requirements for DP IV substrates, although they bear a protonated group and an easy-to-split ester function.

In order to improve the substrate properties of the desired acylation reagents, we synthesized substrate-derived benzoic acid esters, containing a dipeptide structure with protonated amino function and a proline-like residue in the P_1-position. Both substances, *N*-glycyl-*N*-nitrobenzoylpyrazolidide and *N*-glycyl-*O*-benzoylprolinol can be regarded as inverse substrates.[30]

The compounds did not acylate the enzyme. They could only be shown to be weak competitive inhibitors with inhibition constants, K_i around 1 mM.

In summary, none of our attempts to produce benzoyl-DP IV has been successful. This should be interpreted in terms of high substrate specificity. Recently, Heins and coworkers showed in an extended study of DP IV substrate specificity that relatively slight changes in substrate structure lead to drastic reduction of enzymatic activity.[31]

Reaction of DP IV with Azapeptide-4-nitroanilides

In contrast to the instability of *N*-unprotected azadipeptide esters, the anilides are much more stable than their peptide analogues. This strengthens our theory for an intramolecular decay mechanism in buffer solution. In general, azapeptide anilides are very poor substrates of serine proteases. This is also true in the case of DP IV.

Ala-AzaAla-pNA was not cleaved at all, enzyme-catalyzed hydrolysis of Ala-AzaPro-pNA occurred very slowly. Up to a substrate concentration of 2.5 mM we were not able to find any saturation behaviour and no accumulation of acyl-enzyme was detected. The second-order rate constant of enzyme-catalyzed hydrolysis of $334\,M^{-1}s^{-1}$ is about 20,000 times smaller than the constant obtained with Ala-Pro-pNA. The pH-dependence of the second order rate constant resembles those for acylation of DP IV by "normal" substrates. A pK_a of 7.1 was calculated.[32] Summarising, these results indicate a rate-limiting acylation for azapeptide substrates in contrast to a rate-limiting deacylation step for hydrolysis of α-carbon substrates.

Discussion of Azapeptide Structure

A discussion of the above results must explain the fact that deacylation of acyl-DP IV cannot efficiently be slowed down by an aza-substitution at the P_1-position. This is in sharp contrast to the results obtained with other serine proteases (see Table V). Aza-substitution has been shown to be most successful with chymotrypsin- and trypsin-like enzymes, whereas eleastases and subtilisins exhibited higher deacylation rates. This is most likely explained by the ability of chymotrypsin, cathepsin G and trypsin to maintain strong interactions with the side chain of the amino acid in P_1-position. The aza-substitution will certainly change the geometry of the α-carbon atom from tetrahedral to planar. If the P_1-amino acid is held more rigidly due to the strong binding of the side chain, structural changes will lead to an unfavourable orientation of the carbonyl group within the active centre of the enzyme. Attack by a water molecule activated by His 57 (chymotrypsinogen numbering) is more difficult and deacylation is slowed. But, if the enzyme is not able to bind the side chain so strongly, the steric effect of aza-substitution is decreased.[21]

Additionally, the hydrazine-like structure of the peptide backbone will cause further structural changes. Due to the strong interaction of the nitrogen atom's lone pair electrons, conformations with the lone pairs perpendicular to each other are electronically favoured.[34]. If not otherwise constrained, the two nearly planar, peptide bond-like structures will twist up to an angle of nearly 90° to minimize electron orbital interactions. Therefore backbone conformation at the P_2-P_1-amide bond is drastically changed. Especially in the case of dipeptidyl peptidase IV, where conformation of the P_2-residue may be critical for substrate recognition and catalysis, these structural alterations can lead to poorly active compounds. We believe this to be the reason for the drastically reduced acylation rates of the azapeptide-4-nitroanilides.

Considering the deacylation reaction, the rate found for Ala-AzaAla-derivatives is

TABLE V
Reactivation rate constants of azapeptidyl-serine proteases

Protease	Azapeptidyl moiety	Deacylation rate [s^{-1}]
Trypsin[36]	EtOC-AzaOrn-	$< 1 \times 10^{-4}$
Chymotrypsin[33]	Ac-AzaPhe-	1.8×10^{-4}
Substilisin BPN[33]	Ac-Ala-AzaLeu-	25×10^{-4}
HL Elastase[21]	Ac-Ala-Ala-AzaAla-	32×10^{-4}
DP IV	Ala-AzaAla-	$10 \quad 10^{-4}$
DP IV	Ala-AzaPro-	260×10^{-4}

Conditions: trypsin pH 7.6, 24°C; chymotrypsin, subtilisin BPN and HL elastase pH 6.0, 25°C; DP IV pH 6.0, 30°C.

comparable to those of other serine proteases with a small hydrophobic amino acid side chain in P_1-position of the substrate (see Table V). But there is no explanation for the unexpectedly high reactivation rates of acyl-DP IV containing azaproline within the limits of the above structural considerations. To explain the relatively high deacylation rates (3 orders of magnitude reduced compared to conventional substrates, chymotrypsin shows a reduction 5–6 orders of magnitude) it has to be concluded that the structure of the azapeptidyl enzyme cannot so greatly differ from the "normal" acyl-enzyme structure. This may be due to the cyclic structure of azaproline. Since here the nitrogen atoms of *N,N*-diacylhydrazine are linked by an aliphatic bridge, the nitrogen atom lone pair electrons are forced to assume angles far from 90° and the significant lone pair-lone pair interaction results in substantially pyramidal acylated nitrogen atoms.[35] The attached acyl groups are now located in exo-and endo-position above and below the plane of the ring. Im fact, the structure at the α-nitrogen atom of azaproline closely resembles the structure of a conventional amino acid, making azaproline an unsuited amino acid analogue to efficiently decrease deacylation velocity.

SUMMARY

A series of azaanalogues of substrates for dipeptidyl peptidase IV has been prepared and their stability and ability to form a stablized acylenzyme with DP IV were investigated. Compounds containing azaproline showed a rapid decay in buffered solution, interpreted here as due to intramolecular cyclization. Azaanalogues of DP IV substrates did form stable acyl-enzymes, but they were not as effective as has been described for other azapeptide-serine protease systems. A possible explanation would be the inability of azaproline to distort substrate conformation within the active site to an extent preventing the deacylation effectively.

We did not succeed in acylating DP IV by the use of nonspecific or substrate-derived acylating reagents. Further work is needed to clarify whether this is due to the extraordinary high substrate specificity or by a generally different catalytic mechanism of DP IV as compared to other serine proteases.

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