Chapter 1

Detection of the Activity of the Ectopeptidases DPIV and APN Using Sensitive Fluorogenic Substrates

CARMEN MRESTANI-KLAUS*, SUSAN LOREY*, JÜRGEN FAUST*, FRANK BÜHLING#, and KLAUS NEUBERT*

**Department of Biochemistry/Biotechnology, Institute of Biochemistry, Martin-Luther-University Halle-Wittenberg, Kurt-Mothes-Str. 3,D-06120 Halle, Germany; #Institute of Immunology, Otto-von-Guericke-University Magdeburg, Leipziger Str. 44, D-39120 Magdeburg, Germany*

1. INTRODUCTION

Dipeptidyl peptidase IV (DPIV, DPPIV, EC 3.4.14.5) is a membrane-bound serine exopeptidase that has been identified as the leukocyte antigen CD26.

DPIV was first isolated from rat liver by Hopsu-Havu *et al* (1966). It occurs ubiquitously in mammalian organs with a wide tissue expression in high density on epithelial cells of small intestine (Darmoul *et al* 1994), kidney proximal tubules (Kenny *et al* 1976), human placenta (Püschel *et al* 1982) and liver (Ikehara *et al* 1994), but also on the surface of immune cells (Lojda 1977, Schön *et al* 1990, Bühling *et al* 1994, 1995). Furthermore, this enzyme was extracted from microorganisms as *Flavobacterium meningosepticum* (Yoshimoto *et al* 1982), *Lactococcus lactis spp. lactis* (Zevaco *et al* 1990), *Streptococcus thermophilus* ACA-DC (Tsakalidou *et al* 1998), *Saccharomyces cerevisiae* (Bordallo *et al* 1984), *Porphyromonas gingivalis* (Kiyama *et al* 1998), *Aspergillus fumigatus* (Beauvais *et al* 1997), poison of honey bee (Kreil *et al* 1980) and from plants like *Papaver somniferum* (Stano *et al* 1997).

The molecular mass of DPIV is 110-150 kDa dependent on the organism and cell type (Wolf *et al* 1978, Ikehara *et al* 1994). The native enzyme

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consists of two identical subunits each containing an independent active site (Küllertz *et al* 1978). Besides DPIV also other enzymes displaying DPIV activity have been isolated and characterized. A soluble DPIV with a molecular mass of 175 kDa being identified in serum belongs to this group (Duke-Cohan *et al* 1995). The corresponding membrane-associated DPIV equivalent to the soluble form was described as DPPT-L on activated T cells (Duke-Cohan *et al* 1996). Jacotot *et al* (1996) and Blanco *et al* (1998) characterized a DPIV-β with DPIV activity exhibiting however a substantial lower molecular mass of 82 kDa. (Concerning other members of this group of enzymes cf. chapter 7 in this book by Abbot and Gorrell.)

DPIV was characterized as a multifunctional enzyme in mammalian cells. It plays an important role in processing proline-containing bioactive peptides and in the modulation/regulation of physiological activities of these peptides as could be shown for the DPIV-catalyzed hydrolysis of substance P, human neuropeptide Y, somatoliberin, endomorphin, glucagon-like peptide, glucose-dependent insulinotropic polypeptides, chemokines and other Xaa-Pro peptides, respectively (Mentlein 1999, De Meester *et al* 1999). Furthermore, the functionalization of inactive precursors of bioactive peptides as for instance melittin has been reported (Kreil *et al* 1980).

In the immune system, DPIV has been shown to be involved in activation and proliferation of immune cells, particularly T cells (Schön *et al* 1990, Fleischer 1994, Kähne *et al* 1999, cf. chapter 8 of this book by Kähne *et al*). Furthermore, DPIV has been reported to mediate the immunosuppressive effect of the HIV-1 Tat protein (Gutheil *et al* 1994). It was demonstrated that the N-terminal Xaa-Xaa-Pro sequence of this protein is important for DPIV inhibition and for suppression of CD26-dependent T cell growth (Wrenger *et al* 1996). Recently, the N-terminal part of the thromboxane A2 receptor could be identified as an endogenous inhibitory ligand of DPIV. It might modulate T cell activation via DPIV/CD26 inhibition (Wrenger *et al* 2000).

Aminopeptidase N (APN, EC 3.4.11.2) is a membrane-bound, zinc-dependent metallopeptidase. It turned out to be identical with the human cluster differentiation antigen CD13 expressed on the surface of myeloid progenitors, monocytes, granulocytes and myeloid leukemia cells (Look *et al* 1989). APN is a homodimer glycoprotein with a molecular mass of 280 kDa (Pfleiderer 1970, Plakidou-Dymock *et al* 1993). It possesses a single helical transmembrane region and only a short N-terminal cytoplasmic tail (Olsen *et al* 1988; see also chapter 2 of this book by Lendeckel *et al*).

Together with thermolysin, neutral endoprotease-24.11 and angiotensin-converting enzyme APN was integrated in the zinc-peptidase clan (MA) (Helene *et al* 1991, Rawlings *et al* 1995). The catalysis and the mode of zinc-binding of these enzymes take place via the amino acid sequence His-

Glu-Xaa-Xaa-His+Glu. APN is classified together with other exopeptidases to the M1 family (Rawlings *et al* 1995).

APN is widely distributed among species and tissues although it is of greatest abundance in brush border membranes of the kidney, mucosal cells of the small intestine and in the liver. It is also present in the lung (Funkhouser *et al* 1991) and is located on endothelial cells in blood vessels.

A detailed localization of this enzyme has been carried out in the brain because of its potential involvement in regulating the activity of certain neuropeptides, particularly of enkephalins (Matsas *et al* 1985, Lucius *et al* 1995, Xu *et al* 1995). APN also appears to play a role in various virus infections (Delmas *et al* 1992, Yaeger *et al* 1992, Söderberg *et al* 1993) as well as in the formation of tumor cell invasions (Saiki *et al* 1993, Fujii *et al* 1996).

2. SUBSTRATE SPECIFICITY

2.1 Dipeptidyl peptidase IV

The substrate specificity of dipeptidyl peptidase IV has been well characterized. It cleaves dipeptides from the N-terminus of oligo- and polypeptides with proline or, with less efficiency, alanine, pipecolic acid, dehydroproline and hydroxyproline in the penultimate position (P_1 position) (Heins *et al* 1988) or with proline-type residues modified in their ring sizes (Rahfeld *et al* 1991). In addition, Bongers *et al* (1992) found that DPIV from human placenta was also accepted by serine, valine, glycine and α-amino-n-butyric acid in P_1. Furthermore, DPIV from pig kidney can hydrolyze synthetic derivatives of bovine growth hormone-releasing factor (somatoliberine) with threonine in P_1 position (Martin *et al* 1993). Schutkowski (1991) showed that dipeptide-4-nitroanilides with glycine or alanine as well as their N-alkylated derivatives were also cleaved at P_1 position. S configuration of the amino acids in both P_1 and P_2 positions in the case of proline substrates (Heins *et al* 1984, 1988) and in P_1 position in the case of alanine substrates (Heins *et al* 1988), a free and protonated N-terminus as well as *trans* conformation of the peptide bond to be cleaved (Fischer *et al* 1983) are of particular importance for the enzymatic hydrolysis.

The P'_1 position accepts all amino acid residues, except secondary amines such as N-methylated amino acids, proline and hydroxyproline (Kenny *et al* 1976). Brandt *et al* (1995) attributed this effect to the loss of one hydrogen bond from the nitrogen of the amino acid in P'_1 position to the carbonyl oxygen of the P_2 amino acid.

In P_2 position DPIV tolerates any proteinogenic amino acid with a significant preference of aminoacyl residues bearing hydrophobic or branched side chains (Heins *et al* 1988). DPIV can hydrolyze substrates with phosphorylated amino acid residues in P_2 position (Kaspari *et al* 1996) as well as peptides containing one thioxoaminoacyl-prolylpeptide bond (Schutkowski *et al* 1994), but up to three orders of magnitude less efficiently compared to their non-phosphorylated and non-thioxylated analogues. An increase of the distance of the N-terminal amino group to the carbonyl group in P_2 leads to a significant reduction of the enzymatic hydrolysis (Heins *et al* 1988).

The chemokine RANTES(1-68) (regulated on activation normal T cell expressed and secreted) is one of the largest naturally occuring DPIV substrates described by now (Oravecz *et al* 1997, Proost *et al* 1998). Its N-terminal truncation generates peptides important during ant-inflammatory and antiviral responses (chp. 9 this book by De Meester *et al*)

2.2 Aminopeptidase N

APN has a broad substrate specificity removing N-terminal amino acids from almost all unsubstituted oligopeptides and from amid or arylamid. Alanine is the most favored residue to be cleaved. Bulky hydrophobic amino acid derivatives are good substrates but leucine-amide is poorly hydrolyzed. In the P'_1 position proline residues are not accepted by APN (Mentlein 1988). Peptides containing the aminoacyl residues glutamic acid, aspartic acid or proline in the P_1 position are poor substrates.

3. COMMON SUBSTRATES OF DPIV AND APN

A number of dipeptide substrates, such as p-nitroanilides (Nagatsu *et al* 1976, Fischer *et al* 1983), 6-aminoquinolinones (Brynes *et al* 1981), β-naphthylamides (Gossrau 1985), 4-methoxy-β-naphthylamides (Püschel *et al* 1982), 7-amido-4-methylcoumarins (Kojima *et al* 1979), 6-aminonaphthalenesulfonamides (Butenas *et al* 1997), and Ala-Pro-cresyl violet (Van Noorden *et al* 1997), all of them containing fluorophoric groups, have been used for characterization of isolated and cell-bound enzymatic activity.

For APN, assay of activity can be performed using amino acid 7-amido-4-methylcoumarins (McDonald *et al* 1986), 4-methoxy-β-naphthylamides (Miller *et al* 1979), α- as well as β- naphthylamides (Lojda 1979, McDonald *et al* 1986), p-nitroanilides (McDonald *et al* 1986, Gillespie *et al* 1992), and the tetrapeptide H-Gly-Leu-Gly-Gly-OH (Reisenauer *et al* 1985).

4. RHODAMINE 110 SUBSTRATES

4.1 (Xaa-Pro)$_2$-R110 substrates of DPIV

4.1.1 Synthesis and characterization

For sensitive detection of cellular enzymatic activity, the stable cellular association of the released fluorophor of the substrate hence preventing high background fluorescence is of special importance. Rhodamine 110 is a highly fluorescent xanthene dye displaying an excitation wavelength at 494 nm and a quantum yield of 0.91 (Leytus *et al* 1983a) (Fig. 1). It possesses two amino groups suitable for the coupling of protease substrates resulting in a colourless non-fluorescent compound. Hydrolysis of these substrates releases the highly fluorescent rhodamine 110 (Leytus *et al* 1983a). In previous investigations such type of substrates was used for the detection of enzymatic activities of lysosomal localized as well as isolated proteases (Leytus *et al* 1983b, Ulbricht *et al* 1995). In these studies, a stable cellular fluorescence was observed due to the intracellular hydrolysis of the substrates and the accumulation of the fluorescent cleaving products within the cells.

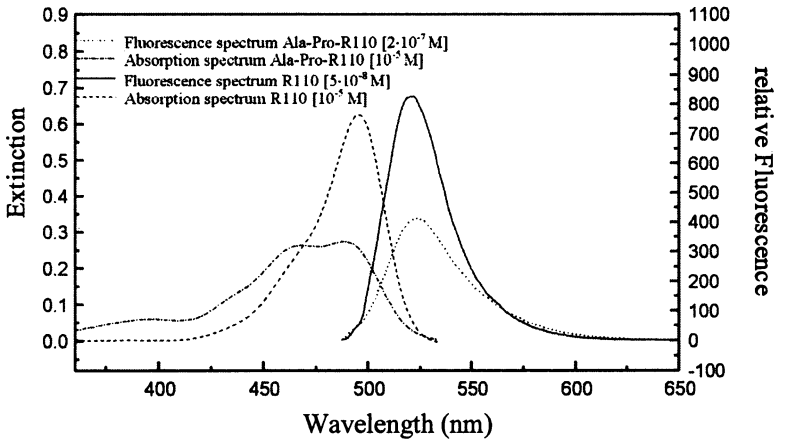

Figure 1: Absorption and emission spectra of R110 and Ala-Pro-R110. Conditions: 40 mM Tris/HCl buffer, I = 0.125 M, pH 7.6, 30 °C; fluorescence measurements: λ_{Ex} = 480 nm, $\lambda_{Exslit,Emslit}$ = 5 nm (Lorey 1999).

To obtain very sensitive fluorogenic substrates for the detection of DPIV on the surface of T cell lines we synthesized rhodamine 110-based bis-substituted substrates of the type $(Xaa\text{-}Pro)_2$-R110 with Xaa = Gly, Ala, Leu, Phe, Cha, Ser, Lys, Abu (Cha = cyclohexylalanine, Abu = α-aminobutyric acid) (Fig. 2). These compounds were synthesized using the mixed anhydride method by coupling Boc-Pro-OH (Boc = tert.-butyloxycarbonyl) with rhodamine 110 and subsequent extension with Boc-Xaa-OH. The truncation of the Boc-protecting group was achieved acidolytically. The corresponding mono-substituted analogues of the type Xaa-Pro-R110 necessary for the characterization of the enzyme-catalyzed substrate hydrolysis were obtained by coupling Boc-Xaa-Pro-OH and rhodamine 110 using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide. The protected compounds were purified by MPLC, the end products after deprotection of the Boc group were purified by RP-HPLC (Lorey *et al* 1997, 1998, 1999).

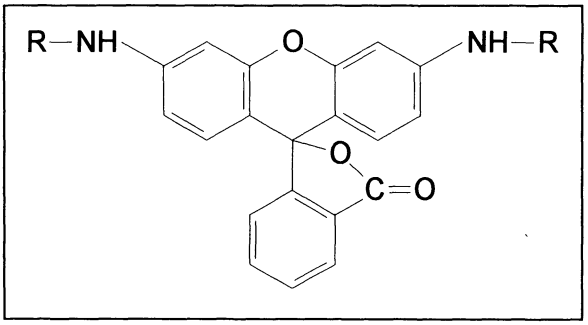

Figure 2: Structure of DPIV substrates with R = Xaa-Pro- and of APN substrates with R = Xaa-.

4.1.2 Enzymatic hydrolysis of $(Xaa\text{-}Pro)_2$-R110

The synthesized substrates of the types $(Xaa\text{-}Pro)_2$-R110 and Xaa-Pro-R110 differ in their size and hydrophobicity. Therefore, it was of interest to observe the kinetic character of their hydrolysis by isolated DPIV in comparison to the cleavage by cell surface DPIV.

The detection of the enzymatic hydrolysis is based on the DPIV catalyzed cleavage of the corresponding dipeptide Xaa-Pro from the colourless and non-fluorescent $(Xaa\text{-}Pro)_2$-R110 leading to the fluorescent Xaa-Pro-R110 derivative. After the enzymatic hydrolysis of the second Xaa-Pro residue the mono-substituted analogue is transformed to the 10fold higher fluorescent free rhodamine 110 (Fig. 1).

The kinetic experiments with isolated DPIV from pig kidney were performed according to Leytus *et al* (1983a) at an enzyme concentration where less than 5 % of the substrates are cleaved within the measuring time. Under these conditions, the mono-substituted analogues are formed almost exclusively after hydrolysis of the $(Xaa\text{-}Pro)_2$-R110 compounds.

All synthesized substrates except $(Phe\text{-}Pro)_2$-R110 are processed by DPIV exhibiting k_{cat}/K_m values in a range beween $1 \cdot 10^6\ M^{-1} \cdot s^{-1}$ and $5 \cdot 10^6\ M^{-1} \cdot s^{-1}$ (Table 1). Apart from $(Gly\text{-}Pro)_2$-R110 and Gly-Pro-R110, a graduation of the kinetic constants is observed in dependence on the size of the amino acid in P_2 position. $(Ala\text{-}Pro)_2$-R110 turned out to be the best substrate with a k_{cat}/K_m value of $4.30 \cdot 10^6\ M^{-1} \cdot s^{-1}$. Analogous to Heins *et al* (1988) concerning the enzymatic hydrolysis of dipeptide-p-nitroanilides, bis-(dipeptideamido)-rhodamine 110 derivatives were hydrolyzed favorably with hydrophobic aminoacyl residues in P_2. $(Lys\text{-}Pro)_2$-R110 and $(Gly\text{-}Pro)_2$-R110 were cleaved less efficiently as demonstrated by k_{cat}/K_m values of $1.48 \cdot 10^6\ M^{-1} \cdot s^{-1}$ and $1.19 \cdot 10^6\ M^{-1} \cdot s^{-1}$, respectively, exhibiting lower affinity to the active site of DPIV reflected by enhanced K_m values of these substrates.

According to Brandt *et al* (1995), the interaction of the aminoacyl side chains of inhibitory product-anologue amino acid pyrrolidides with the aromatic moiety of Trp^{628} of the active site of DPIV is of special importance for the efficiency of the enzyme inhibition. Therefore, the interaction of the amino acid in P_2 position with Trp^{628} seems to be essential for the substrate hydrolysis. Furthermore, Harada *et al* (1984) showed that the modification of the tryptophan residue leads to a decrease in DPIV activity. The interaction with Trp^{628} might be suppressed on the one hand due to the absence of the side chain in the case of $(Gly\text{-}Pro)_2$-R110 and on the other hand due to enhanced steric requirements in the case of $(Lys\text{-}Pro)_2$-R110.

The lowest K_m value was obtained for $(Cha\text{-}Pro)_2$-R110 indicating a high affinity of the substrate to the active site of DPIV. However, the k_{cat} value of this substrate is about one order of magnitude lower than the k_{cat} values of all other rhodamine 110 substrates investigated in this study.

Table 1. Kinetic Constants of the Hydrolysis of Xaa-Pro-R110 and $(Xaa\text{-}Pro)_2$-R110 by Isolated DPIV from Pig Kidney (Lorey 1999)

Compound	K_m [10^5 M]	K_{cat} [s^{-1}]	k_{cat}/K_m [$M^{-1} \cdot s^{-1}$]	K_i [10^4 M]	Kinetics
Ala-Pro-pNA*	1.14 ± 0.03	54.89 ± 4.17	$(4.81 \pm 0.39) \cdot 10^6$		MM
Ala-Pro-R110#	2.50 ± 0.11	72.39 ± 3.46	$(2.90 \pm 0.19) \cdot 10^6$		MM
Gly-Pro-R110*	4.59 ± 1.33	72.02 ± 3.46	$(1.57 \pm 0.47) \cdot 10^6$		MM
Phe-Pro-R110*	4.65 ± 0.58	54.72 ± 4.26	$(1.18 \pm 0.17) \cdot 10^6$		MM
$(Ala\text{-}Pro)_2$-R110	1.47 ± 0.19	63.19 ± 11.38	$(4.30 \pm 0.95) \cdot 10^6$		MM
$(Abu\text{-}Pro)_2$-R110	2.55 ± 0.07	84.84 ± 2.11	$(3.33 \pm 0.12) \cdot 10^6$	5.10 ± 1.00	SI
$(Leu\text{-}Pro)_2$-R110	2.42 ± 0.23	58.36 ± 1.45	$(2.41 \pm 0.24) \cdot 10^6$	4.69 ± 1.82	SI
$(Ser\text{-}Pro)_2$-R110	3.42 ± 1.09	67.73 ± 4.21	$(1.98 \pm 0.68) \cdot 10^6$		MM
$(Cha\text{-}Pro)_2$-R110*	0.40 ± 0.11	6.87 ± 0.15	$(1.72 \pm 0.49) \cdot 10^6$	1.35 ± 0.05	SI
$(Lys\text{-}Pro)_2$-R110	5.42 ± 1.22	80.12 ± 9.59	$(1.48 \pm 0.39) \cdot 10^6$		MM
$(Gly\text{-}Pro)_2$-R110	9.91 ± 1.57	118.15 ± 18.70	$(1.19 \pm 0.27) \cdot 10^6$		MM
$(Phe\text{-}Pro)_2$-R110#			no hydrolysis		

*Compounds available as hydrochlorides, # compounds available as trifluoroacetates. SI Substrate inhibition, MM Michaelis Menten kinetic. Conditions: 40 mM Tris/HCl buffer, I = 0.125 M, pH 7.6, 30°C, measurements over 120 s at 494 nm for R110, at 390 nm for pNA. DPIV was used between $6.85 \cdot 10^{-10}$ M and $2.34 \cdot 10^{-9}$ M for the hydrolysis of Xaa-Pro-R110 to R110, between $1.37 \cdot 10^{-10}$ M and $2.28 \cdot 10^{-10}$ M for the hydrolysis of $(Xaa\text{-}Pro)_2$-R110 to Xaa-Pro-R110 and at $4.56 \cdot 10^{-10}$ M for the hydrolysis of Ala-Pro-pNA.

Furthermore, the enzyme-catalyzed release of the fluorophors of Xaa-Pro-pNA and $(Xaa\text{-}Pro)_2$-R110 enabled the comparison of the detection sensitivity of the hydrolysis of both types of compounds by isolated DPIV from pig kidney. We found that the hydrolysis of the rhodamine substrates is about 4000fold more sensitive compared to the hydrolysis of the commonly used p-nitroanilides mainly because of the strongly fluorescent R110 leaving group (Lorey 1999).

$(Phe\text{-}Pro)_2$-R110 is not bound and not cleaved by DPIV. However, the corresponding non-aromatic analogue $(Cha\text{-}Pro)_2$-R110 turned out to be a DPIV substrate. Obviously, the loss of the aromatic ring system of the phenylalanyl side chain seems to be responsible for the substrate character, not the size of the molecule. Since $(Phe\text{-}Pro)_2$-R110 is not bound by DPIV it is assumed that an unfavorable conformation is formed being unaccessible for DPIV instead of an inhibition of the enzyme-substrate interaction. This assumption is underlined by the finding that the corresponding mono-(dipeptide amido)-rhodamine 110 analogue Phe-Pro-R110 is a DPIV substrate.

To gain more insight into structure-function relationships of the dipeptideamido rhodamines conformational studies were performed using NMR spectroscopy and molecular modelling (Mrestani-Klaus *et al* 1998). These studies revealed low-energy structures compatible with the NMR data of the bis-substituted derivatives that may adopt backbone conformations corresponding to the model of the recognition conformation of DPIV substrates proposed by Brandt *et al* (1995). The most stable conformations of $(Phe\text{-}Pro)_2$-R110 were characterized by sandwich-like interactions between the aromatic moieties of both phenylalanyl side chains (Fig. 3). All conformations without this type of interactions were about 6 kcal/mol higher in energy. In addition, the stability of these conformations was supported by results of molecular dynamics simulations showing that both phenyl rings keep in close contact to each other during the total simulation time of 100 ps at 300 K. In the case of the mono-substituted Phe-Pro-R110, the phenylalanyl side chain could adopt conformations that allow attractive interactions with Trp^{628} of the active site of DPIV being responsible for the substrate character of this analogue. Thus, sandwich-like interactions between the two aromatic rings of both Phe side chains are likely the main reason that $(Phe\text{-}Pro)_2$-R110 could not be recognized as a substrate by DPIV. Steric hindrances with Trp^{628} migh prevent binding to the active site of DPIV.

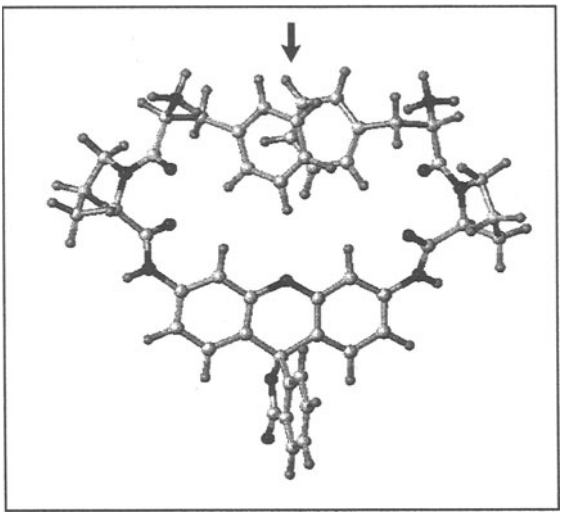

Figure 3: Low-energy structure of $(Phe\text{-}Pro)_2$-R110 obtained by molecular dynamics simulations.

Taken together, the kinetic constants of the rhodamine 110 substrates are comparable with those of the dipeptide-p-nitroanilides (Heins *et al* 1984, 1988). Hence, the size of the rhodamine 110 molecule seems to be of subordinate importance. This can be attributed to the fact that in the case of the hydrolysis of Xaa-Pro substrates the deacylation turns out to be the rate-determining step (Küllertz *et al* 1978).

The compounds $(Xaa\text{-}Pro)_2$-R110 with Xaa = Ala, Ser, Lys, Gly as well as all Xaa-Pro-R110 analogues are hydrolyzed by a classical Michaelis Menten kinetic. $(Abu\text{-}Pro)_2$-R110, $(Leu\text{-}Pro)_2$-R110 and $(Cha\text{-}Pro)_2$-R110 are cleaved according to the model of substrate inhibition (see Table 1), where a second substrate molecule interacts with the enzyme leading to a catalytically inactive SES complex.

The $(Xaa\text{-}Pro)_2$-R110 derivatives with Xaa = Gly, Ala, Abu, Leu, Ser, Cha were also hydrolyzed by soluble recombinant human DPIV (rh DPIV). Analogous to the hydrolysis by DPIV from pig kidney, $(Phe\text{-}Pro)_2$-R110 is not accepted as a substrate by rh DPIV. The k_{cat}/K_m values of the hydrolysis of the bis-(dipeptideamido)-rhodamines by rh DPIV are about two orders of magnitude lower compared to the hydrolysis of identical substrates by DPIV from pig kidney. This significant difference might be due to varied specific amino acid sequences of DPIV isolated from different species such as pig kidney and human kidney.

4.1.3 Detection of DPIV activity on the surface of DPIV-rich cells

For the synthesis of the substrates $(Xaa\text{-}Pro)_2$-R110 we assumed that compounds containing apolar amino acids in P_2 exhibit better penetration hence being particularly available for cytosolic DPIV. Compounds with polar amino acids in the P_2 position should penetrate the cell membrane less efficiently hence being favorably hydrolyzed by membrane-bound DPIV. Our aim was to find a possibility to differentiate between cytosolic and membrane-bound DPIV activities and to quantify them by localization of the fluorescence release. Of the compounds synthesized, $(Xaa\text{-}Pro)_2$-R110 with Xaa = Leu, Cha and Abu as unpolar analogues should penetrate the cell membrane better than those with Xaa = Gly, Ala and Ser. The mono-substituted derivatives as well as $(Lys\text{-}Pro)_2$-R110 could not be utilized for the measurements on cells due to their high net fluorescence and instability.

In our investigations we studied the hydrolysis of $(Xaa\text{-}Pro)_2$-R110 compounds by DPIV-rich U937 cells in comparison with the substrate cleavage by DPIV of U937 cell lysate as well as by isolated recombinant human DPIV (rh DPIV). As a result, all compounds are substrates of the cellular enzyme. However, significant differences in the fluorescence release were obtained beween the experiments with U937 cells, U937 cell lysate and

rh DPIV at identical substrate concentrations although the enzyme activities of cells, lysate and rh DPIV were standardized to an identical activity concerning the hydrolysis of Gly-Pro-pNA (Lorey 1999). For all $(Xaa\text{-}Pro)_2$-R110 substrates (Fig. 4A), except for $(Leu\text{-}Pro)_2$-R110 (Fig. 4B), a 5fold higher fluorescence release was found in the case of lysate and a 20fold higher amount in the case of rh DPIV compared to intact cells.

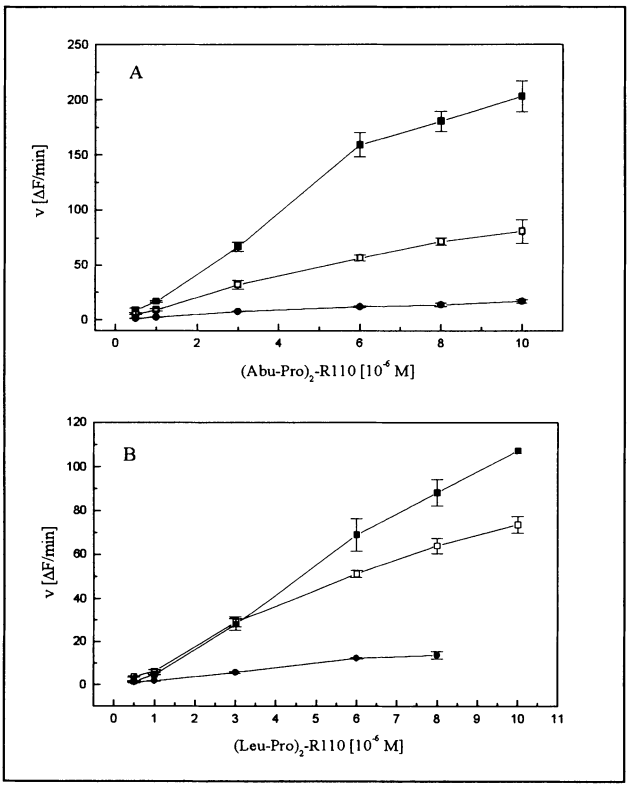

Figure 4: Hydrolysis of the substrates $(Xaa\text{-}Pro)_2$-R110 by DPIV of intact U937 cells (●), by DPIV of U937 lysate (□) and of isolated recombinant human DPIV (■); A: $(Abu\text{-}Pro)_2$-R110, B: $(Leu\text{-}Pro)_2$-R110. Conditions: PBS, pH = 7.2, incubation 5 min (rh DPIV), 20 min (U937 cell lysate), 30 min (U937 cells), 37 °C; U937 cells, U937 cell lysate and rh DPIV of an activity of 25 pkat/ml, fluorescence plate reader: λ_{Ex} = 488 nm, λ_{Em} = 525 nm, lamp voltage 1.8 V (Lorey 1999).

One reason for this effect may be the steric shielding of the active site of DPIV by neighbouring membrane-associated proteins. Therefore, the interaction of the considerably larger bis-(dipeptideamido)-R110 with cellular DPIV might be restricted due to the enhanced space requirements of

the rhodamine derivatives. In the lysate, where the cellular structures consist of fragments, the active site might be better available resulting in a faster enzyme-catalyzed hydrolysis. Similar effects should be responsible for the enhanced substrate hydrolysis of the bis-(dipeptideamido)-rhodamine 110 compounds by rh DPIV. An increase in fluoroescence due to a participation of intracellular enzyme in the substrate hydrolysis in lysate is not taken into account since the enzymatic activities of both intact cells and cell lysate have been adjusted. The decreased fluorescence release on cells and in lysate compared to isolated DPIV can also be associated with a fluorescence quenching by cell components. Thus, in our case the fluorescence of free R110 is about 92 % compared to the fluorescence of a cell-free solution. However, this small fluorescence difference could not be the only reason for the different fluorescence releases after the substrate hydrolysis by isolated DPIV, DPIV of intact cells and lysate.

The comparison of the released fluorescence by substrate hydrolysis on cells of all $(Xaa\text{-}Pro)_2$-R110 compounds used here shows small differences at identical substrate concentrations. The exact kinetic characterization of the substrate hydrolysis could not be performed since measurements of the initial rates in the substrate saturation range are not possible due to the concentration dependence of the fluorescence of R110 and of the Xaa-Pro-R110 derivatives. Moreover, the nearly complete overlap of the fluorescence spectra of both R110 and Xaa-Pro-R110 prevent a quantification of both catalysis products (Fig. 1).

Concerning the detection sensitivity, for the hydrolysis of Xaa-Pro-pNA a detection limit of cellular DPIV activity of 4 pkat/ml was determined. The detection limit for the hydrolysis of the $(Xaa\text{-}Pro)_2$-R110 compounds by cellular DPIV (U937) is between 1 and 2 pkat/ml hence being 400fold more sensitive than the Xaa-Pro-pNA hydrolysis. Compared to the isolated enzyme this result represents an about one order of magnitude lower detection limit indicating that the hydrolysis of rhodamine 110 substrates by DPIV of intact cells is limited due to the molecular size of these compounds (Lorey 1999).

Altogether, the compounds $(Xaa\text{-}Pro)_2$-R110 (Xaa = Gly, Ala Abu, Leu, Ser, Cha) turned out to be specific and sensitive substrates of cell-associated DPIV. A differentiation between both extracellular membrane-bound and intracellular DPIV by the localization of the fluorescence release using fluorescence microscopy was not possible. The reason for this can be seen in the immediate diffusion of the released R110 as well as Xaa-Pro-R110 after substrate hydrolysis from the cell surface into the surrounding medium or into the cell lumen resulting in a high background fluorescence of the medium. Furthermore, perhaps both hydrolysis products penetrate into the intact cells leading to an unspecific fluorescence of the entire cell that does

not allow a differentiation between strong and weak fluorescent regions. Therefore, the substrates (Xaa-Pro)$_2$-R110 are not suitable for differentiation of cellular and membrane-bound intracellular DPIV activity, but very well for the determination of the total DPIV activity within one measurement. On the basis of the distinct detection sensitivity of the hydrolysis of these compounds compared to the hydrolysis of dipeptide-p-nitroanilides used so far, (Xaa-Pro)$_2$-R110 substrates represent a real alternative for qualitative enzyme activity investigations at cellular level.

4.2 (Xaa)$_2$-R110 substrates of APN

A number of fluorogenic substrates containing rhodamine 110 as fluorescent leaving group have also been synthesized for APN (Fig. 2). Among these substrates of the structure (Xaa)$_2$-R110 where Xaa = Gly, Ala, Leu, the (Leu)$_2$-R110 analogue proved to be the best substrate with a k_{cat}/K_m value of $2.94 \cdot 10^6\ M^{-1} \cdot s^{-1}$.

Despite all difficulties in the detection of membrane-bound enzymes mentioned above APN activity at the cell surface of activated T cells could be shown by means of laser-scanning microscopy using these rhodamine substrates (Lendeckel *et al* 1996).

4.3 Xaa-Pro-R110-anchor substrates of DPIV

4.3.1 Synthesis and characterization

Our data presented above underlined the advantages of using DPIV substrates of the structure (Xaa-Pro)$_2$-R110 compared to Xaa-Pro-pNA substrates due to the high sensitivity of R110. However, these measurements using substrates of the type (Xaa-Pro)$_2$-R110 demonstrated that quantification of surface-associated enzymatic activity at single cell level is difficult. This is because diffusion of the enzymatically released R110 into the incubation buffer results in a high background fluorescence and unspecific staining of other cells.

In order to overcome these difficulties we synthesized on the basis of the mono-substituted compounds Gly-Pro-R110 and Ala-Pro-R110 unsymmetrically substituted R110 derivatives of the type Xaa-Pro-R110-Y (Fig. 5), where Y is a functional group bound through a spacer at the free amino group of the xanthene molecule of Xaa-Pro-R110.

The synthesis of these substrates was carried out by coupling Boc-Gly-Pro-R110/Boc-Ala-Pro-R110 with the corresponding halogenalkyl/halogenaryl chlorides as well as in the case of the maleinimide compounds according to

the carbodiimide method. After acidolytical deprotection of the Boc group, the anchor substrates were purified by HPLC (Lorey 1999).

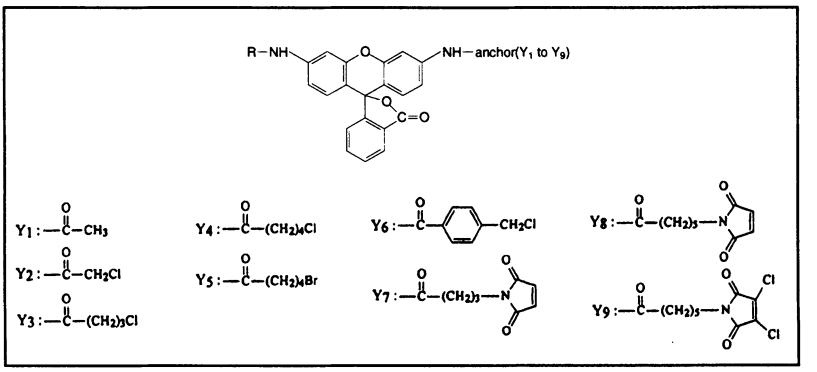

Figure 5: Structure of fluorogenic anchor substrates of DPIV with R = Gly(Ala)-Pro- and of APN with R = Ala-.

The resulting bifunctional compounds contain on the one hand the Xaa-Pro component specific for DPIV recognition and on the other hand the anchoring functional group Y (Y_2 to Y_9) which should allow a covalent binding of the substrates at the cell surface. For this covalent binding of the substrates as well as of the fluorophor R110-Y released at the cell surface after enzyme-catalyzed hydrolysis, both thiol- and amino group-reactive halogenalkyl/halogenaryl as well as N-alkyl maleinimide substituents were used as anchor groups.

4.3.2 Enzymatic hydrolysis of Xaa-Pro-R110-Y

The hydrolysis of the compounds Xaa-Pro-R110-Y with Xaa = Gly, Ala, and Y = Y_1 to Y_9 was studied using DPIV isolated from pig kidney. The enzyme-catalyzed hydrolysis of these analogues leads to the cleavage of the Xaa-Pro dipeptide thus releasing R110-Y displaying neither a substrate nor an inhibitor of DPIV. Although Gly-Pro-R110 and Ala-Pro-R110 are hydrolyzed by a classical Michaelis Menten kinetic (see Table 1), all compounds of the type Xaa-Pro-R110-Y are cleaved according to the model of substrate inhibition. The determined kinetic constants are within one order of magnitude. The k_{cat}/K_m values vary in a range beween $1.14 \cdot 10^6\ M^{-1} \cdot s^{-1}$ and $3.33 \cdot 10^6\ M^{-1} \cdot s^{-1}$ (Table 2) indicating that these compounds are good DPIV substrates.

Table 2. Kinetic Constants of the Hydrolysis of Xaa-Pro-R110-Y by DPIV from Pig Kidney (Lorey 1999)

Compound	K_m [10^5 M]	k_{cat} [s^{-1}]	k_{cat}/K_m [$M^{-1} \cdot s^{-1}$]	K_i [10^4 M]
Gly-Pro-R110-Y				
Y_1 = -CO-CH_3	17.40 ± 6.24	235.60 ± 43.25	(1.35 ± 0.56) · 10^6	2.95 ± 1.04
Y_2 = -CO-CH_2Cl	6.23 ± 0.61	70.84 ± 4.21	(1.14 ± 0.13) · 10^6	2.30 ± 0.25
Y_3 = -CO-$(CH_2)_3Cl$#	3.77 ± 0.40	67.49 ± 3.46	(1.79 ± 0.21) · 10^6	6.05 ± 0.90
Y_4 = -CO-$(CH_2)_4Cl$	3.83 ± 0.31	59.65 ± 2.55	(1.56 ± 0.14) · 10^6	2.24 ± 0.19
Y_5 = -CO-$(CH_2)_4Br$#	1.54 ± 0.17	42.39 ± 1.78	(2.75 ± 0.33) · 10^6	7.87 ± 1.58
Y_6 = -CO-C_6H_4-CH_2Cl	1.10 ± 0.20	36.64 ± 3.74	(3.33 ± 0.69) · 10^6	1.02 ± 0.27
Y_7 = -CO-$(CH_2)_3$-$C_4H_2O_2N$#	2.67 ± 0.21	60.12 ± 2.08	(2.25 ± 0.19) · 10^6	6.32 ± 0.77
Y_8 = -CO-$(CH_2)_5$-$C_4H_2O_2N$#	1.25 ± 0.10	40.41 ± 1.23	(3.23 ± 0.28) · 10^6	3.69 ± 0.39
Ala-Pro-R110-Y				
Y_9 = -CO-$(CH_2)_3$-$C_4NO_2Cl_2$*	0.58 ± 0.07	14.67 ± 0.92	(2.55 ± 0.44)·10^6	0.80 ± 0.13

*Compounds available as hydrochlorides, # compounds available as trifluoroacetates. Conditions: 40 mM Tris/HCl buffer, I = 0.125 M, pH 7.6, 30°C, measurements over 120 s at 494 nm. DPIV was used between 6.85 · 10^{-10} M and 1.37 · 10^{-9} M.

For the Gly-Pro-R110-Y substrates, the increase in hydrophobicity that is connected with an extension of the residue Y resulted in enhanced affinities of the compounds to the active site of DPIV. This could be seen in the systematic decrease of the K_m values. On the basis of these findings we can assume that also in this case hydrophobic interactions between the residue Y and hydrophobic areas of the active site of DPIV exhibit a positive impact on the substrate binding.

4.3.3 Xaa-Pro-R110-Y as substrates of cellular DPIV

The compounds of the type Xaa-Pro-R110-Y were used for investigations on DPIV-rich myeloic U937 cells. Our aim was to determine extracellular membrane-bound DPIV activity. Gly-Pro-R110-Y_1 containing an acetyl residue was used for comparison as the only compound without a functional group. The compounds Xaa-Pro-R110-Y with Y_2 to Y_9 as reactive anchors are assumed to bind preferably thiol groups of the membrane proteins ensuring stable covalent fixation of the R110 derivatives as required for the quantification of DPIV activity.

We could show that all Xaa-Pro-R110-Y compounds were hydrolyzed after incubation with DPIV-rich U937 cells. However, the fluorescence release at identical substrate concentrations varied substantially (Fig. 6) depending on the steric requirements of the anchor group Y. Compounds containing flexible anchor groups were cleaved by cell-associated DPIV

more efficiently than those with large or very short Y residues indicating that the size of the residue Y represents a limiting factor for substrate hydrolysis.

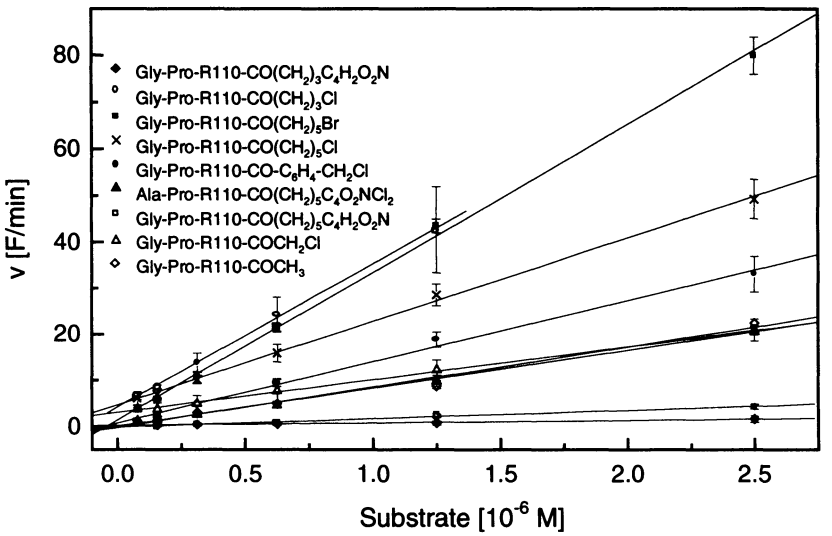

Figure 6: Hydrolysis of the substrates Xaa-Pro-R110-Y with Xaa = Gly and Ala and Y = Y_1 to Y_9 by DPIV of DPIV-rich U937 cells. Conditions: PBS, pH = 7.2, incubation 30 min, 37 °C, U937 cells of an activity of 25 pkat/ml, fluorescence plate reader: λ_{Ex} = 488 nm, λ_{Em} = 525 nm, lamp voltage 2.0 V (Lorey 1999).

Furthermore, the suitability of the anchor groups Y for stable fixation of the substrates Xaa-Pro-R110-Y or the hydrolysis products R110-Y on cells was examined. U937 cells were incubated with identical concentrations of the corresponding substrates and the cell-associated fluorescence of the hydrolysis product R110-Y was measured directly in the solution and after one to four wash steps by means of flow cytometry. There are significant differences between the fluorescence release in the solution and the fluorescence release on the cells allowing to draw conclusions about the reactivity of the anchors Y and about their suitability for quantification of cell-associated DPIV. On the basis of these results the stability of the cell-associated fluorescence was classified according to the reactivity of the residues Y (Fig. 7A-D, Table 3).

Gly-Pro-R110-Y_1 and R110-Y_1 containing one acetyl group can not react with SH groups of the cell surface. Therefore, for this analogue only weak hydrophobic interactions with hydrophobic areas on the cell should be considered. According to this assumption, for the hydrolysis product R110-Y_1 only 3 % of the initially detected cleavage product remained cell-associated (Fig. 7A). The greater proportion of it diffuses into the medium.

The compounds Gly-Pro-R110-Y contain SH-reactive halogenalkyl carbonyl (Y_2 to Y_5) and chloromethyl benzoyl (Y_6) groups, respectively. For the hydrolysis product R110-Y_2 containing a chloroacetyl anchor after four washings a cell-associated fluorescence of about 92 % of the initial fluorescence signal was detected (Fig. 7B) indicating a high anchor reactivity. The substrates with the chloromethyl benzoyl anchor (Y_6) (Fig. 7C) and N-alkyl maleinimide anchors (Y_7 to Y_9) were less reactive showing a cell-associated fluorescence between about 70 % to 80 %. For all other substrates containing halogenalkyl carbonyl residues (Y_3 to Y_5) a significant lower cell-associated fluorescence after four wash steps was detected (Table 3, Fig. 7D). They turned out to be the most ineffective anchors.

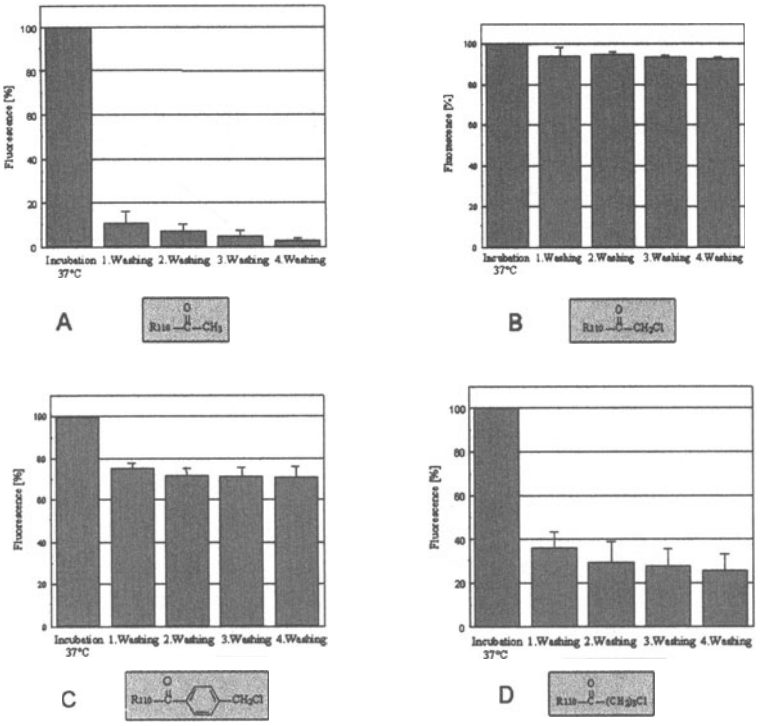

Figure 7: Fluorescence on DPIV-rich U937 cells after incubation with substrates of the structure Xaa-Pro-R110-Y. A to D represent the differences of the directly measured cell-associated fluorescence as well as after one to four wash steps. Substrates: Gly-Pro-R110-Y (Y = Y_1, Y_2, Y_3, Y_6 ; cf. Table 2 and Fig. 5). A: Y = Y_1, B: Y = Y_2, C: Y = Y_6, comparable with Y_7, Y_8 and Y_9, D: Y = Y_3, comparable with Y_4 and Y_5. Conditions: PBS, pH = 7.2, incubation 30 min, 37°C; U937 cells: 10^6 cells/ml, [Xaa-Pro-R110-Y] = $5 \cdot 10^{-6}$ M, cytofluorometer: λ_{Ex} = 488 nm, λ_{Em} = 530/30 nm = FL1 (Lorey 1999).

Table 3. Cellular Fluorescence Relative to the Cumulative Fluorescence [%] after Incubation with Xaa-Pro-R110-Y Substrates (Lorey 1999)

Compound	1. Wash step	2. Wash step	3. Wash step	4. Wash step
Gly-Pro-R110-Y				
Y = Y_1	10.40 ± 5.40	6.84 ± 3.40	4.62 ± 2.67	2.73 ± 1.28
Y = Y_2	93.84 ± 4.43	94.82 ± 1.24	93.50 ± 0.91	92.50 ± 0.78
Y = Y_3	36.18 ± 7.34	29.65 ± 9.04	29.65 ± 9.04	25.72 ± 7.48
Y = Y_4	24.77 ± 5.44	14.07 ± 5.38	11.91 ± 4.26	9.91 ± 3.91
Y = Y_5	42.77 ± 0.82	36.94 ± 5.02	35.36 ± 5.87	32.90 ± 7.02
Y = Y_6	75.23 ± 2.58	71.81 ± 3.29	71.50 ± 3.93	71.03 ± 5.14
Y = Y_7	89.56 ± 8.73	78.09 ± 9.39	71.04 ± 8.28	71.04 ± 8.28
Y = Y_8	88.46 ± 6.26	80.36 ± 9.51	74.78 ± 8.81	72.42 ± 9.40
Ala-Pro-R110-Y				
Y = Y_9	93.59 ± 5.46	85.31 ± 9.83	80.26 ± 9.63	77.39 ± 9.57

Conditions see Fig. 7; Y_1-Y_9 see Fig. 5

Generally, the insertion of a reactive group into a rhodamine 110 peptide substrate results in a stable fixation of the fluorophor on cells preventing high background fluorescence. These substrates are therefore suitable for the use in flow cytometry as well as fluorescence microscopy.

For the determination of DPIV enzyme activities on cellular level it is of particular interest to distinguish between enzymatically active and enzymatically inactive cells within one population of cells. Such an enzyme activity differentiation can complement the differentiation between DPIV-positive and DPIV-negative cells by means of CD26-specific antibody staining. For that purpose, a comparison between CD26 antibody staining using a PE-labeled (R-Phycoerythrin) antibody and staining of the cells after enzymatic cleavage of the substrates Xaa-Pro-R110-Y (Y_2 to Y_9) was carried out. The antibody binding does not influence the enzymatic activity. After CD26 antibody staining of the mononuclear cells isolated from the blood of healthy donors, two lymphocyte populations (CD26-positive and CD26-negative cells) can be detected on the basis of their significantly differing antibody fluorescence. Surprisingly, both cell populations showed nearly the same R110-Y fluorescence as a result of cellular DPIV. On the other hand, in the case of separated cell populations according to their DPIV activity a clear differentiation between CD26 high-expressing and CD26 low-expressing cells was possible using the substrates Xaa-Pro-R110-Y. Therefore, an enzyme activity differentiation turns out to be possible in the case of enzymatically uniform cell populations. In the case of suspensions of cells differing in their DPIV activity the classification of these cells regarding their DPIV activity by use of the substrates Xaa-Pro-R110-Y is impossible.

There are manifold reasons for this limitation, as for instance the mode of substrate hydrolysis and substrate binding to the cells, the mutual influence between the cells as well as the shedding of enzyme from the cells.

Further studies on this subject are in progress.

4.4 Xaa-R110-anchor substrates of APN

Similar rhodamine anchor substrates of the type Ala-R110-Y with Y = Y_2, Y_6 and Y_9 have also been synthesized for APN (Fig. 5). They were successfully used for the detection of APN enzyme activity on endothelial cells by means of fluorescence microscopy. As in the case of DPIV, the differentiation of APN activities turns out to be possible on uniform cell populations using cytofluorometry (Lendeckel *et al*, unpublished results).

5. CONCLUSIONS

In this study we present the synthesis and enzymatic characterization of sensitive fluorogenic rhodamine 110-based substrates for the detection and quantification of DPIV and APN on the surface of immune cells.

For the $(Xaa\text{-}Pro)_2$-R110 substrates of DPIV and $(Xaa)_2$-R110 substrates of APN a detection of cellular protease activity turned out to be impossible since the enzymatically released fluorophor R110 immediately diffuses from the cell surface into the surrounding medium or into the inner of the cell.

To overcome this limitation, we have developed Xaa-Pro- as well as Xaa-rhodamine substrates containing both thiol- and aminogroup-reactive anchor residues thus allowing a stable binding of the fluorescent moiety on the cell surface. The reactivity, length, and hydrophobicity of the anchor groups have been characterized as the decisive factors that facilitate the determination of cellular enzyme activities. Using fluorescence microscopy it was possible to distinguish between cells differing in their enzymatic activity. However, a differentiation of the enzyme activity at single cell level in suspensions of enzyme high-expressing and enzyme low-expressing cells by use of flow cytometry is not possible.

The reason for this might be the different substrate binding due to varied expressions of SH group-bearing proteins of various cell types. Further influencing parameters could be cellular interactions.

As alternative, rhodamine lipid anchor substrates are quite possible where a stable binding of the catalytically released fluorophor R110 in the bilayer of cell membranes could be obtained via hydrophobic interactions. On the basis of such type of anchoring in the lipid bilayer of cells, a uniform substrate binding and a reduction of cellular interactions might be achieved.

The aim of these investigations should be the differentiation/quantification of cells of different enzymatic activity by flow cytometry.

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