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Influence of dipeptidyl peptidase IV on enzymatic properties of adenosine deaminase*

Svetlana Sharoyan[1], Alvard Antonyan[1], Sona Mardanyan[1]✉, Giulio Lupidi[2] and Gloria Cristalli[3]

[1]H.Ch. Buniatyan Institute of Biochemistry, Yerevan, Armenia; [2]Department of Biology M.C.A. Sciences, University of Camerino, Camerino, Italy; [3]Department of Chemical Sciences, University of Camerino, Camerino, Italy; ✉e-mail: biochem@ipia.sci.am

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The importance of ADA (adenosine deaminase) in the immune system and the role of its interaction with an ADA-binding cell membrane protein dipeptidyl peptidase IV (DPPIV), identical to the activated immune cell antigen, CD26, has attracted the interest of researchers for many years. To investigate the specific properties in the structure–function relationship of the ADA/DPPIV-CD26 complex, its soluble form, identical to large ADA (LADA), was isolated from human blood serum, human pleural fluid and bovine kidney cortex. The kinetic constants (K_m and V_{max}) of LADA and of small ADA (SADA), purified from bovine lung and spleen, were compared using adenosine (Ado) and 2′-deoxyadenosine (2′-dAdo) as substrates. The Michaelis constant, K_m, evidences a higher affinity of both substrates (in particular of more toxic 2′-dAdo) for LADA and proves the modulation of toxic nucleoside neutralization in the extracellular medium due to complex formation between ADA and DPPIV-CD26. The values of V_{max} are significantly higher for SADA, but the efficiency, V_{max}/K_m, in LADA-catalyzed 2′-dAdo deamination is higher than that in Ado deamination. The interaction of all enzyme preparations with derivatives of adenosine and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was studied. 1-DeazaEHNA and 3-deazaEHNA demonstrate stronger inhibiting activity towards LADA, the DPPIV-CD26-bound form of ADA. The observed differences between the properties of the two ADA isoforms may be considered as a consequence of SADA binding with DPPIV-CD26. Both SADA and LADA indicated a similar pH-profile of adenosine deamination reaction with the optimum at pHs 6.5–7.5, while the pH-profile of dipeptidyl peptidase activity of the ADA/DPPIV-CD26 complex appeared in a more alkaline region.

Keywords: large and small adenosine deaminases, enzyme–substrate and enzyme–inhibitor interactions, protein–protein interaction, CD26-dipeptidyl peptidase IV

INTRODUCTION

Dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) is a widely distributed multifunctional glycoprotein, expressed as a non-covalently linked homodimer of 210 kDa at the cell surface in nearly all mammalian tissues. It was defined as an antigen CD26 on activated human T lymphocytes and medullary thymocytes, and plays a significant role in the immune and endocrine systems, bone marrow mobilization, cancer growth, cell adhesion, etc. (Gorrell *et al.*, 2001; Boonacker & Van Noorden, 2003; Mentlein,

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Abbreviations: ADA, adenosine deaminase; ADAcp, adenosine deaminase complexing protein; Ado, adenosine; 2′-dAdo, 2′-deoxyadenosine; 1-dAdo, 1-deazaadenosine; 3-dAdo, 3-deazaadenosine, DPP, dipeptidyl peptidase; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; 1-dEHNA, 1-deazaEHNA; 3-dEHNA, 3-deazaEHNA; Gly-Pro-pNA, dipeptide Gly-Pro-p-nitroanilide; LADA, large adenosine deaminase; SADA, small adenosine deaminase.

2004; Gorrell, 2005). DPPIV substrates are a number of chemokines and neuropeptides, the glucagon-like peptide and the glucose-dependent insulinotropic peptide essential in diabetes mellitus, etc. In addition to the integral membrane form, a soluble form of DPPIV-CD26 occurs in the serum, seminal fluid, pleural fluid, bile, and kidney.

Adenosine deaminase (ADA, adenosine aminohydrolase, EC 3.5.4.4), an enzyme involved in the metabolism of purine nucleosides, catalyses the irreversible hydrolytic deamination of adenosine (Ado) and 2′-deoxyadenosine (2′-dAdo) to inosine and 2′-deoxyinosine, respectively. The enzyme is widely distributed in vertebrate tissues and plays a critical role in a number of physiological systems (Senba *et al.*, 1987; Hirschhorn, 1990). In nature, several isoforms of ADA are known that differ by molecular mass, kinetic properties and tissue distribution (Van Der Weyden & Kelley, 1976). These isoforms are: the minor isoenzyme, ADA2, (Shrader *et al.*, 1978), and two molecular forms of the major isoenzyme: small, SADA, a monomer with molecular mass 35–40 kDa, and large, LADA, with molecular mass 280–300 kDa. The latter is formed by complex formation between the catalytic subunit, SADA, with a protein named ADA complexing protein (ADAcp), recently identified as DPPIV-CD26 (Fox *et al.*,1984; Kameoka *et al.*,1993; Dong *et al.*, 1996). First of all, the membrane-localized complex ADA/DPPIV-CD26 protects the cells from Ado- and 2′-dAdo-mediated apoptosis and inhibition of proliferation. Co-localization of this complex and Ado receptor, A1R, has been shown in different cells (Franco *et al.*, 1998; Beraudi *et al.*, 2003). The interaction between these proteins could be a functional basis of the extra-enzymatic role of ecto-ADA in modulating ligand-induced signalling, desensitization and internalization of A1Rs (Gines *et al.*, 2001).

The importance of ADA in the immune system in general (Giblett *et al.*, 1972; Van der Weyden & Kelley, 1977; Hirschhorn, 1990), and the unresolved questions regarding the role of its interaction with ADAcp–CD26-DPPIV, expressed in activated immune cells (De Meester *et al.*, 1994; Martin *et al.*, 1995; Dong *et al.*, 1996), have been in the field of researchers' interest for many years. The 3D-structure of the ADA/DPPIV-CD26 complex shows that their interaction does not result in steric interference and can not modulate the catalytic sites of the enzymes (Ludwig *et al.*, 2004) or affect their enzymatic activities (De Meester *et al.*, 1994). The available information concerning the collation of the two ADA isoforms is contradictory: Akedo *et al.* (1972) observed a larger activity of SADA compared with that of LADA; an increase of the resultant activity at LADA formation by SADA binding with ADA-CP was shown in the works by Schrader and Stacy (1977) and Weisman *et al.* (1988); no differences in LADA and SADA catalytic properties were found by other researchers (Van der Weyden & Kelley, 1976; Trotta *et al.*, 1979; Fonoll *et al.*, 1982).

In the present work we intended to compare the catalytic peculiarities of SADA and its natural complex with DPPIV-CD26, LADA. Hopefully, the obtained information will contribute to the problem of potential therapeutic significance: whether or not the SADA binding with DPPIV-CD26 is involved in regulation of the enzyme interaction with substrates, substrate analogs, and inhibitors. To this end, we isolated SADA from bovine spleen and lung, and LADA — from human blood serum, human pleural fluid, and bovine kidney cortex. Significant differences in the kinetic constants of the two isoforms in the reactions of Ado and 2′-dAdo deamination, and in inhibition of these reactions by the derivatives of adenosine and EHNA, used for the first time in the LADA-catalyzed reactions, were observed. Obviously, it is reasonable to consider the observed differences between the enzymatic properties of the two ADA isoforms as a consequence of the catalytic subunit binding with DPPIV-CD26.

MATERIALS AND METHODS

Materials. The substrate analogs and inhibitors of ADA: 1-dAdo, 3-dAdo, EHNA, 1-dEHNA, and 3-dEHNA were synthesized as described earlier (Cristalli *et al.*, 1993). The substrates of ADA (Ado, 2′-dAdo) and DPPIV, the dipeptide Gly-Pro-*p*-nitroanilide (Gly-Pro-pNA), bovine serum albumin and CNBr-Sepharose were purchased from Sigma Ltd. (USA) DEAE-cellulose – from Whatman (England) Sephadex G-100 and G-200, DEAE-Sephadex A-50 – from Pharmacia Biotech (Uppsala, Sweden). Other reagents were of the highest purity.

Preparation of SADA and LADA. SADA from bovine spleen and lung was isolated and purified as described earlier (Sharoyan *et al.*, 1994). In the present work, electrophoretically homogeneous preparations of specific activity about 350 μmol/min per mg of protein were used. LADA from bovine kidney cortex, human blood serum and pleural fluid was isolated using the chromatographic procedures described below.

Kidney cortex was separated from fat and medulla, washed with 0.15 M NaCl, and homogenized with a PT-1 homogenizer at 8000 r.p.m. for 1.5 min in 10 mM K,Na-phosphate buffer, pH 7.4 (buffer A), at a ratio of 1:5 (w/v). The homogenate was centrifuged at 15000 *g* for 20 min, 4°C. Then the supernatant was batch-bound to DEAE-cellulose (pre-equilibrated in buffer A) for 45 min at 4°C. The settled cellulose was packed into a glass column, washed

with three column volumes of buffer A, and the same containing 20 mM KCl. The proteins adsorbed on the cellulose were eluted with buffer A containing 0.3 M KCl. Fractions of 10 ml were collected and analyzed for both ADA and DPP activities. The blood serum and pleural fluid were subjected to similar procedures of batch-binding and elution on DEAE-cellulose after preliminary dialysis against 20 vol. of buffer A at 4°C, overnight.

The active fractions of DEAE-cellulose eluate were pooled and subjected to gel-filtration in 10 ml portions through Sephadex G-200$_{med}$ (3 × 45 cm) equilibrated with buffer A containing 0.1 M KCl (buffer B). The high molecular mass fractions possessing ADA activity were collected, dialyzed against 10 vol. of buffer A at 4°C, overnight, and put on a column of DEAE-Sephadex A-50 (2 × 12 cm) pre-equilibrated in buffer A. After washing the column with the same buffer, the adsorbed proteins were eluted with a linear gradient of 0.02–0.2 M KCl, 200 ml total volume. The protein fractions eluted at 0.13–0.15 M KCl contained both ADA and DPP activities. They were pooled, dialysed against 10 vol. of 20 mM K,Na-phosphate buffer pH 7.4 containing 0.1 M KCl (buffer C) at 4°C, overnight, and subjected to affinity chromatography on bovine lung SADA-liganded CNBr-Sepharose (pre-equilibrated in buffer C). The column was washed with buffer C containing increasing concentrations of KCl, typically: 0.1, 0.3, 0.5, 0.8 and 1 M, 10 ml of each. Afterwards, protein fractions were eluted with buffer C containing 6 M urea, and pooled and dialysed against buffer A to remove urea. This fraction also possessed both of the enzymatic activities. It was concentrated using a micro-concentrator and subjected to gel-filtration through Sephadex G-200$_{sf}$ column equilibrated in buffer B.

Disc electrophoresis in non-denaturing conditions indicated nearly 90% purity of the obtained preparations. They were kept at –20°C for 2 months without a significant loss of activity.

Enzyme assays. The ADA activity was assayed by determination of ammonia liberated in the reaction of substrate, Ado or 2′-dAdo, deamination, using a phenol-hypochlorite colorimetric method, described earlier (Mardanyan *et al.*, 2001). The enzyme concentrations in the assay mixture were 0.1 μg /ml for SADA from lung and spleen, and 30, 100, and 150 μg/ml for LADA from kidney cortex, pleural fluid and blood serum, respectively.

DPP activity was assayed using Gly-Pro-pNA as a substrate. Usually, 500 μl of assay mixture contained 40 mM K,Na-phosphate buffer, pH 7.5, and an enzyme sample (30–60 μg of protein). The reaction was initiated by addition of a 2 mM stock solution of substrate in water to a final concentration of 0.24 mM. After incubation for 20–30 min at 37°C, the reaction was stopped by cooling the assay mixture in an ice bath. The amount of product was evaluated from differential absorbance at 390 nm of a sample against an identical mixture without the enzyme, using the absorption coefficient of the chromogenic group, nitroaniline, at this wavelength of 9.9 $mM^{-1}cm^{-1}$ (Mentlein & Struckhoff, 1989).

Protein determination was performed by the method of Bradford (1976) using BSA as a standard, and/or spectrophotometrically, from the difference of absorbance of a protein solution at 215 and 225 nm (Murphy & Kies, 1960).

Determination of kinetic constants. Kinetic constants were obtained by analyzing enzyme activity dependences on the substrate concentration using the Michaelis-Menten equation for enzyme kinetics, with Lineweaver-Burk transformation. The inhibition constant, K_i, was determined using the Dixon graphical method described earlier (Mardanyan *et al.*, 2001). These enzymatic parameters were determined using the GraFit software (Leatherbarrow, 2001).

pH-dependences. The ADA and DPP activities were determined in a pH range 5.0–9.3, using 50 mM K,Na-phosphate buffer for the pH interval 5.0–7.5, and 50 mM boric buffer within pHs 7.5–9.3. The enzyme assay mixtures were preincubated at 37°C for 5 min at the appropriate pH before the reaction was started by substrate addition. The pK_a double bell equation of the GraFit software (Leatherbarrow, 2001) was used to analyze the obtained bell-shape pH profiles and to evaluate the apparent pK_a and pK_b values, respectively, in acidic and basic slopes.

Spectral measurements. Spectral measurements were performed at 25°C in UV-VIS spectrophotometers Specord M-40 and 50PC (Germany), equipped with thermostated holders.

Statistical analyses. Statistical analyses were performed using the statistical software InStat, version 3 for Windows (GraphPad Software, Inc., San Diego, CA, USA). Specific differences were tested using Student's *t*-test. Results are expressed as means ± S.D.

RESULTS AND DISCUSSION

Purification of LADA

Figure 1 shows the elution diagram obtained upon gel filtration on Sephadex G-200$_{med}$ of a sample from kidney cortex, eluted from DEAE-cellulose (as described in Materials and Methods). Identical diagrams were obtained for pleural fluid and blood serum samples. Curve 2 in this figure evidences that in these tissues low-molecular SADA is present in a

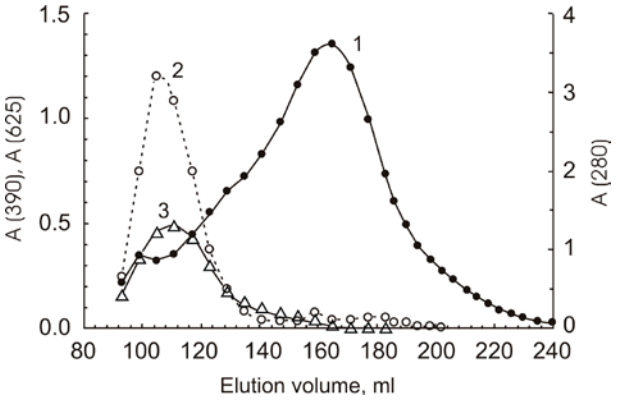

Figure 1. Gel filtration on Sephadex G-200$_{med}$ of kidney cortex homogenate fraction eluted from DEAE cellulose. The protein fractions eluted from DEAE-cellulose by buffer A containing 0.3 M KCl were pooled and subjected to gel filtration as described in Materials and Methods. The obtained fractions were analysed for protein content and ADA and DPP activities. The diagrams represent: 1, protein concentration, absorbance at 280 nm (●); 2, adenosine deaminase activity, absorbance of assay mixture at 625 nm (○); 3, dipeptidyl peptidase activity, absorbance of released nitroaniline chromogenic group at 390 nm (Δ).

negligible amount. The fraction possessing most of the ADA activity fits molecular mass of 280–300 kDa, and we identified it as the LADA isoform. Curve 3 shows that the same protein fractions possess also the DPP activity. We tried to separate the ADA and DPP activities to obtain DPPIV free of ADA activity. For this we used several treatments: thermal (incubation at 56°C), acidic (incubation at pH 3.5), and urea (incubation in 6 or 8 M urea). After passing all of these samples through identical Sephadex G-100 superfine columns equilibrated with buffer B, the same protein fraction, eluted in the void volume of the column, had both the ADA and DPP activities.

Table 1 shows the specific activity of LADA from the three sources at all purification stages, following gel filtration through G-200$_{med}$. It is worth to note that the specific activities of the obtained enzyme preparations are tissue-dependent: they differ both at the initial and the final stages (Table 1, rows 1 and 4, respectively).

The DPP activity of the final LADA preparations from bovine kidney cortex and human blood serum in the reaction of chromogenic group depletion from Gly-Pro-pNA were characterized by the approximate values for K_m, 105 and 80 μM, and by V_{max} values differing by one order, 10.0 and 1 μmol/min per mg, respectively.

We should note that in the obtained preparations of LADA, the 'ADA complexing protein' DPPIV-CD26 was not saturated with SADA; it could bind an additional amount of SADA: after 2 h of incubation of LADA at room temperature in the presence of SADA and passing the mixture through a Sephadex G-100 column, the specific ADA activity of the high-molecular mass protein fraction eluted in the void volume was higher than that of the initial LADA sample (not presented). This observation confirms LADA formation as a SADA complex with a multisubunit protein that contains unoccupied sites (Trotta, 1982).

Differences in the catalytic properties of LADA and SADA

In Table 2, the values of Michaelis constant, K_m, and the activity of the enzyme at saturation with substrate, V_{max}, for two SADA and three LADA preparations in reactions of both Ado and 2′-dAdo deamination are reported. At a glance, we can see the similarity of the parameters for SADA samples from bovine lung and spleen (rows 1 and 2, respectively) and of those for LADA samples from bovine kidney cortex, human pleural fluid and blood serum (respectively, rows 3, 4, 5), showing at the same time significant differences between the parameters for the two isoforms.

The data for K_m demonstrate a higher affinity of both substrates for LADA compared with SADA: the K_m values of Ado in the reaction of deamination by SADA are almost twice as high as those for LADA, while the K_m values of 2′-dAdo for SADA are approximately four times as high as those for LADA. These differences prove that the affinity for both substrates (especially 2′-dAdo) are higher when SADA is in complex with DPPIV-CD26.

The V_{max} values for SADA, compared with those for LADA from kidney, are higher by nearly two orders, and compared to those for LADA from pleural fluid and blood serum — by three orders, for both substrates. Such significant differences cannot be considered as a consequence of the difference in the molecular masses of the two isoforms only (no more than one order of magnitude, 35 and 300 kDa, respectively, for SADA and LADA,).

It is worth to note that the above mentioned observations and other publications (Daddona & Kelley, 1978; Trotta, 1982) allow one to conclude that SADA does not saturate all the binding sites of DPPIV-CD26 in LADA, and the enzyme preparation usually represents a mixture of molecules of DPPIV-CD26 differently saturated by the catalytic subunit SADA. This circumstance hinders the evaluation of the number of catalytic units in the assay mixture and the evaluation of the enzyme catalytic centre activity. Therefore, we were limited in the evaluation of the reaction efficiency, V_{max}/K_m. The data in the last two columns of Table 2 indicate that this parameter for SADA is 20–400 times as high as those for LADA. Let's note that it does not differ significantly in SADA-catalyzed deamination of the two substrates, but the efficiency of LADA-

Table 1. Specific activity of LADA from three tissues at different purification stages in deamination of adenosine

Purification step	Specific activity, μmol NH_3/min/mg		
	Human blood serum	Human pleural fluid	Bovine kidney cortex
Gel filtration, G-200$_{med}$*	0.0011	0.004	0.02
Ion-exchange chromatography, DEAE-Sephadex A-50	0.01	0.065	0.2
Affinity Chromatography, SADA1-linked CNBr-Sepharose	0.2	-----**	2.6
Gel filtration, G-200$_{sf}$	0.45	0.65	5.4

*Since SADA content in tissues used as sources for LADA purification was negligible (Fig. 1), we present the enzyme activity at different purification stages after removing of SADA traces by gel filtration on G-200$_{med}$. **Because of limited availability of tuberculous pleural fluid, we omitted the affinity chromatography step at enzyme purification from this fluid. Otherwise, the amount of the final product would not allow completing the kinetic properties study.

catalyzed 2′-dAdo deamination is higher than that for Ado.

The observed differences in the kinetic constants for the two molecular forms of ADA reflect the importance of neutralization of toxic nucleosides in the extracellular medium by DPPIV-CD26-bound ADA, and of modulation of this function by the complex formation between them.

Interaction of LADA and SADA with inhibitors

In Table 3, the inhibition constants, K_i, are reported for derivatives of adenosine (1-dAdo and 3-dAdo), and for EHNA and its derivatives (1-dEHNA and 3-dEHNA), for SADA and LADA in the reaction of Ado deamination. These data show similar inhibitory efficiencies of 1-dAdo, 3-dAdo, and EHNA for the two ADA molecular forms. In contrast, the K_i values of 1-dEHNA and 3-dEHNA for LADA are 2–3 times lower than those for SADA, evidencing a higher affinity of these compounds for LADA. In accordance with the proposed mechanism of ADA interaction with EHNA (Frieden *et al.*, 1980), the binding of EHNA derivatives to the enzyme complexed with DPPIV-CD26 results in a more pronounced conformational rearrangement near the active centre, leading to the formation of more stable enzyme–inhibitor complexes than in the case of SADA. Probably, if ADA is bound to DPPIV -CD26, its hydrophobic binding site becomes more exposed and binds the hydrophobic erythro-nonyl moiety of EHNA derivatives more strongly. This suggestion correlates with our previous observation, when 3-dEHNA more effectively prevented SADA from chemical modification by *N*-bromosuccinimide than EHNA did (Mardanyan *et al.*, 2001).

In Table 4, K_i values are presented of the same inhibitors for SADA from lung and LADA from kidney cortex and blood serum in the reaction of 2′-dAdo deamination. These data do not show significant differences in the K_i values for all five inhibitors for the two ADA isoforms. However, the K_i values for EHNA and its derivatives for SADA indicate that the deamination of 2′-dAdo could be inhibited with these compounds about three times more effectively than Ado deamination. Apparently, the 2′-deoxyribose moiety of 2′-dAdo hinders its competition for the SADA binding site with EHNA and its derivatives.

pH dependences of ADA and DPP activities

The pH dependences of SADA and LADA activities in the deamination of Ado were studied in pH interval 5.0–9.3, as described in Materials and Methods. Bell-shaped pH profiles were obtained

Table 2. Kinetic constants of LADA and SADA from different tissues.

Kinetic constants for Ado and 2′-dAdo deamination reactions catalysed by LADA and SADA from different tissues were calculated with GraFit software using Michaelis-Menten equation with Lineweaver-Burk transformation. Statistical analyses were performed with InStat software. Significance was determined by Student's *t*-test. Data represent means of five independent experiments ± S.D.

Tissue	ADA form	K_m [μM]		V_{max} [μmol/min per mg]		V_{max}/K_m	
		Ado	2′-dAdo	Ado	2′-dAdo	Ado	2′-dAdo
Lung	SADA	129 ± 15.5	189 ± 25.5	500 ± 15	540 ± 13	3.9	2.9
Spleen	SADA	93 ± 2.1	135 ± 4.5	350 ± 10	410 ± 11	3.8	3.04
Kidney cortex	LADA	56 ± 5.0	39 ± 4.0	6 ± 0.17	5.8 ± 0.17	0.1	0.15
Pleural fluid	LADA	70 ± 7.0	40 ± 5.0	0.7 ± 0.15	0.71 ± 0.14	0.01	0.017
Blood serum	LADA	53 ± 5.0	42 ± 4.8	0.6 ± 0.022	0.64 ± 0.017	0.01	0.015
P^* <		0.05	0.01	0.005	0.005	0.0001	0.0001

*The significance of difference between mean values for two SADA and three LADA preparations

Table 3. Inhibition constants for adenosine- and EHNA-derivatives in reaction of adenosine deamination catalysed by LADA and SADA from different tissues.

K_i was determined using graphical method of Dixon: the constant is equal to the abscissa of intercept of linear plots of reciprocal velocity dependence on inhibitor concentration at two concentrations of substrate. The plots were obtained with GraFit software using linear regression equation. Statistical analyses were performed with InStat software. Significance was determined by Student's *t*-test. Values are means of five independent experiments ± S.D.

Tissue	Form of ADA	K_i [μM]				
		1-dAdo	3-dAdo	EHNA	1-dEHNA*	3-dEHNA*
Lung	SADA	2.9 ± 0.5	810 ± 33	0.04 ± 0.004	1.2 ± 0.13	0.12 ± 0.005
Spleen	SADA	5.7 ± 1.6	>1000	0.057 ± 0.006	1.1 ± 0.05	0.11 ± 0.005
Kidney cortex	LADA	1.6 ± 0.13	690 ± 25	0.021 ± 0.011	0.4 ± 0.04	0.05 ± 0.005
Pleural fluid	LADA	2.5 ± 0.1	>1000	0.03 ± 0.01	0.45 ± 0.11	0.036 ± 0.006
Blood serum	LADA	2.9 ± 0.13	520 ± 20	0.06 ± 0.01	0.6 ± 0.15	0.025 ± 0.008

*The significance of differences between K_i values for 1-dEHNA and 3-dEHNA for SADA and LADA is statistically significant, $P < 0.005$. The differences between K_i values for 1-dAdo, 3-dAdo and EHNA for SADA and LADA isoenzymes are not statistically significant.

Table 4. Inhibition constants for adenosine- and EHNA-derivatives in reaction of 2′-dAdo deamination catalysed by LADA and SADA from different tissues.

K_i was determined as above (Table 3). Values are means of three independent experiments ± S.D.

Tissue	Form of ADA	K_i [μM]				
		1-dAdo	3-dAdo	EHNA	1-dEHNA	3-dEHNA
Lung	SADA	2.05 ± 0.13	>1000	0.015 ± 0.003	0.44 ± 0.057	0.043 ± 0.019
Kidney cortex	LADA	2.27 ± 0.56	650 ± 27	0.021 ± 0.005	0.56 ± 0.13	0.043 ± 0.013
Blood serum	LADA	1.7 ± 0.27	800 ± 30	0.045 ± 0.013	0.33 ± 0.03	0.028 ± 0.044

The differences between K_i values for SADA and LADA are not statistically significant.

(not shown), demonstrating a similar broad pH optimum for the two isoforms of ADA. In Table 5, the obtained pK_a and pK_b values are presented in the acidic and basic sides, respectively, for SADA from lung (for the enzyme from spleen identical data were obtained) and for LADA from kidney and blood serum (the data for the enzyme from pleural fluid were identical).

The observed identity of the pK_a and pK_b values of SADA and LADA indicates that the rearrangement of SADA tertiary structure at its complexation with DPPIV-CD26 does not influence the role of essential amino acids (acidic, Glu217 and Asp295, and basic, His238 (Wilson *et al.*, 1991)) in the enzymatic activity.

In the last row of Table 5, the pH parameters of LADA-catalysed dipeptidyl peptidase activity are presented. The spontaneous depletion of the substrate restricted the investigation of this reaction in the region of the pH higher than 9. Nevertheless, an analysis of the obtained data with the GraFit software shows that the pH optimum of DPP activity of LADA is in a more alkaline and wider region than that of its ADA activity.

In conclusion, we can state that the detailed comparison of the kinetic parameters of the two molecular forms of ADA shows several significant differences between free SADA and that in a soluble complex with DPPIV-CD26 (LADA). Obviously, the kinetic characteristics of SADA (actually, the catalytic subunit of ADA), are modulated by the interaction with DPPIV-CD26 in favour of neutralization of toxic nucleosides (especially 2′-dAdo) in the extracellular medium. The observed higher affinity

Table 5. pH-parameters of Ado deamination catalysed by LADA and SADA.

pK_a and pK_b values in the acidic and basic sides, respectively, were evaluated using GraFit software. Statistical analyses were performed with InStat software by Student's *t*-test. Values are means ± S.D. of three independent experiments in triplicate.

Enzyme	Activity	pH-optimum	pK_a	pK_b
SADA from lung	ADA	6.5–8.1	5.9 ± 0.08	9.0 ± 0.11
LADA from blood serum	ADA	6.5–7.8	5.7 ± 0.12	9.0 ± 0.12
LADA from kidney	ADA	6.5–7.6	5.6 ± 0.12	8.4 ± 0.11
LADA from kidney	DPP	7.0~9.0	5.9 ± 0.11	------- *

*The spontaneous depletion of the substrate restricted the investigation of this reaction in the region of pH higher than 9.0

of EHNA derivatives for LADA could be important both for a selective therapeutic use of these inhibitors, and for obtaining new information concerning the interaction of SADA with the ADA-complexing protein, DPPIV-CD26.

The physiological meaning of the observed differences could be important for the regulation of various functions in cell environment that depend on the presence of purine nucleosides.

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