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BINDING OF THE BOVINE AND PORCINE PANCREATIC SECRETORY TRYPSIN INHIBITOR (KAZAL) TO HUMAN LEUKOCYTE ELASTASE: A THERMODYNAMIC STUDY†

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The effect of pH and temperature on the apparent association equilibrium constant (K_a) for the binding of the bovine and porcine pancreatic secretory trypsin inhibitor (Kazal-type inhibitor, PSTI) to human leukocyte elastase has been investigated. At pH 8.0, values of the apparent thermodynamic parameters for human leukocyte elastase: Kazal-type inhibitor complex formation are: bovine PSTI – $K_a = 6.3 \times 10^4\,M^{-1}$,

†This paper is dedicated to Professor Mario Guarneri on the occasion of his 70th birthday.

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$\delta G^\circ = -26.9\,kJ/mol$, $\delta H^\circ = +11.7\,kJ/mol$, and $\delta S^\circ = +1.3 \times 10^2$ entropy units; porcine PSTI – $K_a = 7.0 \times 10^3\,M^{-1}$, $\delta G^\circ = -21.5\,kJ/mol$, $\delta H^\circ = +13.0\,kJ/mol$, and $\delta S^\circ = +1.2 \times 10^2$ entropy units (values of K_a, δG° and δS° were obtained at 21.0°C; values of δH° were temperature independent over the range (between 5.0°C and 45.0°C) explored). On increasing the pH from 4.5 to 9.5, values of K_a for bovine and porcine PSTI binding to human leukocyte elastase increase thus reflecting the acidic pK-shift of the His57 catalytic residue from $\simeq 7.0$, in the free enzyme, to $\simeq 5.1$, in the serine proteinase : inhibitor complexes. Thermodynamics of bovine and porcine PSTI binding to human leukocyte elastase has been analyzed in parallel with that of related serine (pro)enzyme/Kazal-type inhibitor systems. Considering the known molecular models, the observed binding behaviour of bovine and porcine PSTI to human leukocyte elastase was related to the inferred stereochemistry of the serine proteinase/inhibitor contact region(s).

KEY WORDS: Human leukocyte elastase, bovine and porcine pancreatic secretory trypsin inhibitor (Kazal-type inhibitor, PSTI), serine proteinase : inhibitor complex formation, thermodynamics (of serine proteinase : inhibitor complex formation), pH and temperature effects (on serine proteinase : inhibitor complex formation).

INTRODUCTION

Human leukocyte elastase is a serine proteinase that degrades not only elastin, but also collagens, proteoglycans, hemoglobin, fibrinogen, fibrin and anti-thrombin III. Furthermore, human leukocyte elastase plays a relevant role in the digestion of damaged tissues and bacterial degradation products.[1,2] Excessive leakage of the serine proteinase from leukocytes and/or the reduced levels of the α_1-proteinase inhibitor (the most important physiological inhibitor of human leukocyte elastase)[3–5] can lead to tissue damage, as observed in pulmonary emphysema and cystic fibrosis.[1–5] The enzyme action may be also modulated by binding of eglin *c* (the serine proteinase inhibitor from leech *Hirudo medicinalis*, belonging to the potato inhibitor I family), and by the third domain of avian ovomucoid serine proteinase inhibitors (belonging to the Kazal family).[6–11] Strong macromolecular inhibitors of human leukocyte elastase contain an apolar aminoacid residue (i.e. Leu, Met or Val) at their P_1 position.[6–11]

In order to shed more light on the role of the amino acid residue present at the P_1 position of macromolecular inhibitors on recognition phenomena related to the complex (de)stabilization, thermodynamics for the association of bovine and porcine PSTI (Kazal-type inhibitors displaying an arginyl and a lysyl residue at their P_1 position, respectively) to human leukocyte elastase has been determined between pH 4.5 and 9.5 (I = 0.1 M) and from 5.0°C to 45.0°C.

The human leukocyte elastase : bovine and porcine PSTI complex formation has been analyzed in parallel with the molecular and binding properties of related serine (pro)enzyme/Kazal-type inhibitor systems.[6–8,11–13]

MATERIALS AND METHODS

Materials

Human leukocyte elastase was isolated from granule extracts of normal human granulocytes as previously reported.[14] Bovine and porcine PSTI (type I) were prepared as detailed elsewhere.[15–17] Methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the other products were from Merck AG (Darmstadt, FRG). All chemicals were of analytical grade and used without further purification. The characterization of

human leukocyte elastase, bovine and porcine PSTI as well as methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide was previously reported.[14-18]

Determination of the Apparent Thermodynamic Parameters for the Serine Proteinase : Inhibitor Complex Formation

Values of the apparent association equilibrium constant (K_a) for the serine proteinase : inhibitor complex formation were determined, between pH 4.5 and 9.5 (I = 0.1 M) and between 5.0°C and 45.0°C, by the evaluation of the inhibitory effect of bovine and porcine PSTI on the hydrolysis of methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide catalyzed by human leukocyte elastase.[18] Values of the apparent free energy ($\delta G°$) for the serine proteinase : inhibitor complex formation were calculated, at pH 8.0 (I = 0.1 M) and 21.0°C, from values of K_a.[19,20] The temperature-independent values of the apparent enthalpy variation ($\delta H°$) accompanying the serine proteinase : inhibitor complex formation were obtained, at pH 8.0 (I = 0.1 M) and between 5.0°C and 45.0°C, from the linear dependence of log K_a on 1/T by van't Hoff plots.[19-20] Values of the apparent entropy variation ($\delta S°$) for the serine proteinase : inhibitor complex formation were calculated, at pH 8.0 (I = 0.1 M) and 21.0°C, from values of $\delta G°$ and $\delta H°$.[19,20] Average error values of $\pm 8\%$ (for K_a and $\delta G°$ values) and of $\pm 12\%$ (for $\delta H°$ and $\delta S°$ values) were evaluated as the standard deviation.[20]

Preparation of Computer-Generated Molecular Models of the Serine Proteinase : Inhibitor Complexes

On the basis of the high sequence homology which characterizes human leukocyte elastase and bovine trypsinogen, as well as bovine and porcine PSTI,[21-23] the three dimensional structure of the contact region(s) of the bovine trypsinogen : porcine PSTI complex,[24] with proper amino acid substitutions,[21-23] has been taken as a putative model for the interpretation of thermodynamics of the serine proteinase : inhibitor complex formation, together with the molecular structure of human leukocyte elastase.[25] The atomic coordinates used for computer graphics modeling of the serine proteinase : inhibitor complexes discussed have been recovered from the Brookhaven Protein Data Bank distribution tape.[26,27]

Preparation of the Hydropathy Profiles of Bovine and Porcine PSTI

The hydropathy profiles of bovine and porcine PSTI were calculated according to Kyte and Doolittle.[28] The plots utilize a window size of 7, as previously described.[29]

RESULTS AND DISCUSSION

As shown in Figure 1 and Table I, the affinity of porcine PSTI for human leukocyte elastase is systematically lower than that reported for the serine proteinase : bovine PSTI complex formation. This finding may reflect the direct influence on binding exerted by residues at sites P_8, P_5 and P_1, which host different amino acid residues in the two inhibitors, and which are potential contributions to the molecular recognition interface. Site P_8, which does not form direct interactions in the trypsin(ogen) : porcine PSTI complexes,[13,24] may host a substitution with a bulkier residue (Asn in

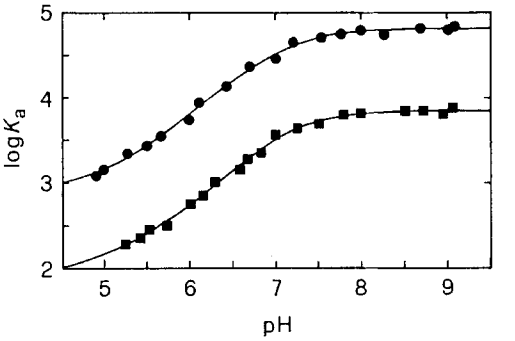

FIGURE 1 pH Dependence of the apparent association equilibrium constant (K_a; M^{-1}) for bovine (●) and porcine (■) PSTI binding to human leukocyte elastase at 21.0°C. The lines were calculated from the following equation[20]

$$\log K_a = C - \log \{([H^+] + K_{UNL})/([H^+] + K_{LIG})\} - \log (K_{LIG}/K_{UNL})$$

where C is a constant that corresponds to the alkaline asymptote of log K_a, and K_{UNL} and K_{LIG} are the apparent proton dissociation equilibrium constants for the inhibitor-free (K_{UNL}) and the inhibitor-bound (K_{LIG}) serine proteinase, respectively. The lines, generated with the following sets of parameters: bovine PSTI – C = 4.81, pK_{UNL} = 7.00, and pK_{LIG} = 5.10; and porcine PSTI – C = 3.85, pK_{UNL} = 7.10, and pK_{LIG} = 5.20, were obtained with an iterative non-linear least-squares curve fitting procedure which also ascribed an average error value of ± 12% to K_{UNL} and K_{LIG} values, as the standard deviation.[20] The pH profile was explored using the following buffers: acetate (pH 4.5 to 6.0); phosphate (pH 6.0 to 8.5); and borate/glycine (pH 8.5 to 9.5); all at I = 0.1 M (sodium salts). According to Menegatti *et al.*,[21] no specific ion effects were found using different buffers with overlapping pH values. For further details, see text.

bovine PSTI *versus* Ser in porcine PSTI), providing an easier way to bridge with polar contact(s) between the inhibitor and the enzyme 98-99-99A region. The same holds for site P_5, where an Asn residue replaces Ser of porcine PSTI. In this case, the contact is tighter and the substitution, as judged from molecular modeling, potentially gives rise to hydrogen bonds to the serine proteinase Arg 177 NEH1 or Tyr224 OH atom. Evaluation of the contribution to binding provided by the replacement of Lys18 (in porcine PSTI) with Arg18 (in bovine PSTI) at the P_1 site is complicated by the

TABLE I
Values of the apparent thermodynamic parameters for bovine and porcine PSTI binding to serine (pro)enzymes (pH 8.0, phosphate buffer, I = 0.1 M)

(Pro)enzyme/Inhibitor System	K_a[a] (M^{-1})	$\delta G°$[a] (kJ/mol)	$\delta H°$[b] (kJ/mol)	$\delta S°$[a] (kJ/mol)
Human leukocyte elastase/bovine PSTI	6.3×10^4	−26.9	+11.7	$+1.3 \times 10^2$
Human leukocyte elastase/porcine PSTI	7.0×10^3	−21.5	+13.0	$+1.2 \times 10^2$
Bovine α-chymotrypsin/bovine PSTI[c]	1.0×10^7	−39.3	+7.5	$+1.6 \times 10^2$
Bovine α-chymotrypsin/porcine PSTI[c]	1.4×10^6	−34.3	+9.2	$+1.5 \times 10^2$
Bovine β-trypsin/bovine PSTI[c]	4.0×10^{10}	−59.4	0.0	$+2.0 \times 10^2$
Bovine β-trypsin/porcine PSTI[c]	1.0×10^{10}	−56.1	+2.1	$+2.0 \times 10^2$
Bovine trypsinogen/bovine PSTI[c]	2.5×10^5	−30.1	+10.5	$+1.4 \times 10^2$
Bovine trypsinogen/porcine PSTI[c]	3.6×10^4	−25.5	+13.0	$+1.3 \times 10^2$

[a] Values of K_a, $\delta G°$ and $\delta S°$ were obtained at 21.0°C. Average error values of ± 8% (for K_a and $\delta G°$ values) and of ± 12% (for $\delta S°$ values) were evaluated as the standard deviation.

[b] The temperature-independent values of $\delta H°$ were obtained between 5.0°C and 45.0°C. An average error value of ± 12% (for $\delta H°$ values) was evaluated as the standard deviation.

[c] Data were obtained from Menegatti *et al.*[21]

necessity of displacing water molecule(s) tightly bound to the primary specificity site of the enzyme (S_1),[7,24] and by allowing for small structural perturbations (mostly residues Val190 and Val216), induced by the presence of the bulky guanidino group in the bovine PSTI P_1 arginyl residue. No other inhibitor side chain contact appears to contribute to the enzyme/inhibitor recognition process, besides some weak van der Waals interactions involving the site P'_{18} of bovine PSTI (Leu in bovine PSTI *versus* Val in porcine PSTI) and the proteinase 147–148 region.

As shown in Figure 2, the hydropathy profiles of Kazal-type inhibitors indicate that the hydrophilicity of bovine PSTI, in the P_{10} . . . P'_2 contact region (i.e. between Cys9 and Tyr20), is higher than that observed for porcine PSTI. This finding is in agreement with the possibility for bovine PSTI to make a number of polar interactions (i.e. hydrogen bonds) with the target serine proteinases and bovine trypsinogen larger than that observed in the serine (pro)enzyme : porcine PSTI adducts.[13,21,24] Accordingly, the affinity of bovine PSTI for human leukocyte elastase as well as for bovine α-chymotrypsin, bovine β-tyrpsin and bovine trypsinogen is higher than that observed for the association of the homologous serine preoteinase inhibitor from porcine pancreas (see Table I).

The pH-dependent change in affinity of bovine and porcine PSTI binding to human leukocyte elastase, shown in Figure 1, is strictly reminescent of those obtained for inhibitor binding to bovine α-chymotrypsin, bovine β-trypsin and bovine trypspinogen,[21,30] and therefore may be described in identical terms. Thus, the increase in the K_a values (i.e. in affinity) for bovine and porcine PSTI binding to human leukocyte elastase increasing the pH from 4.5 to 9.5, may be interpreted as reflecting

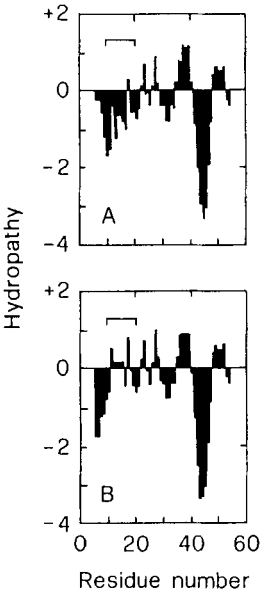

FIGURE 2 Comparison of the hydropathy profile of bovine (panel A) and porcine (panel B) PSTI. The symbols indicate the inhibitor *N*-terminal region (between sites P_{10} and P'_2, i.e. between Cys9 and Tyr20), involved in the proteinase/inhibitor contact area, were major differences for hydropathy have been observed between bovine and porcine PSTI. The plots utilize a window size of 7. For further details, see text.

the acidic pK-shift of the His57 catalytic residue from $\simeq 7.0$, in the free enzyme, to $\simeq 5.1$, in the serine proteinase : inhibitor complexes. The calculated pK-shift of the His57 catalytic residue could reflect the burial of the enzyme active site residues upon the proteinase : bovine and porcine PSTI complex formation, with change in the local dielectric constant and concomitant strengthening of the human leukocyte elastase Ser195 OG–His57 NE2 hydrogen bond;[7] this interaction is very weak, or absent, in the inhibitor-free serine (pro)enzymes.[8,13,25]

Comparison of the apparent $\delta H°$ and $\delta S°$ values, given in Table I, indicate that the human leukocyte elastase : bovine and porcine PSTI complex formation is an entropy-driven process; in fact, no contribution to the negative free energy is provided by the enthalpy change.[31] The positive apparent $\delta S°$ values (see Table I) could reflect the increased degrees of freedom gained by the solvent system when water molecules are removed from the serine proteinase and/or inhibitor surface(s) during complexation.[31]

As previously reported for the (de)stabilization of the adducts formed from the third domain of the avian ovomucoid proteinase inhibitors (belonging to the Kazal family) and serine enzymes acting on noncationic substrates,[6,11] the affinity of bovine and porcine PSTI for human leukocyte elastase is about two orders of magnitude lower than that observed for the formation of the bovine α-chymotrypsin: inhibitor complexes[21] (see Table I). This functional behaviour is likely to be related to the structural differences in the serine proteinase active center, in the surrounding regions, and to their varied interactions with bovine and porcine PSTI.[12,13,21,24,32] In particular, regions 20–30, 45–54, 88–93, 139–152, 167–177, 190–198, 215–224 and 227–232 show sequence hypervariability, different dimensions and, in part, different structures.[7,25,33]

Finally, the affinity of bovine and porcine PSTI for human leukocyte elastase is lower than that generally observed for the stabilization of the complexes formed by the third domain of the avian ovomucoid proteinase inhibitors (belonging to the Kazal family) and serine enzymes acting on noncationic substrates, i.e. human leukocyte elastase/third domain of the turkey ovomucoid proteinase inhibitor system – $K_a = 5.7 \times 10^{10} M^{-1}$; pH 8.3 (0.2 M Tris/HCl buffer)[6,11] and 21.0°C (for comparison see Table I). This finding may reflect the adverse interaction of the positively charged Arg18 and Lys18 aminoacid residues, present at the P_1 position of bovine and porcine PSTI respectively, in the apolar primary specificity site S_1 of target serine proteinases which accomodate preferentially noncationic aminoacid side chains.

As a whole, the reported data indicate that the different affinity of homologous macromolecular inhibitors for homologous serine (pro)enzymes can vary substantially as a result of structural perturbations at the interacting surfaces, altering specific recognition contact(s) but leaving the inhibitor polypeptide backbone structure (i.e. the molecular scaffold) virtually unaltered. In addition, previous results[34] have shown that important factors in specificity are steric repulsion and unsolvated charges at the contact regions; the reported results outline the relevance of uncharged hydrogen bonds in making a contribution to the discrimination energy (i.e. both binding energy and specificity).

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