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BINDING OF THE BOVINE BASIC PANCREATIC TRYPSIN INHIBITOR (KUNITZ) TO THE 33,000 M_r AND 54,000 M_r SPECIES OF HUMAN UROKINASE: 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 basic pancreatic trypsin inhibitor (BPTI, Kunitz inhibitor) to the 33,000 M_r and 54,000 M_r species of human urokinase (EC 3.4.21.31) has been investigated. Under all the experimental conditions, values of K_a for BPTI binding to the 33,000 M_r and 54,000 M_r species of human urokinase are identical. On lowering the pH from 9.5 to 4.5, values of K_a (at 21.0°C) for BPTI binding to human urokinase (33,000 M_r and 54,000 M_r species) decrease thus reflecting the acidic pK-shift of the His-57 catalytic residue from 6.9, in the free enzyme, to 5.1, in the proteinase:inhibitor complex. At pH 8.0, values of the apparent thermodynamic parameters for BPTI binding to human urokinase (33,000 M_r and 54,000 M_r species) are: $K_a = 4.9 \times 10^4\,M^{-1}$, $\Delta G° = -6.3$ kcal/mol, and $\Delta S° = +37$ entropy units (all at 21.0°C); and $\Delta H° = +4.6$ kcal/mol (temperature independent over the explored range, from 5.0°C to 45.0°C).

Thermodynamics of BPTI binding to human urokinase (33,000 M_r and 54,000 M_r species) have been analyzed in parallel with those of related serine (pro)enzyme/Kazal- and /Kunitz-type inhibitor systems. Considering the known molecular models, the observed binding behaviour of BPTI to human urokinase (33,000 M_r and 54,000 M_r species) was related to the inferred stereochemistry of the proteinase/inhibitor contact region.

KEY WORDS: Human urokinase (33,000 M_r and 54,000 M_r species), bovine basic pancreatic trypsin inhibitor (Kunitz inhibitor, BPTI), proteinase:inhibitor complex formation, thermodynamics (of proteinase:inhibitor complex formation), pH and temperature effects (on proteinase:inhibitor complex formation).

Abbreviation: BPTI, bovine basic pancreatic trypsin inhibitor (Kunitz inhibitor).

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INTRODUCTION

Urokinase is a trypsin-like serine proteinase, present in human urine, that catalyzes the plasminogen-to-plasmin conversion.[1-3] Considering the presence in human urine of the specific trypsin inhibitor, composed by two Kunitz-type domains homologous to the bovine basic pancreatic trypsin inhibitor (BPTI, Kunitz inhibitor),[4] it appeared of interest to investigate thermodynamics of BPTI binding to the 33,000 M_r and 54,000 M_r species of human urokinase. The human urokinase (33,000 M_r and 54,000 M_r species):BPTI complex formation has been analyzed in parallel with the molecular and binding properties of related serine (pro)enzyme/Kazal- and /Kunitz-type inhibitor systems (see Barrett and Salvesen,[5] Amiconi *et al.*[6] and Bolognesi *et al.*[7] for reviews).

MATERIALS AND METHODS

Materials

Human urokinase (33,000 M_r and 54,000 M_r species), from urine, and BPTI were kindly provided by Lepetit S.p.A. (Milano, I), and further purified as previously reported.[8,9] *N*-α-carbobenzoxy-L-lysine *p*-nitrophenyl ester 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 urokinase (33,000 M_r and 54,000 M_r species), BPTI and *N*-α-carbobenzoxy-L-lysine *p*-nitrophenyl ester was previously reported.[8-14]

Determination of the Apparent Enzyme:Inhibitor Association Equilibrium Constant (K_a)

Values of the apparent association equilibrium constant (K_a) for the 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 BPTI on the hydrolysis of *N*-α-carbobenzoxy-L-lysine *p*-nitrophenyl ester catalyzed by human urokinase (33,000 M_r and 54,000 M_r species).[13] The detailed biochemical procedure has been published previously.[13] An average error value of ±8% was ascribed to K_a values, as the standard deviation.[13]

RESULTS AND DISCUSSION

Under all the experimental conditions, BPTI binding to human urokinase (33,000 M_r and 54,000 M_r species) conforms to a simple equilibrium as indicated by the Hill coefficient (n) always being equal to 1.00 ± 0.02. Moreover, K_a values were always independent of the enzyme concentration, and were in excellent agreement with published data.[13]

As shown in Figure 1, values of K_a for BPTI binding to the 33,000 M_r and 54,000 M_r species of human urokinase are identical. This finding is in line with published data,[10,11,13] outlining that the proteinase A chain, present only in the 54,000 M_r species

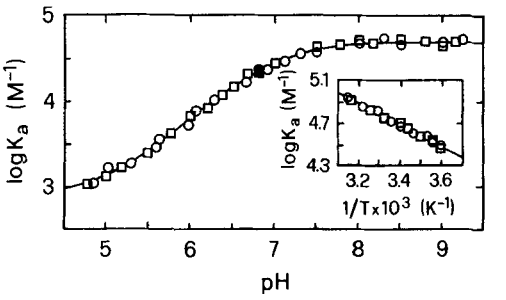

FIGURE 1 pH Dependence of the apparent association equilibrium constant (K_a) for BPTI binding to the 33,000 M_r (○, ●) and the 54,000 M_r (□, ■) species of human urokinase at 21.0°C. Values of K_a (at pH 6.8 and 21.0°C) for BPTI binding to the 33,000 M_r (●) and the 54,000 M_r (■) species of human urokinase, from Ascenzi *et al.*,[13] are reported for comparison. The unbroken line, calculated taking into account the acidic pK-shift of the His-57 catalytic residue from 6.9, in the inhibitor-free proteinase, to 5.1, in its complex with BPTI,[6] was obtained with an iterative non-linear least-squares curve fitting procedure.[20] The inset shows the van't Hoff plot describing the temperature dependence, between 5.0°C and 45.0°C, of K_a for the human urokinase (33,000 M_r and 54,000 M_r species):BPTI complex formation at pH 8.0; from the slope of this plot, the apparent $\Delta H°$ value ($= +4.6$ kcal/mol) was determined.[25,31] 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 Ascenzi *et al.*,[10,11] no specific ion effects were found using different buffers with overlapping pH values.

of human urokinase,[14–17] does not affect the proteinase active center and its interaction with substrates and inhibitors.

The pH-dependent change in affinity of BPTI binding to human urokinase (33,000 M_r and 54,000 M_r species), shown in Figure 1, is strictly reminiscent of that obtained for the binding of Kazal- and/or Kunitz-type inhibitors to bovine α- and/or β-trypsin, bovine trypsinogen, bovine α-chymotrypsin, porcine pancreatic β-kallikreins A and B, human urinary kallikrein, human Lys[77]-plasmin and human leukocyte elastase,[6,19–24] and thus may be described in identical terms. Therefore, the decrease in the K_a values for BPTI binding to human urokinase (33,000 M_r and 54,000 M_r species) on lowering the pH from 9.5 to 4.5, can be interpreted as reflecting the acidic pK-shift of the His-57 catalytic residue from 6.9, in the free enzyme, to 5.1, in the proteinase:inhibitor complex. The calculated pK-shift of the His-57 catalytic residue could reflect the strengthening of the human urokinase (33,000 M_r and 54,000 M_r species) Ser-195 OG — His-57 NE2 hydrogen bond, upon BPTI binding, being very weak, or absent, in the inhibitor-free proteinase.[5,7]

At pH 8.0, values of the apparent thermodynamic parameters for BPTI binding to human urokinase (33,000 M_r and 54,000 M_r species) are: $K_a = 4.9 \times 10^4\ M^{-1}$, $\Delta G° = -6.3$ kcal/mol, and $\Delta S° = +37$ entropy units (all at 21.0°C); and $\Delta H° = +4.6$ kcal/mol (temperature independent over the explored range, from 5.0°C to 45.0°C; see Figure 1, inset). The $\Delta S°$ and $\Delta H°$ values indicate that the complex formation is an entropy-driven process.[25] Next, the positive value of $\Delta S°$ could reflect the removal of the proteinase- and/or inhibitor-bound water molecules during complexation.[5,7,24]

In spite of the evident primary structure homology of human urokinase (33,000 M_r and 54,000 M_r species) with serine proteinases of the trypsin family,[15] the affinity of BPTI for human urokinase (33,000 M_r and 54,000 M_r species) is about eight orders of magnitude lower than that observed for the formation of the bovine β-trypsin:BPTI

complex ($K_a = 3.1 \times 10^{12}\,M^{-1}$, at pH 7.5 and 25.0°C).[26] On the other hand, the affinity of BPTI for human urokinase (33,000 M_r and 54,000 M_r species) is only slightly higher than that reported for the inhibitor association to human α-thrombin ($K_a = 1.2 \times 10^{3}\,M^{-1}$, at pH 7.5 and 21.0°C)[26] (see Figure 1 for comparison). This functional behaviour is likely to be related to the broad structural differences in the proteinase active center and surrounding regions, and to their varied interactions with BPTI. In particular, regions 20–30, 45–54, 88–93, 139–152, 167–177, 190–198, 215–224 and 227–232 can be considered as potential domains exerting steric hindrance on the incoming inhibitor in human urokinase (33,000 M_r and 54,000 M_r species) and in bovine α-thrombin because of their altered structural properties. Moreover, the amino acid substitution at the proteinase subsite S'_4 (Tyr-39 in bovine β-trypsin *versus* Ser-39 in human urokinase (33,000 M_r and 54,000 M_r species) and Glu-39 in bovine α-thrombin) may additionally affect the proteinase:BPTI complex (de)stabilization by modification of the hydrogen bonding pattern at the contact area.[7,14–17,26–30]

Finally, from the physiological viewpoint, the reported data obtained *in vitro*, together with the low levels[4] of the Kunitz-type trypsin inhibitor present in urine (about 0.01 μM in normal individuals; up to 50 μM in cancer patients) do not favour the hypothesis of any productive interaction with human urokinase (33,000 M_r and 54,000 M_r species).

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