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BINDING OF THE TRYPSIN INHIBITOR FROM WHITE MUSTARD (*Sinapis alba* L.) SEEDS TO BOVINE β-TRYPSIN: THERMODYNAMIC STUDY

ENEA MENEGATTI* and MARISA BOGGIAN

Department of Pharmaceutical Sciences, University of Ferrara, Via Scandiana 21, 44100 Ferrara, Italy

PAOLO ASCENZI

Department of Biochemical Sciences, University of Rome "La Sapienza", Piazzale Aldo Moro 5, 00185 Roma, Italy

and

PIER LUIGI LUISI

Institut für Polymere, ETH — Zentrum, CH — 8092 Zürich, Switzerland

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The effect of pH and temperature on the association equilibrium constant (K_a) for the binding of the trypsin inhibitor from white mustard (*Sinapis alba* L.) seeds (MTI) to bovine β-trypsin (EC 3.4.21.4) has been investigated. On lowering the pH from 9 to 3, values of K_a for MTI binding to bovine β-trypsin decrease thus reflecting the acid-pK and -midpoint shifts, upon inhibitor association, of two independent ionizable groups, and of a three-proton transition, respectively. At pH 8.0, values of thermodynamic parameters for MTI binding to bovine β-trypsin are: $K_a = 4.5 \times 10^8\,M^{-1}$, $\Delta G^0 = -11.6$ kcal/mol, and $\Delta S^0 = +53$ entropy units (all at 21 °C); and $\Delta H^0 = +4.1$ kcal/mol (temperature independent between 5 °C and 45 °C). Binding properties of MTI to bovine β-trypsin have been analyzed in parallel with those concerning macromolecular inhibitor association to serine (pro)enzymes.

KEY WORDS: Bovine β-trypsin, trypsin inhibitor (from white mustard, *Sinapis alba* L., seeds), bovine β-trypsin:MTI complex formation, thermodynamics (of bovine β-trypsin: MTI adduct formation), pH effects (on bovine β-trypsin:MTI complex formation).

ABBREVIATIONS: MTI, trypsin inhibitor from white mustard (*Sinapis alba* L.) seeds (Kunitz-type inhibitor); STI, soybean trypsin inhibitor (Kunitz-type inhibitor).

INTRODUCTION

Protein proteinase inhibitors are widespread in the plant kingdom being found particularly in seeds and tubers of Graminaceae, Leguminosae (Fabaciae) and Solanaceae families.[1] Among possible roles suggested for plant proteinase inhibitors, the prevailing ones seem to be the control of endogenous proteinases in the metabolism of storage proteins and during seed dormancy, as well as protection against proteolytic enzymes of parasites.[1,2] The last mentioned role appears to be the most important for the improvement of plant resistance to microbial and insect damage.[1]

*Correspondence.

The molecular properties and specificity behaviours of well over 100 plant proteinase inhibitors have been well studied, taking the soybean trypsin inhibitor (STI; Kunitz-type inhibitor) as the prototype of this class of macromolecules.[1,2] In general, most plant proteinase inhibitors are specific for serine endopeptidases, while only few plant inhibitors react with cysteine enzymes, metalloexopeptidases and acid proteinases. To date, plant inhibitors of aminopeptidases have not been reported to the best of the authors' knowledge.[1,2]

Among plant serine proteinase inhibitors, the trypsin inhibitor from white mustard (*Sinapis alba* L.) seeds (MTI) appears to be the most specific trypsin inhibitor known, showing very little or no reactivity towards proteinases of different origin.[3] MTI is a member of the STI (Kunitz) family, showing some analogies with STI particularly in the molecular weight (18,500 $\pm$ 1,000 daltons), amino acid composition ($\simeq$ 70% in homology), number of disulfides (2 *per* molecule) as well as spectral properties.[3]

In view of the specificity of MTI, thermodynamics for inhibitor binding to bovine β-trypsin has been determined between pH 3 and 9, and from 5 °C and 45 °C.

MATERIALS AND METHODS

Materials

Bovine β-trypsin (treated with diphenylcarbamyl chloride in order to abolish chymotryptic activity) was purified from commercial preparations (from Sigma Chemical Co., St. Louis, U.S.A.) according to Luthy *et al.*[4] MTI was isolated from white mustard (*Sinapis alba* L.) seeds cv. Albatros (from SIS Foraggera, Bologna, Italy) as previously detailed.[3] *N*-α-benzoyl-L-arginine *p*-nitroanilide was purchased from Sigma Chemical Co. (St. Louis, U.S.A.). All the other reagents were from Merck AG (Darmstadt, F.R.G.). All chemicals were of analytical grade and used without further purification. The characterization of bovine β-trypsin, MTI and *N*-α-benzoyl-L-arginine *p*-nitroanilide has been reported elsewhere.[3,4]

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

Values of the association equilibrium constant (K_a) for MTI binding to bovine β-trypsin were determined between pH 3 and 9 (I = 0.1 M), and 5($\pm$0.5) °C and 45($\pm$0.5) °C, by the assay system using *N*-α-benzoyl-L-arginine *p*-nitroanilide as substrate, according to Menegatti *et al.*[3] The biochemical procedure has been published previously.[3] An average error value of $\pm$ 8% was evaluated for K_a values according to the fitting procedure.[5]

RESULTS AND DISCUSSION

Under all the experimental conditions, MTI binding to bovine β-trypsin conforms to a simple equilibrium, as indicated by the Hill coefficient (n) always being equal to 1. Moreover, values of K_a for the enzyme:inhibitor adduct formation are always independent of the proteinase concentration.

The pH-profile of K_a for MTI binding to bovine β-trypsin is similar in magnitude to that reported for STI association,[6] and thus may be described with the same model. Therefore, the simplest mechanism (i.e., with the fewest ionizations) that will account

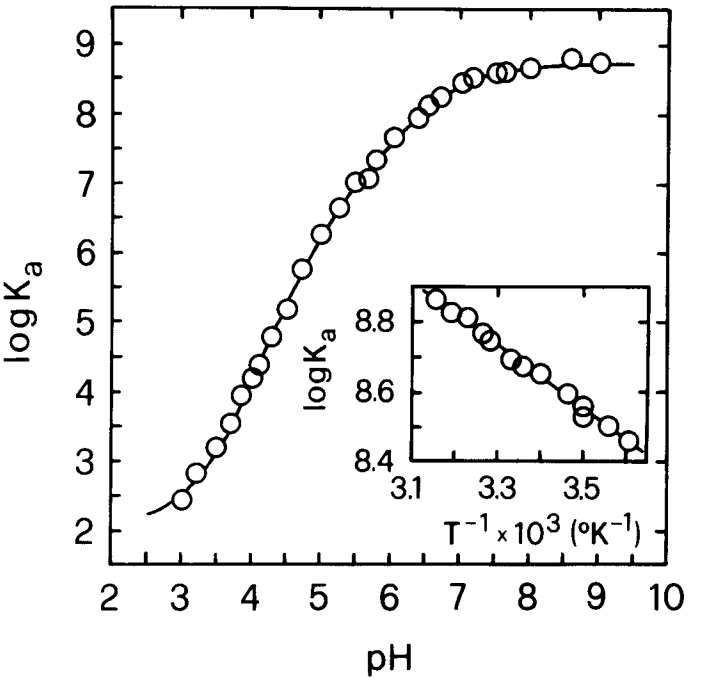

FIGURE 1 pH dependence of the association equilibrium constant (K_a; M^{-1}) for the binding of MTI to bovine β-trypsin, at 21 °C. The continuous line, calculated from Eq. 1 with the following set of parameters: C = 8.70, pK'_{UNL} = 7.0, pK''_{UNL} = 5.3, pK'''_{UNL} = 4.5, pK'_{LIG} = 5.0, pK''_{LIG} = 3.8 and pK'''_{LIG} = 3.5, was obtained with an iterative non-linear least-squares curve-fitting program. The inset shows the van't Hoff plot describing the temperature dependence, between 5 °C and 45 °C, of K_a (M^{-1}) for bovine β-trypsin:MTI complex formation at pH 8.0; from the slope of this plot, the ΔH^0 value (= +4.1 kcal/mol) was determined. The pH profile was explored using the following buffers: phosphate (pH 3 to 3.5); acetate (pH 3.5 to 6.0); phosphate (pH 6.0 to 8.5); and borate/glycine (pH 8.5 to 9); all at I = 0.1 M (sodium salts). No specific ion effects were found using different buffers with overlapping pH values.

for the data may be formulated as follows; on lowering the pH from 9 to 3 the decrease of K_a values (i.e., of the affinity) for MTI binding to bovine β-trypsin reflects, upon inhibitor association, the acid-perturbation of two independent ionizing groups, and the acid-midpoint shift of a three-proton transition. According to linkage relations, this model leads to the following expression:

$$\log K_a = C - \log \frac{([H^+] + K'_{UNL}) \cdot ([H^+] + K''_{UNL}) \cdot ([H^+] + K'''_{UNL})^3}{([H^+] + K'_{LIG}) \cdot ([H^+] + K''_{LIG}) \cdot ([H^+] + K'''_{LIG})^3} - |\Delta pK' + \Delta pK'' + 3\Delta pK'''|, \quad (1)$$

where C is a constant that corresponds to the alkaline asymptote of log K_a, and pK'_{UNL}, pK''_{UNL}, pK'''_{UNL}, pK'_{LIG}, pK''_{LIG} and pK'''_{LIG} are the pK values of proton dissociation constants for the inhibitor-free (K_{UNL}) and inhibitor-bound (K_{LIG}) bovine β-trypsin, respectively. Eq. 1 has been used to generate the continuous line shown in Figure 1; the agreement with the experimental data is satisfactory.

Some information about the chemical identity of ionizable residues modulating

bovine β-trypsin:MTI adduct formation may be gained from comparative analysis with the binding behaviour of proteinase:inhibitor systems, as well as values of pK'_{UNL}, pK''_{UNL}, pK'''_{UNL}, pK'_{LIG}, pK''_{LIG} and pK'''_{LIG} (see Figure 1), which appear to be closely related to those calculated from the pH dependencies of: (i) kinetics for the hydrolysis of synthetic and natural substrates catalyzed by bovine β-trypsin, (ii) kinetics and thermodynamics for ligand and inhibitor binding to bovine β-trypsin, and (iii) spectral properties of bovine β-trypsin and MTI.[1-3,5-11]

Thus, among aminoacid side chains capable of affecting MTI binding to bovine β-trypsin, the His57 residue,[12] involved in the catalytic triad of the serine proteinase,[9,14] has a pK value comparable to pK'_{UNL} (= 7.0) in the inhibitor-free enzyme (see Figure 1). In this respect, the calculated acid-pK shift of this one-proton binding residue (2 pH units; see Figure 1) could be interpreted as reflecting strengthening of the enzyme Ser195 OG — His57 NE2 hydrogen bond which, by analogy with what has been reported in proteinase:inhibitor systems,[9,15-18] may occur in bovine β-trypsin upon MTI binding.

As suggested for bovine β-trypsin:STI adduct formation,[6] the second one-proton binding group modulating the affinity (i.e., K_a values) of MTI for bovine β-trypsin (see Figure 1) may be contributed by the inhibitor, such residue possibly corresponding to His71 of STI.[6,15] As previously reported for STI,[6] this amino acid side chain undergoes, upon proteinase:MTI adduct formation, an acid-pK shift of 1.5 pH units, from pK''_{UNL} = 5.3, in the inhibitor-free enzyme, to pK''_{LIG} = 3.8, in the enzyme:inhibitor adduct (see Figure 1). The modulation of the pH dependence of K_a by the acid-base equilibrium of residue(s) contributed by the inhibitor seems to be a peculiarity of soybean-Kunitz trypsin inhibitors, notably STI[6] and MTI (present study). In fact, such behaviour has not been reported for the adduct formation of serine (pro)enzymes with the bovine basic pancreatic trypsin inhibitor (Kunitz-type inhibitor) and the secretory pancreatic trypsin inhibitors (Kazal-type inhibitors).[5,18-20]

Below pH 5, the titration curve shown in Figure 1 is more difficult to interpret, since values of pK'''_{UNL} and pK'''_{LIG} are midpoint values that average the ionization constants of three equivalent dissociable side chains. As previously reported for STI binding to bovine β-trypsin,[6] one of the residues likely to affect the proteinase:MTI adduct formation may be Asp189, which is present in the primary specificity subsite of the enzyme,[9-14] modulating its catalytic behaviour as well as spectral, ligand and inhibitor binding properties.[1,2,5-11] By analogy with STI,[6] the ionization of Asp102, involved in the catalytic triad of bovine β-trypsin,[9,14] may affect proteinase:MTI association (see Figure 1). Such a finding has been suggested for the complex of bovine β-trypsin with the bovine basic pancreatic trypsin inhibitor (Kunitz-type inhibitor) by the pH dependence of the ^1H nuclear magnetic resonance signal assigned to the proton hydrogen-bonded between residues Asp102 and His57 ND1 of the proteinase; this signal disappears when the pH is lowered to 3.5.[7] Finally, as previously reported for the association of macromolecular inhibitors to serine (pro)enzymes,[5,6,19,20] no plausible assignment is possible for the third acidic ligand-linked ionizable group, which could reflect pH-dependent conformational changes, outside the enzyme:inhibitor contact region, accompanying bovine β-trypsin:MTI adduct formation.

At pH 8.0 and 21 °C, values of K_a, ΔG^0 and ΔS^0 for MTI binding to bovine β-trypsin are; $4.5 \times 10^8\ M^{-1}$, -11.6 kcal/mol and $+53$ entropy units, respectively. Values of log K_a for MTI binding to bovine β-trypsin, obtained at pH 8.0 between 5 °C and 45 °C, are linearly dependent (within experimental error) with respect to T^{-1}

(indicating ΔC_p^0 values equal to 0) and allow the determination of the enthalpy variation associated with the formation of the enzyme: inhibitor adduct ($\Delta H^0 = +4.1$ kcal/mol) (see Figure 1, inset). Both the ΔH^0 and ΔS^0 values (the latter obtained at 21 °C) for MTI binding to bovine β-trypsin, (i) are comparable with those reported for the association of macromolecular inhibitors to serine (pro)enzymes acting on cationic and non-cationic substrates,[2,5,19–21] and (ii) indicate that the complex formation is an entropy-driven process. In agreement with crystallographic analysis,[9,14–18] the positive ΔS^0 value should reflect the expulsion of the enzyme- and/or inhibitor-bound water molecules during bovine β-trypsin:MTI association.

As a whole this study reports detailed thermodynamics concerning the interaction of bovine β-trypsin with MTI, the most selective trypsin inhibitor known.[3] In view of the peculiar specificity,[3] MTI may be interesting for pharmaceutical applications; in fact, the greater the capability of MTI to modulate selectively the activity of bovine β-trypsin in the presence of related proteinases, the greater is, in principle, its possible therapeutic value as a drug.

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