# Binding of Bovine Pancreatic Trypsin Inhibitor to Trypsinogen: Spectroscopic and Volumetric Studies†

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ABSTRACT: We have investigated the binding of bovine pancreatic trypsin inhibitor (BPTI) to bovine trypsinogen by combining ultrasonic velocimetry, high precision densimetry, and fluorescence spectroscopy. We report the changes in volume, adiabatic compressibility, van't Hoff enthalpy, entropy, and free energy that accompany the association of the two proteins at 25 °C and pH 8.0. We have used the measured changes in volume and compressibility in conjunction with available structural data to characterize the binding-induced changes in the hydration properties and intrinsic packing of the two proteins. Our estimate reveals that 110 ± 40 water molecules become released to the bulk from the hydration shells of BPTI and trypsinogen. Furthermore, we find that the intrinsic coefficient of adiabatic compressibility of the two proteins decreases by 14 ± 2%, which is suggestive of the binding-induced rigidification of the proteins' interior. BPTI−trypsinogen association is an entropy-driven event which proceeds with an unfavorable change in enthalpy. The favorable change in entropy results from partial compensation between two predominant terms. Namely, a large favorable change in hydrational entropy slightly prevails over a close in magnitude but opposite in sign change in configurational entropy. The reduction in configurational entropy and, consequently, protein dynamics is consistent with the observed decrease in intrinsic compressibility. In general, results of this work emphasize the vital role that water plays in modulating protein recognition events.

Bovine pancreatic trypsin inhibitor (BPTI)[1] is a small globular protein with 58 amino acid residues and a molecular mass of 6.5 kDa (*1*). It is a member of the Kunitz family that includes serine protease inhibitors. BPTI is characterized by a pearlike shape measuring ∼29 Å in its longest dimension and a diameter of ∼19 Å at its largest width (*2*). BPTI consists of a long twisted (by ∼180°) double-stranded antiparallel β-sheet (residues 17−24 and 29−35) and two α-helical segments (residues 2−6 and 47−56). The protein is stabilized by three disulfide bridges (Cys5−Cys55, Cys30−Cys51, and Cys14−Cys38) (*2*, *3*).

BPTI binds with high affinity to trypsin ($1.7 \times 10^{13}$ $M^{-1}$) and significantly less tightly to its precursor trypsinogen ($5 \times 10^5$ $M^{-1}$) (*4*−*13*). A distinctive feature of BPTI association with trypsinogen is that the zymogen undergoes a disorder-to-order transition upon the binding (*5*). Specifically, the so-called "activation domain" (residues 10−19, 142−152, 184−193, and 216−223) that is essentially disordered in free trypsinogen becomes essentially ordered upon its association with BPTI (*7*−*10*). This transition converts trypsinogen into a conformation similar but not identical to that normally found after its activation to trypsin (*5*). This disorder-to-order transition leads to an entropic penalty that reduces the affinity of BPTI to trypsinogen with the resulting association constant being 8 orders in magnitude smaller than that of BPTI−trypsin binding.

The energetics of the binding of BPTI to trypsinogen has been previously studied by Vincent and Lazdunski (*12*) and Bode (*13*). At room temperature, the association of the inhibitor with the zymogen is entropy driven and proceeds with an unfavorable change in enthalpy (*12*). Vincent and Lazdunski (*12*) suggested that the favorable change in entropy might originate from the release of water molecules from the hydration shells of the two proteins to the bulk following complex formation. It is intuitively clear that the favorable change in hydrational entropy should be opposed by an unfavorable change in configurational entropy resulting from the binding-induced decrease in the protein's dynamics. This notion follows, in particular, from the results of our previous paper in which we thermodynamically characterized the binding of turkey ovomucoid third domain (OMTKY3), a serine protease inhibitor, to α-chymotrypsin (*14*). We found that the favorable change in entropy associated with OMTKY3−α-chymotrypsinogen binding (74 cal $mol^{-1}$ $K^{-1}$) mainly reflects the compensation between the highly favorable hydrational contribution (590 ± 30 cal $mol^{-1}$ $K^{-1}$) and the highly unfavorable configurational contribution (−508 ± 33 cal $mol^{-1}$ $K^{-1}$) (*14*). The unfavorable effect of configurational entropy may be even stronger in the case of BPTI−trypsinogen association since the binding of OMTKY3 to α-chymotrypsin is of the rigid body type and proceeds without any major structural transition. By contrast, as mentioned above, BPTI−trypsinogen complexation is

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; OMTKY3, turkey ovomucoid third domain; PDB, Protein Data Bank.

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accompanied by a disorder-to-order transition of the zymogen that should bring about an additional configurational penalty.

In the present work, we study the binding of BPTI to trypsinogen. To this end, we combine ultrasonic velocimetric, densimetric, and spectroscopic measurements to characterize the binding-induced changes in hydration, intrinsic packing, and dynamics of BPTI and trypsinogen upon their complex formation. By combining our volume and compressibility results with available structural information, we estimate the number of water molecules that become released to the bulk from the hydration shells of BPTI and trypsinogen. Furthermore, our results indicate that the intrinsic coefficient of adiabatic compressibility of the two proteins decreases, an observation that is suggestive of the binding-induced rigidification of the proteins' interior. This finding is in qualitative agreement with our determined reduction in conformational dynamics of the two proteins as manifested in a diminution in configurational entropy.

## MATERIALS AND METHODS

*Materials.* BPTI and bovine trypsinogen of the highest purity commercially available were purchased from Sigma-Aldrich Canada (Oakville, Ontario, Canada) and used without further purification. All measurements reported here were performed in a pH 8.0 buffer consisting of 50 mM Tris-HCl and 20 mM NaCl. All solutions were prepared using doubly distilled deionized water.

The proteins' concentrations were determined spectrophotometrically using extinction coefficients, $\epsilon_{280}$, of $1.08 \pm 0.02$ and $1.43 \pm 0.02$ L g$^{-1}$ cm$^{-1}$ for BPTI and trypsinogen, respectively. Both extinction coefficients were obtained from dry weight analysis and are somewhat higher than $\epsilon_{280} = 0.83$ L g$^{-1}$ cm$^{-1}$, the extinction coefficient of BPTI, and $\epsilon_{280} = 1.39$ L g$^{-1}$ cm$^{-1}$, the extinction coefficient of trypsinogen, reported by Vincent and Lazdunski (*12*). All UV light absorption measurements were conducted using an Aviv model 14 DS UV/Vis light absorption spectrophotometer (Aviv Associates, Lakewood, NJ).

*Fluorescence.* Fluorescence intensity measurements were performed in a 10 mm path-length cuvette using an Aviv model ATF 105 spectrofluorometer (Aviv Associates, Lakewood, NJ). Fluorescence titration profiles were measured by incrementally adding aliquots of BPTI to a cell containing a known amount of trypsinogen. When calculating the relative fluorescence intensity of trypsinogen, we took into account the change in trypsinogen concentration upon each addition of the titrant (BPTI). Titration measurements were performed at temperatures 20, 25, 30, and 35 °C. At each experimental temperature, the buffer was adjusted to pH 8.0, thereby ensuring a constant pH across the temperature range studied. The protein samples were excited at 295 nm, and the intensity of emitted light was recorded through a monochromator at 339 nm. For all fluorescence measurements, the protein concentration was about ~0.2 mg/mL.

*Densimetry.* All densities were measured at 25 °C with a precision of $\pm 1.5 \times 10^{-6}$ g cm$^{-3}$ using a vibrating tube densimeter (DMA-5000; Anton Paar, Graz, Austria). The partial molar volume, $V^\circ$, of the proteins was calculated from density values using the relationship (*15*):

$$V^\circ = M/\rho_0 - (\rho - \rho_0)/(\rho_0 C) \quad (1)$$

where $\rho$ and $\rho_0$ are the densities of the protein solution and the neat solvent, respectively, $C$ is the molar concentration of a protein, and $M$ is the protein's molecular mass.

Densimetric titration profiles were measured by incrementally adding aliquots of BPTI to a densimetric cell containing a known amount of trypsinogen. Volume changes, $\Delta V$, accompanying inhibitor binding to the zymogen were calculated using the equation:

$$\Delta V = [(\rho - \rho_0) - (\rho' - \rho'_0)(1 + V''/V'_0)]/(\rho_0 C) \quad (2)$$

where $V'_0$ is the initial volume of the trypsinogen solution with a concentration $C$ or the buffer solution in which the same volume of the titrant solution, $V''$, has been added, $\rho$ and $\rho_0$ are the densities of the initial free, unbound protein and buffer solutions, respectively, and $\rho'$ and $\rho'_0$ are respectively the densities of the initial protein and the buffer solutions to which the titrant solution has been added.

The titrations were performed in a specially designed vial connected via Tygon tubings to the densimeter as described previously (*16*).

*Ultrasonic Velocimetry.* Solution sound velocity measurements were carried out at 25 °C using the resonator method (*17*). All measurements were conducted at ~7.2 MHz by analyzing the amplitude−frequency characteristics of an ultrasonic resonator as described previously (*13*−*20*). We employed an ultrasonic resonator cell with lithium niobate piezotransducers and a minimum sample volume of 0.8 mL (*18*). For this type of acoustic resonators, the relative precision of sound velocity measurements at frequencies near 7 MHz is at least $\pm 0.15$ cm s$^{-1}$ (*21*−*22*). The resonator cells were designed and manufactured in collaboration with Dr. Wladimir Urbach at the University of Paris V. The analysis of the frequency characteristics of the resonator was performed by a Hewlett-Packard Model HP4195A network/spectrum analyzer (Mississauga, Ontario, Canada).

The key characteristics of a solute directly derived from ultrasonic velocimetric measurements is the relative molar sound velocity increment, [U]:

$$[\mathrm{U}] = (U - U_0)/(U_0 C) \quad (3)$$

where $U$ and $U_0$ are the sound velocities in the protein solution and the neat solvent, respectively.

Acoustic titration experiments were performed at 25 °C by adding to both the sample and the reference cells, each containing 0.8 mL of the initial trypsinogen solution and the solvent, respectively, an equal amount of BPTI solution. In calculating [U], we took into account the changes in sound velocity in the solvent, $U_0$, and in the molar concentration of trypsinogen, $C$, that result from the addition of the titrant (BPTI). Additions were made using a 10 $\mu$L Hamilton syringe equipped with a Chaney adaptor (Hamilton Co., Reno, NV) which allows titrant delivery with a relative accuracy of 0.1%. For all ultrasonic velocimetric and densimetric measurements, the initial trypsinogen concentration was 0.040−0.045 mM, while the concentration of BPTI was 2−3 mM.

*Determination of the Partial Specific Adiabatic Compressibility.* Values of the relative molar sound velocity increment, [U], were used in conjunction with the measured partial molar volume data, $V^\circ$, to calculate the partial molar adiabatic

compressibility, $K°_S$, of the proteins using the relationship (*23*, *24*):

$$K°_S = \beta_{S0}(2V° - 2[U] - M/\rho_0) \quad (4)$$

where $\beta_{S0}$ is the coefficient of adiabatic compressibility of the solvent. The volumetric and ultrasonic velocimetric experiments have been performed at least three times with the average values of [U] and $V°$ being used in the equation. Errors in the reported values of the relative molar sound velocity increment, [U], partial molar volume, $V°$, and partial molar adiabatic compressibility, $K°_S$, were estimated as the maximum uncertainties due to the concentration determination, temperature drifts, and apparatus limitations.

*Determination of Intrinsic Volumes and Solvent-Accessible Surface Areas.* The atomic coordinate sets of free BPTI, free trypsinogen, and the BPTI−trypsinogen complex needed for calculating their intrinsic molecular volumes and solvent-accessible surface areas were obtained from the Protein Data Bank (*25*, *26*): 4PTI for free BPTI (*5*), 1TGB for free trypsinogen (*8*), and 2TGP for the BPTI−trypsinogen complex (*5*). When calculating solvent-accessible areas, we used the most recent version of the original Lee and Richards algorithm (*27*, *28*) developed by Gerstein and co-workers (*29*−*31*). For molecular volume calculations, we used Connolly's molecular surface algorithm (*32*, *33*).

In our analyses below, we do not consider errors when calculating the intrinsic volumes and solvent-accessible surface area of BPTI, trypsinogen, and their complex. These errors are related to accuracy of the X-ray crystallographically determined three-dimensional structures of the proteins that is not easy to quantify.

## RESULTS

*Fluorescence Titration Profiles.* To confirm that BPTI in fact binds to trypsinogen at our experimental conditions, we measured the fluorescence intensity of the zymogen in the absence and presence of the inhibitor at various BPTI-to-trypsinogen ratios, $r$ = [BPTI]/[trypsinogen]. Figure 1 presents the relative fluorescence intensity profile of the binding of BPTI to trypsinogen at 25 °C in a pH 8.0 Tris-HCl buffer. The dependence shown in Figure 1 was fitted using the following equation derived for a 1 to 1 stoichiometric binding:

$$X = X_0 + \alpha\Delta X \quad (5)$$

where $X$ is a binding-dependent observable (in this case, relative fluorescence intensity), $X_0$ is the initial value of $X$ in the absence of the inhibitor, $\Delta X$ is the maximum change in $X$ when the zymogen is saturated with the inhibitor, $\alpha$ = [PI]/[$P_T$] is the fraction of trypsinogen molecules associated with BPTI, [PI] is the concentration of the complex, and [$P_T$] is the total concentration of the zymogen. The value of $\alpha$ is given by the relationship:

$$\alpha = 0.5(r + 1) + Y^{-1} - [0.25(r - 1)^2 + (r + 1)/Y + Y^{-1}]^{1/2}$$

$Y = 2K_b[P_T]$, $K_b$ = [PI]/([P][I]) is the binding constant, [P] is the concentration of free zymogen, and [I] is the concentration of the free inhibitor.

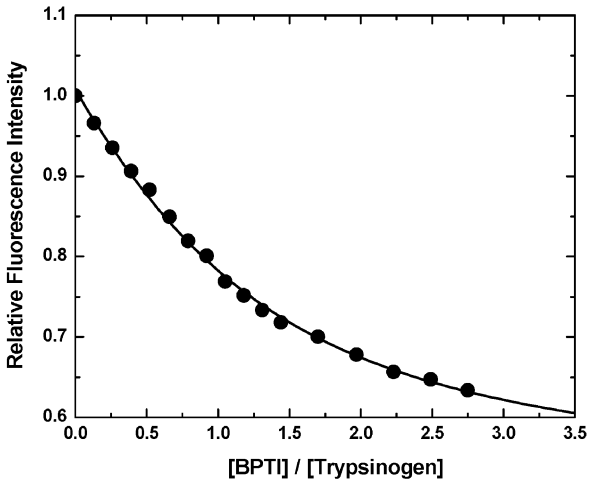


FIGURE 1: Relative fluorescence intensity of trypsinogen plotted against the BPTI−trypsinogen molar ratio, $r$, in a pH 8.0 Tris-HCl buffer at 25 °C. The excitation and emission wavelengths are 295 and 339 nm, respectively. When calculating the relative fluorescence intensity of trypsinogen, the change in the concentration of the protein upon each addition of the titrant (BPTI) was taken into account. The initial concentration of trypsinogen is 10 $\mu$M. The experimental points were fitted using eq 5 (solid lines).

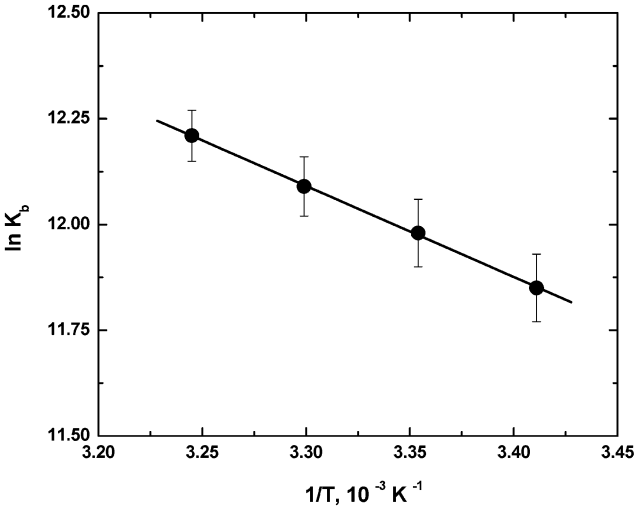


FIGURE 2: Temperature dependence of the binding constant of BPTI to trypsinogen in a pH 8.0 Tris-HCl buffer.

Using eq 5 in conjunction with the binding profile shown in Figure 1, we evaluate an association constant, $K_b$, of (1.60 ± 0.10) × $10^5$ $M^{-1}$ for the binding of BPTI to trypsinogen at 25 °C and pH 8.0. Our value of $K_b$ is somewhat smaller than (5.6 ± 0.3) × $10^5$ $M^{-1}$, the value previously reported by Vincent and Lazdunski (*12*) and Bode (*13*). This discrepancy is not unexpected since the previous studies were conducted in the presence of $CaCl_2$ (*12*, *13*). $Ca^{2+}$ ions are known to preferentially bind to the BPTI−trypsinogen complex, thereby stabilizing it and increasing the apparent association complex (*34*, *35*).

*Determination of the van't Hoff Enthalpy.* We have carried out fluorescence titration experiments at 20, 25, 30, and 35 °C and determined the binding constant, $K_b$, at each experimental temperature. The values of $K_b$ are (1.40 ± 0.11) × $10^5$, (1.60 ± 0.12) × $10^5$, (1.78 ± 0.10) × $10^5$, and (2.00 ± 0.10) × $10^5$ $M^{-1}$ at 20, 25, 30, and 35 °C, respectively. Figure 2 graphically presents the temperature dependence of $K_b$. From this dependence, we calculate the binding

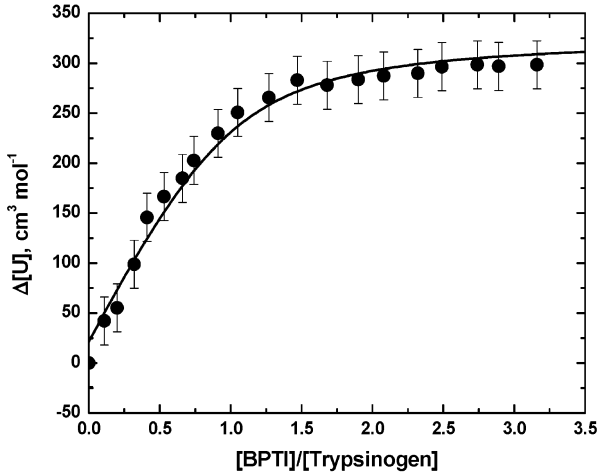


FIGURE 3: Change in the relative molar sound velocity increment of trypsinogen plotted against the BPTI−trypsinogen molar ratio, *r*, in a pH 8.0 Tris-HCl buffer at 25 °C. The initial concentration of trypsinogen is ~0.04 mM. The experimental points are fitted using eq 5 (solid lines).

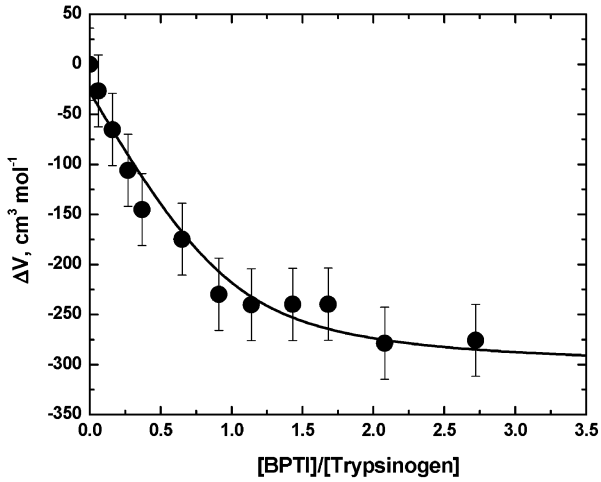


FIGURE 4: Change in the partial molar volume of trypsinogen plotted against the BPTI−trypsinogen molar ratio, *r*, in a pH 8.0 Tris-HCl buffer at 25 °C. The initial concentration of trypsinogen is ~0.04 mM. The experimental points are fitted using eq 5 (solid lines).

enthalpy, $\Delta H_b$, using the van't Hoff relationship:

$$\Delta H_b = -R\left(\frac{\partial \ln K_b}{\partial 1/T}\right) \quad (6)$$

where $R$ is the universal gas constant and $T$ is the absolute temperature.

Our calculated value of $\Delta H_b$ equals 4.3 ± 0.5 kcal mol$^{-1}$, which is in good agreement with 3.8 kcal mol$^{-1}$, the value reported by Vincent and Lazdunski (*12*).

*Volumetric Properties.* Figures 3 and 4, respectively, present changes in the relative molar sound velocity increment, Δ[U], and volume, Δ*V*, for trypsinogen in the absence and presence of the titrant (BPTI) at various BPTI-to-trypsinogen binding ratios, *r*, at 25 °C. The relative molar sound velocity increment, $[u]_{BPTI}$, partial molar volume, $V°_{BPTI}$, and partial molar adiabatic compressibility, $K°_{SBPTI}$, of BPTI are equal to 1180 ± 20 cm$^3$ mol$^{-1}$, 4280 ± 20 cm$^3$ mol$^{-1}$, and 0.021 ± 0.003 cm$^3$ mol$^{-1}$ bar$^{-1}$, respectively. If expressed in terms of specific quantities (per gram rather than per mole of protein), the values of $[u]_{BPTI}$, $v°_{BPTI}$, and $k°_{SBPTI}$ are equal to 0.182 ± 0.003 cm$^3$ g$^{-1}$, 0.720 ± 0.003 cm$^3$ g$^{-1}$, and (3.3 ± 0.5) × 10$^{-6}$ cm$^3$ g$^{-1}$ bar$^{-1}$, respectively. These specific quantities are well within the range of the values typical for globular proteins (*36*−*41*). The relative specific sound velocity increment, $[u]_{Trp}$, partial specific volume, $v°_{Trp}$, and partial specific adiabatic compressibility, $k°_{STrp}$, of trypsinogen are equal to 0.185 ± 0.002 cm$^3$ g$^{-1}$, 0.725 ± 0.003 cm$^3$ g$^{-1}$, and (3.4 ± 0.4) × 10$^{-6}$ cm$^3$ g$^{-1}$ bar$^{-1}$, respectively. These values coincide with our previous measurements (*39*).

Table 1: Summary of Thermodynamic Data on the Binding of BPTI to Trypsinogen at 25 °C

| | |
|---|---|
| $\Delta G_b$, kcal mol$^{-1}$ | −7.1 ± 0.2 |
| $\Delta H_b$, kcal mol$^{-1}$ | 4.3 ± 0.5 |
| $\Delta S_b$, cal mol$^{-1}$ K$^{-1}$ | 38.3 ± 1.8 |
| $\Delta[U]_b$, cm$^3$ mol$^{-1}$ | 300 ± 35 |
| $\Delta V_b$, cm$^3$ mol$^{-1}$ | −278 ± 45 |
| $\Delta K_{Sb}$, 10$^{-4}$ cm$^3$ mol$^{-1}$ bar$^{-1}$ | −518 ± 51 |

The sound velocity, Δ[U], and volume, Δ*V*, data presented in Figures 3 and 4, respectively, have been fitted with eq 5. The observed changes in the relative molar sound velocity increment, $\Delta[U]_b$, and volume, $\Delta V_b$, accompanying saturation of trypsinogen by BPTI represent the differences between the values corresponding to the complex and each of the two associating proteins: $\Delta[U]_b = [U]_{complex} - [U]_{BPTI} - [U]_{Trp}$ and $\Delta V_b = V°_{complex} - V°_{BPTI} - V°_{Trp}$.

The change in adiabatic compressibility, $\Delta K_{Sb}$, accompanying the binding of the inhibitor to the zymogen can be calculated from the values of $\Delta[U]_{obs}$ and $\Delta V_{obs}$ by differentiating eq 4, $\Delta K_{Sb} = 2\beta_{so}(\Delta V_b - \Delta[U]_b)$. Our determined values of $\Delta[U]_b$, $\Delta V_b$, and $\Delta K_{Sb}$ are 300 ± 35 cm$^3$ mol$^{-1}$, −278 ± 45 cm$^3$ mol$^{-1}$, and (−518 ± 51) × 10$^{-4}$ cm$^3$ mol$^{-1}$ bar$^{-1}$, respectively. These values are tabulated in Table 1.

## DISCUSSION

*Binding Energetics.* For a protein−protein association event, the observed binding enthalpy, $\Delta H_b$, depends on the enthalpy of buffer ionization, $\Delta H^b_i$, as given by the relationship (*42*):

$$\Delta H_b = \Delta H°_b + N_{H^+}\Delta H^b_i \quad (7)$$

where $\Delta H°_b$ is the binding enthalpy in a buffer with $\Delta H^b_i$ equal to zero and $N_{H^+}$ is the number of protons released to the bulk upon the binding (*42*).

However, as recently shown by Horn et al. (*43*), in an "open system" the binding enthalpy obtained from the van't Hoff analysis lacks the buffer ionization component (the second term in eq 7). A proton-linked binding system is considered open if its pH is adjusted so as to be the same at each experimental temperature. This criterion corresponds to our experimental conditions. Thus, our system is open, while our measured $\Delta H_b$ does not contain the enthalpic contribution from buffer ionization.

The free energy of BPTI−trypsinogen complexation, $\Delta G_b$, can be calculated from the binding constant, $K_b$, using $\Delta G_b = -RT \ln K_b$. At 25 °C, the value of $\Delta G_b$ is −7.1 ± 0.2 kcal mol$^{-1}$. The binding entropy, $\Delta S_b$, can be determined using $\Delta S_b = (\Delta H_b - \Delta G_b)/T$. At 25 °C, $\Delta S_b$ equals 38.3 ±

1.8 cal $mol^{-1}$ $K^{-1}$. The values of $\Delta G_b$, $\Delta H_b$, and $\Delta S_b$ are listed in Table 1.

*Volumetric Properties of BPTI Association with Trypsinogen.* Buffer ionization generally contributes to the observed changes in volume, $\Delta V_b$, and compressibility, $\Delta K_{Sb}$, accompanying a protein−protein association event. However, we can safely ignore the buffer ionization terms, since the volume and compressibility of ionization of Tris-HCl are small (*44*, *45*). At 25 °C, the volume and compressibility of ionization of Tris-HCl are 1 $cm^3$ $mol^{-1}$ and 0 $cm^3$ $mol^{-1}$ $bar^{-1}$, respectively (*44*, *45*). These values are smaller than the errors of our determined values of $\Delta V_b$ and $\Delta K_{Sb}$.

Changes in protein volume and adiabatic compressibility associated with protein binding or folding have been traditionally interpreted in terms of the intrinsic and hydration contributions (*14*, *40*, *41*, *46*−*49*). For a protein binding event, $\Delta V_b$ and $\Delta K_{Sb}$ can be presented as (*14*, *48*)

$$\Delta V_b = \Delta V_M + \Delta\Delta V_h \quad (8)$$

$$\Delta K_{Sb} = \Delta K_M + \Delta\Delta K_h \quad (9)$$

where $\Delta V_M$ is the change in the intrinsic volume of the proteins, $V_M$; $\Delta\Delta V_h$ is the change in the hydration contribution to volume, $\Delta V_h$; $\Delta K_M$ is the change in the intrinsic compressibility of the proteins, $K_M = \beta_M V_M$; $\beta_M$ is the coefficient of adiabatic compressibility of the protein interior; and $\Delta\Delta K_h$ is the change in the hydration contribution to compressibility, $\Delta K_h$.

A change in the hydration contribution to volume, $\Delta\Delta V_h$, can be presented as the sum of two terms: $\Delta\Delta V_h = \Delta V_T + \Delta V_I$, where $\Delta V_T$ is the change in the thermal volume, $V_T$, which originates from thermally activated mutual vibrational motions of solute and solvent molecules and $\Delta V_I$ is the change in the interaction volume, $V_I$, which represents solvent contraction due to solute−solvent interactions around charged and polar solute groups (*14*, *38*, *41*, *48*, *50*, *51*). Consequently, eq 8 can be rearranged to an expanded form:

$$\Delta V_b = \Delta V_M + \Delta V_T + \Delta V_I \quad (10)$$

The change in the intrinsic volume, $\Delta V_M$, can be calculated on the basis of X-ray crystallographic structures of the BPTI−trypsinogen complex and the two free proteins. In our calculations, we used the definition of molecular volume in contrast to a more frequently used definition of Voronoi volume (*28*, *32*, *33*, *41*, *52*). We have recently discussed and compared the applications of Voronoi and molecular volumes to protein-related investigations (*41*). When calculating Voronoi volume, all the space within a protein is assigned to its constituent atoms by constructing around each atom a so-called Voronoi polyhedron, an irregularly shaped polyhedron of limiting size (*28*, *52*). The faces of a Voronoi polyhedron are formed by planes which are drawn as perpendicular bisectors of the vectors connecting each pair of atoms, while the edges of the polyhedron represent the intersections of these planes. Hence, there is a uniquely defined set of polyhedrons surrounding each protein atom. The Voronoi volume of a protein is calculated as a sum of the volumes of all the constituent polyhedra.

Molecular volume is defined as the volume enclosed by the molecular surface of a protein (*28*). The molecular surface of a protein consists of the part of the protein surface which contacts a rolling probe solvent molecule plus the reentrant surface, which corresponds to a series of patches formed by the interior-facing domain of the probe when it simultaneously contacts more than one atom on the protein surface (*28*).

Although, the molecular volume of a globular protein is roughly 15% smaller than its Voronoi volume, both definitions of intrinsic volume are valid and their use in protein studies is justified insofar as their differences are clearly understood and taken into account (*41*). However, strictly speaking, the Voronoi polyhedra are unambiguously defined only for atoms that are surrounded by other atoms. This means that the Voronoi procedure is accurate when treating atoms in the protein interior and is somewhat vague when applied to analyzing surface atoms. From this viewpoint, the use of molecular volume in studying protein recognition events (that are generally accompanied by burial of previously solvent-exposed surface atoms) could be slightly more advantageous.

Our calculated values of the intrinsic (molecular) volume, $V_M$, of the complex, free trypsinogen, and free BPTI (using the PDB entries 2TGP, 1TGB, and 4PTI, respectively) are 34537, 27216, and 7114 $Å^3$, respectively. The change in the intrinsic volume, $\Delta V_M$, is equal to 207 $Å^3$ (34537 − 27216 − 7114) or 124 $cm^3$ $mol^{-1}$.

The terms $\Delta V_T$ and $\Delta V_I$ in eq 10 can be discriminated only on the basis of structural data on the two interacting proteins and the complex. Specifically, as a first approximation, the thermal volume, $V_T$, of a globular protein is roughly proportional to its solvent-accessible surface area, $S_A$, with the proportionality coefficient, $\delta$, of 1.0 ± 0.1 Å (*39*, *41*). Consequently, the value of $\Delta V_T$ can be calculated as the product of $\delta$ and the binding-induced change in solvent-accessible surface area of the two proteins, $\Delta S_A$. Our calculated values of $S_A$ for the complex, free trypsinogen, and free BPTI (using the PDB entries 2TGP, 1TGB, and 4PTI, respectively) are 12099, 9087, and 4009 $Å^2$, respectively. The change in solvent-accessible surface area, $\Delta S_A$, equals −997 $Å^2$ (12099 − 9087 − 4009), while the change in $V_T$, $\Delta V_T = \delta\Delta S_A$, equals −997 ± 100 $Å^3$ [−(1.0 ± 0.1) × 997] or −600 ± 60 $cm^3$ $mol^{-1}$. Armed with the values of $\Delta V_M$ and $\Delta V_T$, we now use eq 10 to estimate $\Delta V_I = \Delta V_b - \Delta V_M - \Delta V_T$ = 198 ± 75 $cm^3$ $mol^{-1}$ [−(278 ± 45) − 124 + (600 ± 60)]. Such a large increase in $V_I$ is suggestive of significant dehydration of the interacting surfaces of the two proteins. The value of $\Delta V_I$ equals $\Delta n_h(V_0 - V_h)$, where $\Delta n_h$ is the number of water molecules released from the proteins' hydration shells to the bulk and $(V_0 - V_h)$ is the average difference in the partial molar volume between bulk water and water of protein hydration. The average value of $(V_0 - V_h)$ for a globular protein is 1.8 $cm^3$ $mol^{-1}$ (∼10% of the partial molar volume of bulk water) (*53*).

The number of water molecules released to the bulk, $\Delta n_h$, upon the formation of the BPTI−trypsinogen complex can be evaluated by dividing $\Delta V_I$ by $-(V_h - V_0)$. We calculate $\Delta n_h$ of 110 ± 40 [(198 ± 75)/1.8]. Error of $\Delta n_h$ determination may increase if one takes into account error of the estimate of $(V_0 - V_h)$. However, the value of 1.8 $cm^3$ $mol^{-1}$ was obtained on the basis of model-dependent calculations, which makes it difficult to determine its associated uncertainty (*53*). It should be noted although that empirical

estimates also yield the value of 1.8 $cm^3$ $mol^{-1}$ for ($V_0$ − $V_h$) (*54*).

Thus, the binding of BPTI to trypsinogen results in a release of 110 ± 40 water molecules to the bulk state. Significantly, this number corresponds to the number of water molecules within the first coordination layer of the interfacial area that can be estimated by dividing $\Delta S_A$ by 9 $Å^2$, the effective cross-section of a water molecule (998/9 = 111). This coincident finding is suggestive of the interacting surfaces of the two proteins being solvated by a single layer of water molecules. This observation contradicts the notion that the hydration shell of a native globular protein consists of two to three layers of water molecules (*39*, *41*, *53*). Multiple layers of protein hydration were invoked to rationalize the data on the partial molar volume and adiabatic compressibility of 12 globular proteins (*39*). In addition, our previously determined numbers of water molecules released to the bulk upon the binding of 2′-CMP and 3′-CMP to ribonuclease A and OMTKY3 to α-chymotrypsin are two to three times larger than the number of water molecules within the first coordination layer of the respective interacting surfaces (*14*, *48*). These observations have provided a further support to the notion that ribonuclease A, OMTKY3, and α-chymotrypsin (more precisely, their binding sites) are solvated by two to three layers of water molecules. By contrast, 110 ± 40 water molecules released to the bulk upon the binding of BPTI to trypsinogen correspond to a single layer of hydration. As a working hypothesis, the smaller than expected number of water molecules released to the bulk upon BPTI−trypsinogen association can be rationalized if one takes into account that free trypsinogen is partially unfolded. In contrast to native globular proteins, small molecules and unfolded polypeptides are solvated by a single layer of water molecules (*41*). By extension, the disordered domain (the activation domain) of trypsinogen should be solvated by a single layer of water molecules (*9*, *10*). Since BPTI−trypsinogen association involves part of the activation domain, reduced dehydration of the proteins can be expected. In addition, due to the binding-induced disorder-to-order transition of the activation domain of trypsinogen, the local hydration of the solvent-exposed regions of the domain should increase (from a single to a double or triple layer). This enhancement of trypsinogen hydration may be manifested as an apparent reduction in the net dehydration effect of BPTI−trypsinogen complexation. Other rationalizations of the observed lower than expected number of water molecules released to the bulk may be put forward. For example, one may suggest that not all water molecules that are released upon the binding of BPTI to trypsinogen exhibit an altered density. Alternatively, the BPTI−trypsinogen interface may retain some water molecules that are not localized and, therefore, cannot be detected by X-ray crystallography. Even though our opinion is that these possibilities are rather unlikely, further studies are required to elucidate the origins of the small value of $\Delta n_h$. To this end, osmotic stress measurements might prove particularly useful and informative (*55*−*58*).

Armed with the value of $\Delta n_h$, we evaluate the change in the hydration contribution to compressibility, $\Delta\Delta K_h$, in eq 9. The value of $\Delta\Delta K_h$ equals the product of $\Delta n_h$ and ($K_{S0}$ − $K_{Sh}$), where ($K_{S0}$ − $K_{Sh}$) is the average difference in the partial molar adiabatic compressibility between bulk water and water of protein hydration. The value of ($K_{S0}$ − $K_{Sh}$) has been estimated to be $1.3 \times 10^{-4}$ $cm^3$ $mol^{-1}$ $bar^{-1}$ (∼20% of the partial molar adiabatic compressibility of bulk water) (*53*). Our calculated value of $\Delta\Delta K_h$ is 0.0143 ±0.0052 $cm^3$ $mol^{-1}$ $bar^{-1}$ [(110 ± 40) × $1.3 \times 10^{-4}$]. The change in the intrinsic compressibility of the proteins, $\Delta K_M$, can be estimated from eq 9 as $\Delta K_M = \Delta K_{Sb} - \Delta\Delta K_h = -0.0661$ ± 0.0073 $cm^3$ $mol^{-1}$ $bar^{-1}$ [−(0.0518 ± 0.0051) − (0.0143 ± 0.0052)]. By differentiating $K_M = \beta_M V_M$, the following relationship can be derived:

$$\Delta K_M = \beta_M \Delta V_M + \Delta\beta_M V_M \qquad (11)$$

For globular proteins, the intrinsic coefficient of adiabatic compressibility, $\beta_M$, is $\sim 25 \times 10^{-6}$ $bar^{-1}$ (*39*−*41*, *46*). Using this value and eq 11, we calculate the binding-induced change in the intrinsic coefficient of adiabatic compressibility, $\Delta\beta_M$, of BPTI and trypsinogen to be $-(3.5 \pm 0.4) \times 10^{-6}$ $bar^{-1}$. This value signifies a decrease of 14 ± 2% in the intrinsic coefficient of adiabatic compressibility of the two proteins upon their association.

In the aggregate, our volumetric results suggest that BPTI−trypsinogen complexation results in a release to the bulk of 110 ± 40 water molecules and rigidification of the proteins' interior as manifested in a 14 ± 2% decrease in $\beta_M$. Significantly, the latter result is in qualitative agreement with the previous NMR and X-ray crystallographic data, which also suggest that the two proteins become more tightly packed upon their association (*9*, *10*).

*Resolving Entropy Data in Terms of Configurational and Hydrational Contributions.* The binding entropy, $\Delta S_b$, for a protein association event can be presented as the sum of the intrinsic (configurational), $\Delta S_{conf}$, hydrational, $\Delta S_{hyd}$, and translational/rotational, $\Delta S_{trans}$, terms (*59*):

$$\Delta S_b = \Delta S_{conf} + \Delta S_{hyd} + \Delta S_{trans} \qquad (12)$$

For 1:1 binding stoichiometry, the value of $\Delta S_{trans}$ is roughly −8 cal $K^{-1}$ $mol^{-1}$ (*59*). The hydrational change in entropy, $\Delta S_{hyd}$, associated with the binding of BPTI to trypsinogen can be estimated by multiplying the number of water molecules released to the bulk, $\Delta n_h$, by the average difference in the partial molar entropy between bulk water and water of protein hydration, ($S_0$ − $S_h$). The data on entropy of hydration of various amino acid residues (*60*) suggest that, at 25 °C, the value ($S_0$ − $S_h$) is essentially independent of the chemical nature of a solvent-exposed atomic group and, on average, equals 1.3 ± 0.3 cal $mol^{-1}$ $K^{-1}$. With this value, we estimate the value of $\Delta S_{hyd}$ to be positive and equal to 143 ± 52 cal $mol^{-1}$ $K^{-1}$ [1.3 × (110 ± 40)]. The configurational change in entropy, $\Delta S_{conf}$, can be calculated from eq 12. In contrast to $\Delta S_{hyd}$, $\Delta S_{conf}$ is negative and equal to −97 ± 54 cal $mol^{-1}$ $K^{-1}$ [(38.3 ± 1.8) + 8 − (143 ± 52)].

The absolute value of the configurational entropy, $S_{conf}$, of a globular protein is defined as the entropy difference between the folded and fully unfolded conformations in a vacuum. In a previous work (*14*), we have analyzed the data on the absolute values of configurational entropies of globular proteins, $S_{conf}$, presented by Makhatadze and Privalov (*60*). At 25 °C, the value of $S_{conf}$ (in kcal $mol^{-1}$ $K^{-1}$) of a globular protein correlates with its molecular mass, $M$ (kDa), according to $S_{conf} = -0.54 + 0.17M - 0.0014M^2$ (with a correlation

coefficient of 0.98). On the basis of this relationship, we estimate the sum of the configurational entropies of free BPTI (6.5 kDa) and trypsinogen (24 kDa), $S_{conf}$, to be 3.24 kcal mol$^{-1}$ K$^{-1}$ (0.51 + 2.73). However, the real value of $S_{conf}$ should be smaller since free trypsinogen is partially disordered. The change in configurational entropy, $\Delta S_{conf}$, accompanying the binding of BPTI to trypsinogen constitutes ~3% (0.097/3.24) (or, possibly, more) of the initial value of $S_{conf}$ of the two unassociated proteins. A 3% decrease in configurational entropy signifies a reduction in the dynamics of the inhibitor and the enzyme upon their complexation. This entropy-detected diminution in protein dynamics is consistent with the observed tightening of the proteins' interior, as suggested by our measured decrease in the intrinsic coefficient of adiabatic compressibility, $\beta_M$ (14 ± 2%).

*Role of Water in the Binding.* At our experimental conditions, the binding of BPTI to trypsinogen is an entropy-driven event that proceeds with an unfavorable change in enthalpy. The relative order of the values of $\Delta S_{conf}$, $\Delta S_{hyd}$, and $\Delta S_{trans}$ suggests that the hydrational and configurational terms represent the major contributors to the binding entropy. The favorable change in hydrational entropy, $\Delta S_{hyd}$ (143 ± 52 cal mol$^{-1}$ K$^{-1}$), prevails over the unfavorable change in configurational entropy, $\Delta S_{conf}$ (−97 ± 54 cal mol$^{-1}$ K$^{-1}$), thereby providing the driving force for BPTI association with trypsinogen. We made a similar observation for the binding of turkey ovomucoid third domain (OMTKY3) to α-chymotrypsin (*14*).

It is instructive to compare the value of $\Delta G_b$ (−7.1 kcal mol$^{-1}$) with $-T\Delta S_{hyd}$ (−42.6 ± 15.5 kcal mol$^{-1}$). This comparison reveals that $\Delta G_b$ constitutes only ~17 ± 6% of $-T\Delta S_{hyd}$ and underscores the vital role of hydration in the energetics of BPTI association with trypsinogen. The importance of hydration for determining the affinity and specificity of protein recognition has been recognized and emphasized by a number of researchers (*61*−*67*). Results of the present work as well as our previous results (*14*) provide a quantification of the role of water in modulating protein−protein recognition events while also underscoring the fact that hydration must be carefully taken into account in any rigorous analysis and prediction of the energetics of protein recognition.

## CONCLUDING REMARKS

We report changes in spectroscopic and volumetric properties accompanying the binding of BPTI to trypsinogen at pH 8.0. The binding of the inhibitor to the zymogen at 25 °C results in an increase in volume, $\Delta V_b$, of 278 ± 45 cm$^3$ mol$^{-1}$ and a decrease in adiabatic compressibility, $\Delta K_{Sb}$, of (−518 ± 51) × 10$^{-4}$ cm$^3$ mol$^{-1}$ bar$^{-1}$. We interpret these changes in conjunction with three-dimensional structures of the complex and the free proteins in terms of the binding-induced changes in hydration and intrinsic packing of the two proteins. BPTI binding to trypsinogen brings about a release to the bulk of 110 ± 40 water molecules from the proteins' hydration shells, while the coefficient of adiabatic compressibility of the proteins' interior diminishes by 14 ± 2%.

Our van't Hoff analysis of the temperature-dependent binding profiles suggests that BPTI association with trypsinogen is an entropy-driven event with an unfavorable enthalpic contribution. At 25 °C, BPTI association with trypsinogen causes changes in free energy, $\Delta G_b$, van't Hoff enthalpy, $\Delta H_b$, and entropy, $\Delta S_b$, of −7.1 ± 0.2 kcal mol$^{-1}$, 4.3 ± 0.5 kcal mol$^{-1}$, and 38.3 ± 1.8 cal mol$^{-1}$ K$^{-1}$ respectively. We use the measured change in entropy in conjunction with our volumetric results to evaluate the hydrational, $\Delta S_{hyd}$, and configurational, $\Delta S_{conf}$, contributions to $\Delta S_b$. The values of $\Delta S_{hyd}$ and $\Delta S_{conf}$ are equal to 143 ± 52 and −97 ± 54 cal mol$^{-1}$ K$^{-1}$, respectively. Thus, a highly favorable change in hydrational entropy, $\Delta S_{hyd}$, prevails over an unfavorable change in configurational entropy, $\Delta S_{conf}$, thereby providing the thermodynamic impetus for the binding of BPTI to trypsinogen. The relative magnitude of $\Delta G_b$ is only 17 ± 6% of $-T\Delta S_{hyd}$, which suggests that, if only ~17% fewer water molecules were released to the bulk, the binding of BPTI to trypsinogen would not occur.

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