

Oligomerization affects the kinetics and thermodynamics of the interaction of a Bowman-Birk inhibitor with proteases

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ABSTRACT

The black-eyed pea trypsin/chymotrypsin inhibitor (BTCI) forms concentration dependent homomultimers, as previously demonstrated by Light scattering and Atomic Force Microscopy. Considering that these self-aggregates might influence their binding to cognate enzymes, we investigated the interaction of BTCI at picomolar concentrations using surface immobilized Chymotrypsin (α -CT) and Trypsin (T) by Surface Plasmon Resonance. Our results indicate that BTCI has subnanomolar affinity to both immobilized enzymes, which is approximately two orders of magnitude higher than previously reported. Moreover, we probed the influence of temperature on protein binding equilibria in order to investigate their interaction energetics. While the BTCI/T interaction concurs with the canonical entropy-driven mechanism described for BBI interactions with serine proteinases, the BTCI/ α -CT interaction does not. Our measurements indicate that bimolecular BTCI/ α -CT complexes form with a negative enthalpy change and a moderate entropic increase. Direct calorimetric evaluation is in accord with the van't Hoff approximation obtained by SPR. We demonstrate that as protein concentrations increase to the micromolar range, secondary endothermic events become prevalent and affect both the kinetics and thermodynamics of protein associations. Our study reinforces that BBI interactions with serine proteinases should be studied in dilute solutions to abridge often neglected secondary interactions.

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1. Introduction

Bowman-Birk inhibitors (BBIs) and their interactions with proteolytic enzymes have been the subject of intense research since the first reports of enzyme activity depletion due to inhibitor complexation [1]. In dicotyledonous plants, BBIs are present as double-headed inhibitors having molecular masses ranging from 6 to 9 kDa, seven disulphide bonds, and two homologous and independent enzyme-specific binding loops [2]. Although it has been

thoroughly reported that these molecules form simple bimolecular heterocomplexes with chymotrypsin (α -CT) and trypsin (T), the acquisition of accurate kinetic and thermodynamic parameters is challenging. Besides the specific interactions between inhibitory loops and enzyme active sites, secondary binding sites and self-aggregates have been described, which result in complex binding equilibria [3–5].

The black-eyed pea trypsin/chymotrypsin inhibitor (BTCI) is an 83-residue BBI (9.1 kDa) isolated from the seeds of *Vigna unguiculata* (c.v. Seridó) [6]. It presents rich biotechnological potential as an anticarcinogenic agent, as a guanylin-enhancing natriuresis and renal functional response in rats, as a bradykinin-potentiating molecule, and as an insecticide, inhibiting the growth of larvae with agricultural importance [7–12]. BTCI is composed mainly of anti-parallel β -sheets containing reactive sites for T (Lys26) and α -CT (Phe53) in two independent canonical loops connected by the β -hairpin motif [13]. Moreover, it forms stable binary and tertiary

Abbreviations: BTCI, black-eyed pea trypsin/chymotrypsin inhibitor; SPR, Surface Plasmon Resonance; ITC, Isothermal Titration Calorimetry; BBI, Bowman-Birk inhibitor; MUTMAC, 4-Methylumbelliferyl p-(NNN-trimethylaminonium)-cinnamate chloride; 4-MU, 4-methylumbelliferone.

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complexes with T and α -CT presenting binding constants from 10^7 to 10^9 M $^{-1}$ [14,15]. BTCI has an unusual solvent exposed hydrophobic patch with buried hydrophilic residues at subdomain 2, a characteristic feature of BBIs that is considered the source of their self-association tendencies [13,16,17]. BTCI forms concentration dependent self-oligomers, ranging from monomers to dimers, trimers and hexamers as demonstrated by light scattering and atomic force microscopy [18,19].

The present work explores the kinetics and thermodynamics of the BTCI interaction with surface immobilized α -CT and T at physiological pH by Surface Plasmon Resonance (SPR). This technique monitors the formation and dissolution of protein complexes in nanomolar/picomolar concentrations, where BTCI is thought to be predominantly in monomeric form according to light scattering measurements [18]. Direct calorimetric evaluation by isothermal titration calorimetry (ITC) is performed to validate SPR thermodynamic data. According to our measurements, BTCI interacts with α -CT and T with higher affinity and with different interaction energetics than previously estimated [14,15]. To our best judgment, it is feasible to attribute the observed discrepancies in kinetic and thermodynamic constants between our data and previous assessments to be a result of numerous secondary concentration-dependant binding events.

2. Material and methods

2.1. Proteins

α -CT from bovine pancreas was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany) and used without further purification. Sequencing grade modified T from Promega (Madison, WI, USA) was used due to pronounced autolysis observed in preliminary tests with unmodified trypsin, which precluded data acquisition (data not shown). Methylated T is referred plainly as T throughout the whole manuscript. BTCI was purified from the seeds of *Vigna unguiculata* as previously described [6]. Protein concentrations were determined by spectrophotometry (Shimadzu, Tokyo, Japan) according to previously published works [13].

2.2. Surface plasmon resonance and isothermal titration calorimetry

BTCI interactions with surface-immobilized serine proteinases were evaluated in a BIACORE 3000 (GE Healthcare, Buckinghamshire, UK) biosensor. HBS-EP buffer, CM5 sensor chips, amine coupling kits and immobilization and regeneration buffers were purchased from GE Healthcare. A MicroCal VP-ITC (GE Healthcare, Buckinghamshire, UK) was used to evaluate the BTCI/ α -CT interaction in solution. Filtered and degassed HBS-E buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA) was used for calorimetric evaluations.

2.3. α -CT and T sensor chip immobilization

CM5 sensor chips were docked and hydrated for about 24 h before protein immobilization. T and α -CT were immobilized by standard amine coupling using HBS-EP as running buffer. Both serine proteinases were individually resuspended in acetate buffer pH 5.5 at a concentration of 5 μ g.mL $^{-1}$ and injected in the flow cells activated with NHS/EDC until the desired immobilization levels were attained (approximately 700 RU for α -CT and 1200 for T). Experimental and control cells were deactivated with 1 M Ethanolamine. Lysine-methylated T was probably immobilized in its free N-terminus using standard amine coupling chemistry.

2.4. BTCI interaction analyses with α -CT and T by SPR

SPR analyses were performed at a 50 μ L.min $^{-1}$ flow rate. Regeneration conditions consisted in one 30s injection of 10 mM glycine buffer pH 2.4 for the BTCI/T interaction, and two injections for the BTCI/ α -CT interaction. BTCI was dissolved in HBS-EP buffer in serial two-fold dilutions, from 250 to 3.9 nmoles.L $^{-1}$. Concentration series were injected over surface immobilized enzymes. Each concentration series consisted of BTCI injected twice at each concentration at each evaluated temperature (15–35 °C in 5° intervals). Data corresponding to the association phase of interactions were acquired for 4 min, and the dissociation phase was monitored for 25 min due to the slow dissociation kinetics. Bulk refractive index changes were corrected using a reference flow cell and double reference buffer injections were performed. Sensorgrams were fit to a simple bimolecular model ($A + B \leftrightarrow AB$) using BIAevaluation 3.1. Global data fittings were performed for each concentration series.

2.5. Thermodynamic analysis of SPR binding data

Equilibrium kinetics data for the BTCI/ α -CT and BTCI/T interactions were fit to the non-linear form of the Gibbs-Helmholtz equation [20] using Origin 7.0 (Origin Lab, MA, USA).

$$\Delta G^0 = \Delta H_0 - T\Delta S_0 + \Delta C_p(T - T_0) - T\left(\Delta C_p \cdot \ln\left(\frac{T}{T_0}\right)\right) \quad (1)$$

Transition state analysis was carried out using the Eyring equations, which assume an active state in equilibrium with reactants ($A + B \leftrightarrow AB^* \leftrightarrow AB$) whose concentration determines the velocity of the reaction [21]. This should be considered simply as a convenient semi-empirical model in the current context.

2.6. Isothermal titration calorimetry

BTCI was submitted to 3 cycles of buffer exchange with HBS-E using Amicon 10 kDa centrifugal filters (Milipore, MA, USA), quantified and stored at –80 °C until use. The enzyme α -CT was prepared fresh just before titrations and submitted to 3 cycles of buffer exchange with HBS-E before protein quantification by spectrophotometry [13]. The operational molarity of α -CT after buffer exchange was estimated using a spectrofluorimetric method [22]. Briefly, a 5-fold molar excess of MUTMAC was incubated with α -CT in HBS-E buffer for 10 min following the quantification of 4-MU compared to a 1 nmole.L $^{-1}$ standard solution. Spectrofluorimetric measurements were performed in octuplicates on a Spectramax M2 instrument (Molecular Devices, CA, USA). Microcalorimetric reaction cells were filled with BTCI and titrated with α -CT; Protein concentrations are indicated in each experiment. Syringe rotor speed was set to 300 rpm and 28 α -CT injections were performed, 10 μ L each, following standard instrument procedures. α -CT dilution heats were determined in independent experiments and subtracted from the titration data. The BTCI/T interaction could not be evaluated due to trypsin autolysis, and the methylated T was not used due to high sample requirements. Calorimetric data were obtained and evaluated using the Origin software 7.0 (Origin Lab, MA, USA).

3. Results

3.1. BTCI has subnanomolar affinity to α -CT and T

The BTCI interaction kinetics with α -CT and T was evaluated by SPR at nanomolar concentrations from 15 to 35 °C. The enzymes α -

CT and T, the latter derivatized by reductive methylation, were immobilized on the dextran surface of CM5 sensor chips using standard amine coupling chemistry. Mass transport effects were considered negligible at 50 $\mu\text{L}/\text{min}$, and the linked reaction test detected no obvious intermediate state for both reactions (data not shown). Binding curves resulting from the injection of 250 to 3.9 nmol L^{-1} BTCI over surface-immobilized α -CT and T were fit to a simple bimolecular interaction model at the evaluated temperatures. Sensorgrams and their corresponding fittings (in red) are displayed in Fig. 1. Data for the BTCI/T interaction presented higher deviations from the model, possibly due to residual enzyme

autolysis [1]. BTCI both associates and dissociates with T twice as fast as it does with α -CT at 25 $^{\circ}\text{C}$, but both inhibitor/enzyme complexes present undistinguishable equilibrium binding affinities (Table 1). Indeed, the equilibrium dissociation constants (K_D) of the BTCI/ α -CT and BTCI/T complexes at 25 $^{\circ}\text{C}$ are 0.41 ± 0.00 and 0.42 ± 0.07 nM, respectively. Temperature had opposite effects on the stability of protein complexes: while the BTCI/ α -CT affinity decreased nearly three times as the reaction temperature shifted from 15 to 35 $^{\circ}\text{C}$, the formation of the BTCI/T complex was favored roughly by the same amount (Table 1).

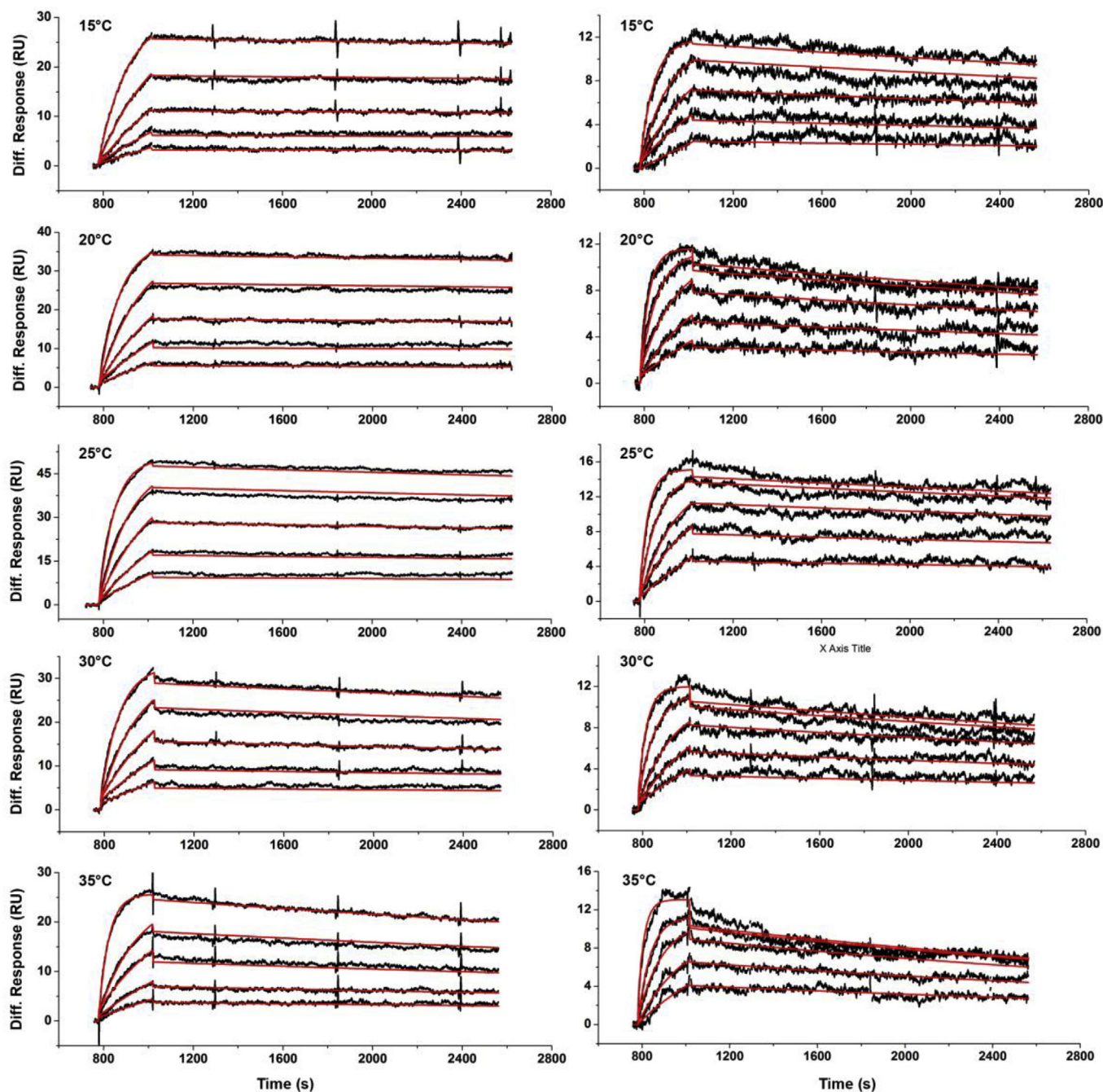


Fig. 1. Temperature dependence of the BTCI binding to surface-immobilized α -CT and T by surface plasmon resonance. Black lines correspond to the binding responses obtained by the injection of a concentration series of BTCI, ranging from 250 to 3.9 nM, over α -CT (Left column) and T (Right column) derivatized dextran sensor chips at the temperatures shown at the sensorgrams. Red lines correspond to data fittings obtained by the adjustment to the simple bimolecular model ($A + B \leftrightarrow AB$).

Table 1
Kinetic parameters of the BTCl/ α -CT and BTCl/T interactions as a function of temperature. Data are presented as mean \pm standard deviation for 2 independent concentration series, each concentration injected in duplicate.

Temp (K)	BTCl: α -CT			BTCl:T		
	$k_a \times 10^4 \text{ M}^{-1}\text{s}^{-1}$	$k_d \times 10^{-5} \text{ s}^{-1}$	$K_D \times 10^{-10} \text{ M}$	$k_a \times 10^4 \text{ M}^{-1}\text{s}^{-1}$	$k_d \times 10^{-5} \text{ s}^{-1}$	$K_D \times 10^{-10} \text{ M}$
288	6.3 \pm 0.4	2.4 \pm 0.1	3.83 \pm 0.00	11 \pm 1	10 \pm 2	9.6 \pm 0.9
293	8.57 \pm 0.04	3.1 \pm 0.5	3.6 \pm 0.6	19.2 \pm 0.1	11.7 \pm 0.5	6 \pm 2
298	11.3 \pm 0.03	4.6 \pm 0.1	4.07 \pm 0.04	21 \pm 3	9 \pm 1	4.2 \pm 0.7
303	15.6 \pm 0.1	9.5 \pm 0.8	6 \pm 1	44 \pm 6	18 \pm 6	4.0 \pm 0.7
308	17.70 \pm 0.02	14.6 \pm 0.2	8 \pm 1	44.05 \pm 0.05	16.0 \pm 0.8	4 \pm 2

3.2. BTCl interacts with α -CT and T with distinct thermodynamic profiles

SPR derived equilibrium dissociation constants were used to calculate the Gibbs free energy of binding (ΔG) of the BTCl/ α -CT and BTCl/T complexes and the Gibbs-Helmholtz equation was fit to the data (Eq. (1)). A linear dependency was observed between the ΔG and reaction temperature for the BTCl/T interaction, indicating a negligible heat capacity change ($\Delta C_{p_{vh}}$) upon complex formation (Fig. 2). On the other hand, the complexation between BTCl and α -CT occurred with a negative $\Delta C_{p_{vh}}$, as it is observed in other protein-protein interactions (Table 2) [23]. While the formation of the BTCl/ α -CT complex is exothermic and favored by an entropy increase ($\Delta H^\circ_{vh} = -7.5 \pm 0.9 \text{ kcal.mol}^{-1}$ and $\Delta S^\circ_{vh} = 18 \pm 3 \text{ cal.mol}^{-1}\text{K}^{-1}$), the BTCl/T interaction is endothermic and therefore driven by a large entropy increase ($\Delta H^\circ_{vh} = 8 \pm 2 \text{ kcal.mol}^{-1}$ and $\Delta S^\circ_{vh} = 70 \pm 6 \text{ cal.mol}^{-1}\text{K}^{-1}$). Eyring equations were used to dissect the reaction pathways by introducing a theoretical transient state (Fig. 2A and B) [21]. Theoretical reaction pathways were built based on transition state theory and they are depicted in Fig. 3.

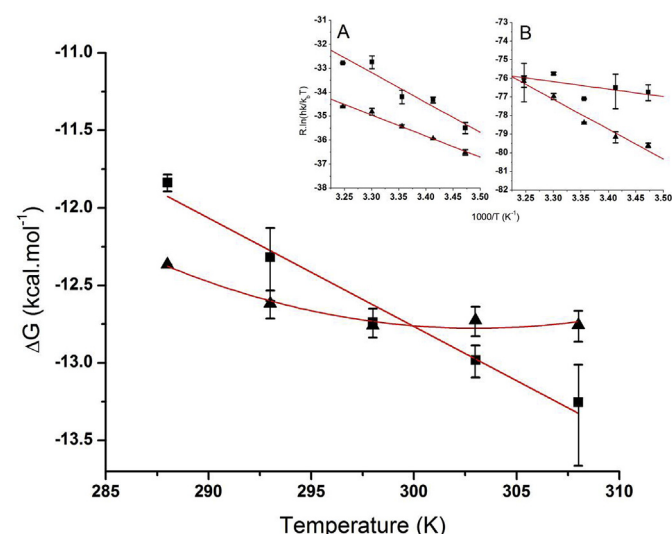


Fig. 2. Gibbs free energy of binding of the BTCl/ α -CT and/T complexes and the temperature dependence of rate constants. Equilibrium dissociation constants obtained for the BTCl/ α -CT (▲) and /T (■) interactions were used for the calculation of the Gibbs free energy of binding as a function of temperature. Data were fit to a non-linear form of the Gibbs-Helmholtz equation (red line) resulting in the thermodynamic parameters listed in Table 2. Insets A and B correspond to the Eyring plots generated for k_a and k_d for BTCl/ α -CT (▲) and /T (■). Pearson correlation coefficients were higher than 0.95, except for the k_d of the BTCl/T complex ($R = 0.65$). Each data point is the average of duplicates and error bars correspond to standard deviation.

Table 2
Thermodynamics aspects of the formation of the BTCl/ α -CT and BTCl/T complexes.

	BTCl/ α -CT	BTCl/T
Gibbs-Helmholtz		
$\Delta G \text{ (kcal.mol}^{-1}\text{)}^a$	-12.76 ± 0.01	-12.74 ± 0.09
$\Delta H \text{ (kcal.mol}^{-1}\text{)}$	-7.5 ± 0.9	8 ± 2
$\Delta S \text{ (cal.mol}^{-1}\text{K}^{-1}\text{)}$	18 ± 3	70 ± 6
$\Delta C_{p_{vh}} \text{ (kcal.mol}^{-1}\text{K}^{-1}\text{)}$	-1.0 ± 0.3	
Transition State Theory		
$\Delta H_a \text{ (kcal.mol}^{-1}\text{)}$	8.8 ± 0.6	12 ± 2
$\Delta H_d \text{ (kcal.mol}^{-1}\text{)}$	16 ± 2	4 ± 3
$\Delta S_a \text{ (cal.mol}^{-1}\text{K}^{-1}\text{)}$	-6 ± 2	8 ± 7
$\Delta S_d \text{ (cal.mol}^{-1}\text{K}^{-1}\text{)}$	-24 ± 6	-63 ± 9

^a ΔG obtained from $-RT \ln(K_A)$.

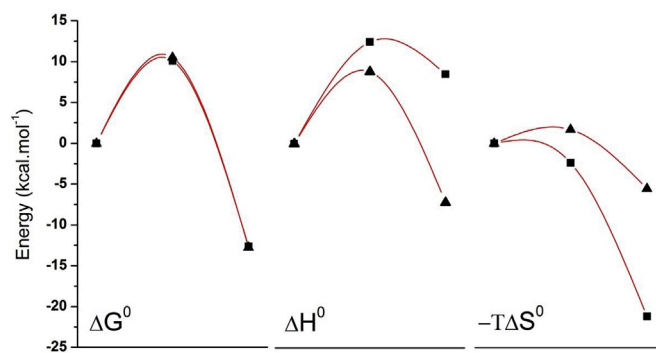


Fig. 3. Energy profiles for the BTCl interaction with serine proteinases at pH 7.4. Theoretical minimum reaction paths for the BTCl/ α -CT (▲) and/T (■) interactions. Plots demonstrate ΔG° , ΔH° and $-T\Delta S^\circ$. Continuous red lines are used as a guide to the eye.

3.3. BTCl interaction with α -CT in solution by ITC

Considering that the BTCl/ α -CT thermodynamics described herein contradicts previously published data [14], we sought to validate results by direct thermodynamic measurements. For such, the BBI was loaded in the ITC microcalorimeter cell at 3 μM and titrated at 25 $^\circ\text{C}$ with α -CT at 20 μM , in HBS-E buffer, pH 7.4. The operational molarity of α -CT following sample preparation was $94 \pm 3\%$, indicating that the enzyme remains competent after buffer exchange. The steepness of the binding curve in the titration isotherm prevented an accurate estimation the association constant (K_A) for these proteins by ITC, therefore the kinetic parameters obtained by SPR were used. The measured BTCl/ α -CT enthalpy and entropy of interaction at 25 $^\circ\text{C}$ were $\Delta H^\circ_{cal} = -7.0 \pm 0.1 \text{ kcal.mol}^{-1}$ and $\Delta S^\circ_{cal} = 19 \text{ cal.mol}^{-1}\text{K}^{-1}$ (Fig. 4). Increasing the BTCl concentration in the reaction cell to 5 μM introduced non-negligible endothermic events following the association between the inhibitor and the enzyme (Fig. 4B). Further six-fold increase in BTCl concentration (Fig. 4C) introduced

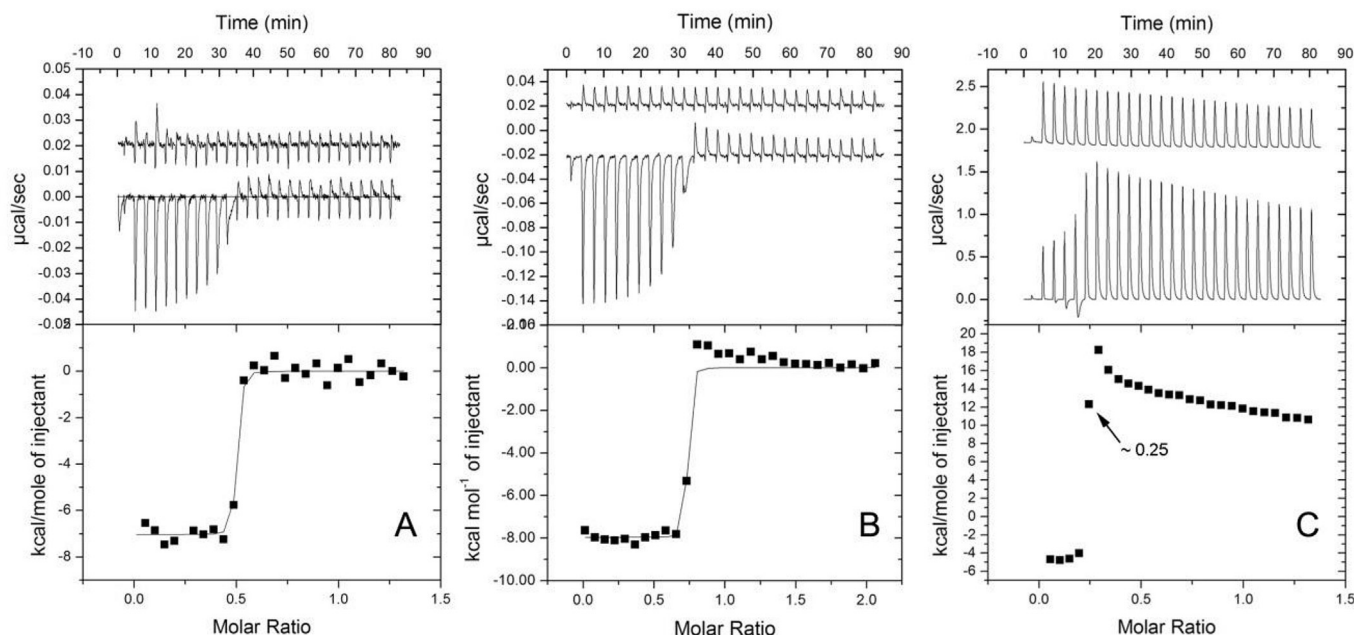


Fig. 4. Calorimetric data for the interaction of BTCl with α -CT with varying protein concentrations. Raw data and integrated calorimetric traces for the injection $28 \times 10 \mu\text{L}$ A) $20 \mu\text{M}$ α -CT into $3 \mu\text{M}$ BTCl, B) $50 \mu\text{M}$ α -CT into $5 \mu\text{M}$ BTCl and C) $200 \mu\text{M}$ α -CT into $30 \mu\text{M}$ BTCl. Raw data of control α -CT injections in HBS-E buffer are depicted above BTCl- α -CT calorimetric traces.

significant post-active site titration endothermic events, producing a complex titration data. At the concentration of $30 \mu\text{M}$, the association between BTCl and α -CT active sites was followed by more significant endothermic events. Moreover, the reaction stoichiometry shifted from 1:~2 to 1:~4 α -CT:BTCl as BTCl concentrations increased from 3 to $30 \mu\text{M}$ in the reaction cell (Fig. 4A–C).

4. Discussion

The present work reports the kinetics and thermodynamics of the interaction between BTCl, a double-headed BBI, with the serine proteinases α -CT and T by SPR. Dilute protein solutions were used as an effort to abridge the concentration-dependant multiple linked chemical equilibria that are characteristic to BBI interactions with serine proteinases [4]. The agreement between SPR sensorgrams and the bimolecular model observed in Fig. 1 indicates that simple protein associations occurred in this experimental setup, without appreciable parallel reactions (Table 1, Fig. 1). SPR data specifies that, at nanomolar concentrations, BTCl has an undistinguishable affinity to both surface-immobilized enzymes (Table 1). The K_D of the BTCl interaction with α -CT and T were previously estimated as $1.15 \times 10^{-7} \text{ M}$ and $6.25 \times 10^{-9} \text{ M}$ at 298 K , respectively [14,15]. Data obtained by SPR represents approximately a 300-fold higher affinity for α -CT and a 15-fold higher affinity for T than previously assessed by indirect assays using samples at micromolar concentrations [14,15]. We propose that BTCl multimers, which become more prevalent with increasing protein concentration, constitute less or even unreactive inhibitor species by hindering the enzyme access to the inhibitor enzyme-binding loops, as previously indicated by AFM data [13]. Evaluation of the BTCl molecule X-ray structure suggest that the α -CT binding loop should indeed be more influenced by protein aggregation, in accordance with our observations. The BTCl α -CT binding loop (Phe53) is encoded within the subdomain 2 region, recognized as the hydrophobic core that drives BBI multimerization [13]. Further evidence is obtained by AFM with the detection of an unreactive BTCl fraction following the inhibitor incubation with

equimolar amounts of α -CT, while only bimolecular complexes are detected after its incubation with T [13].

Distinct thermodynamic profiles were obtained for the BTCl interaction with α -CT and T by SPR at 25°C (Table 2, Fig. 3). While the BTCl/T interaction is endothermic and driven by entropy, which concurs with previously obtained data [15,24], the BTCl/ α -CT interaction is exothermic, in disagreement with previous assays [14]. The ITC data in Fig. 4 sheds light in these discrepancies. Previous BTCl/ α -CT thermodynamic analyses were conducted with a minimum BTCl concentration of $6.2 \mu\text{M}$ [14], a concentration where secondary binding events, characterized by endothermic peaks, were detected by ITC (Fig. 4B). Indeed, at $30 \mu\text{M}$ these endothermic events become not only detectable, but constitute the main source of heat exchange in the titration of BTCl with α -CT (Fig. 4C). We cannot, at present, define the specific molecular events that result in such endothermic heat exchanges. However, we hypothesize that they are related to the dissolution of pre-formed BTCl self-aggregates and/or α -CT dimers [25], in an event that precedes the formation of specific enzyme/inhibitor complexes. Further experiments will be conducted to investigate such hypotheses. Moreover, unreactive BTCl self-aggregates can also explain the disparities in the reaction stoichiometry measured by SPR and ITC experiments. While the first detects only bimolecular complexes (Table 1), the latter shows a concentration dependent reaction stoichiometry (Fig. 4A–C). Possible explanations reside in the SPR inherent insensitivity to pre-formed unreactive BTCl molecular species, which, if present at the assayed concentrations, are flowed-through the chip surface without producing measurable changes in refractive index.

The present paper demonstrates that BTCl forms simple bimolecular complexes with T and α -CT with similar affinity but distinct thermodynamic profiles when probed by Surface Plasmon Resonance. Moreover, the stoichiometry, kinetics and thermodynamics of the BTCl complexation with α -CT are consistently influenced by the increment in protein concentrations from the nanomolar to the micromolar range [13,14,26]. Our study indicates that secondary interactions should play an important role in the kinetic and

thermodynamic parameters in protein interactions in certain experimental setups. Moreover, it reinforces that BBI interactions with serine proteinases should be studied in dilute solutions to abridge secondary interactions, which are often overlooked in biophysical investigations, and are much closer to the actual physiological scenario of these proteins.

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