Complexation of the Tissue Plasminogen Activator Protease with Benzamidine-type Inhibitors: Interference by the Kringle 2 Module†

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ABSTRACT: Well-resolved high-field ^{1}H NMR signals between −0.1 and −0.7 ppm afford convenient probes to monitor the conformational state of the tissue plasminogen activator (tPA) protease, modulated by covalent inhibitor binding or activation cleavage [Hu, C.-K., Kohnert, U., Wilhelm, O., Fischer, S., & Llinás, M. (1994) *Biochemistry 33,* 11760−11766]. We have investigated recombinant BM 06.022 (a domain-deletion variant mutant from *Escherichia coli* comprising the kringle 2 and protease modules) and protease constructs of tPA in both single-chain (sc) and two-chain (tc) forms. The two proteins were studied when confronted with the noncovalent (i.e., reversible) active site inhibitors benzamidine and a series of bisbenzamidine derivatives: 2,5-bis(4-amidinobenzylidene)cyclopentanone, 2,6-bis(4-amidinobenzylidene)cyclohexanone, 2,7-bis(4-amidinobenzylidene)cycloheptanone, and 2,8-bis(4-amidinobenzylidene)cyclooctanone. At pH* 4.6, the ^{1}H NMR spectrum is sensitive to complexation of the protease module with the various effectors. The amplitude of the inhibitor-shifted resonances is more pronounced for the tc-protease than for the sc-protease, suggesting that access of inhibitors to the protease catalytic site is facilitated upon conversion to the tc form. The effects detected by the NMR spectrum suggest a biphasic process, involving stronger (primary) and weaker (secondary) bindings to a single protease active site. Binding to the protease module in tc-BM 06.022 essentially generates the same spectral characteristics as detected upon binding to the isolated tc-protease construct. In contrast, a negligible perturbation by the inhibitors is observed on the (sc) BM 06.022. Hence, in the intact BM 06.022 the kringle 2 is structurally coupled to the protease module thus interfering with inhibitor molecules from accessing the protease active site. These domain−domain interactions relax upon conversion to the catalytically active tc form, thus decoupling the kringle 2 from the protease module in BM 06.022 while simultaneously exposing the active site to become accessible to effectors or substrates.

The tissue-type plasminogen activator (tPA)[1] is a trypsin-like proteinase (Strassburger *et al.*, 1983) with Asp371, His322, and Ser478 configuring the catalytic triad. Plasmin (Wallén *et al*., 1981), tissue kallikrein, and blood coagulation factor Xa (Ichinose *et al.*, 1984) catalyze hydrolysis of the Arg275−Ile276 peptide bond of tPA thereby converting the latter single-chain (sc-) configuration to a two-chain (tc-) form (Wallén *et al*., 1982, 1983) in which the two polypeptides remain linked through the Cys264−Cys395 bridge. The protease module of human tPA has been expressed in *Escherichia coli* as a recombinant protein (tPA*del*4-261; Hu *et al.*, 1994). Recombinant BM 06.022 is a non-glycosylated, single-chain polypeptide (tPA*del*4-175) that comprises the human tPA kringle 2 and protease modules (Kohnert *et al.*, 1992). BM 06.022 has been developed as a fibrinolytic agent of clinical potential, and its biochemical properties are similar to those of tPA (Kohnert *et al.*, 1992, 1993, Stürzebecher *et al.*, 1992). BM 06.022 affords a convenient model system to investigate potential kringle 2-protease domain−domain interactions in tPA (Hu *et al.*, 1994).

1,5-Dansyl-L-glutamylglycyl-L-arginylchloromethyl ketone (EGRck) is a serine protease inhibitor which reacts to form a covalent linkage with His322 of tPA (Kettner & Shaw, 1979). No structural difference between sc and tc forms of tPA have been found by monitoring EGRck fluorescence (Higgins & Lamb, 1986) or sulfonate nitroxide spin labels and fluorophores (Nienaber *et al.*, 1992). In contrast, intrinsic (Loscalzo, 1988) and anthranilate fluorophore (Nienaber *et al.*, 1992) fluorescence studies have indicated that sc- and tc-tPA may exist in different conformations in the absence of fibrin. In addition, the former study (Loscalzo, 1988) suggested that binding of sc-tPA to fibrin might alter its conformation to become closer to that of tc-tPA. This is consistent with the observation that, while in the absence of fibrin the kinetics of plasminogen activation by sc-tPA is at least one order of magnitude slower than by

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[1] Abbreviations: bbzi, bisbenzamidine inhibitor; bbzi-12, 2,5-bis(4-amidinobenzylidene)cyclopentanone; bbzi-13, 2,6-bis(4-amidinobenzylidene)cyclohexanone; bbzi-14, 2,7-bis(4-amidinobenzylidene)-cycloheptanone; bbzi-15, 2,8-bis(4-amidinobenzylidene)cyclooctanone; BM 06.022, recombinant human tPA*del*4-261 from transformed *E. coli* cells; CHO-tPA, recombinant human tPA from transformed Chinese hamster ovary cells; EGRck, 1,5-dansyl-L-glutamylglycyl-L-arginyl-chloromethyl ketone; K2, kringle 2; K_d, ligand−protein equilibrium dissociation constant; K_i, inhibition constant; P, protease; pH*, glass electrode pH reading uncorrected for deuterium isotope effects; sc-, single-chain; tc-, two-chain; tPA, human tissue plasminogen activator; 7AHA, 7-aminoheptanoic acid.

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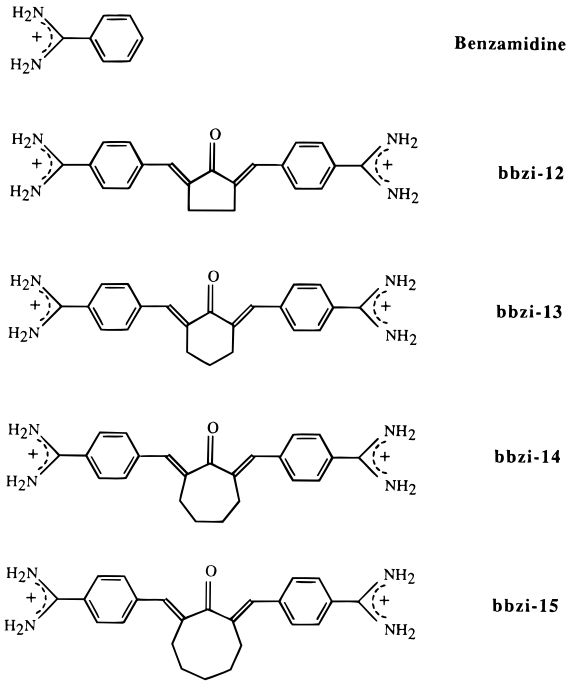
Chart 1. Benzamidine and Related Inhibitors of the tPA Protease[a]



tc-tPA, in the presence of fibrin activation of plasminogen by sc-tPA occurs at a rate that is comparable to that of tc-tPA [reviewed by Andreasen *et al.* (1991)].

Benzamidine and structurally related bisbenzamidine derivatives are well known competitive inhibitors for enzymes of the trypsin family (Stürzebecher *et al.*, 1976, 1992). The same inhibitors are variously effective on tPA and the truncated recombinant BM 06.022 mutant. The inhibition constant (K_i) values for the sc forms of both enzymes are higher by one order of magnitude when compared to tc forms. For bisbenzamidine inhibitors (bbzi's), the K_i values for the tc forms range between 1 and 10 μM at pH 8.0 (Stürzebecher *et al.*, 1992). Thus, it is justified to assume that the various bbzi analogs effectively interact with the catalytic site of tPA and BM06.022.

In this paper, we report a ^{1}H NMR study of the effects of sc-to-tc conversion on BM 06.022 and on the intact, recombinant protease module. The study hinges on characterizing the state of the various constructs through their interactions with the noncovalent benzamidine-type protease inhibitors (Chart 1) as specific active site probes. Large protein size (M_r ~30 160 for recombinant protease and M_r ~39 589 for BM 06.022) and low solubility (≤0.6 mM at pH* ~4.6, and mostly insoluble in the ~5−8 pH* range) of the investigated proteins preclude, as yet, exploiting homonuclear multidimensional spectroscopic experiments on the available samples. However, although the ^{1}H NMR spectra are broad, they prove to be sensitive to the conformational state of the proteins, particularly to changes accompanying the activation cleavage or those resulting from EGRck binding (Hu *et al.*, 1994). Both mechanisms induce shifts of high-field resonances of the protease (Figure 1a,b) or of BM 06.022 (Figure 1c,d) while leaving, in the latter, the kringle 2 spectrum essentially unchanged (notice, e.g., the kringle 2 Leu226 δ-methyl signal in Figure 1c,d). Here, we show that the protease conformational state is distinctively perturbed by the various benzamidine inhibitors and present

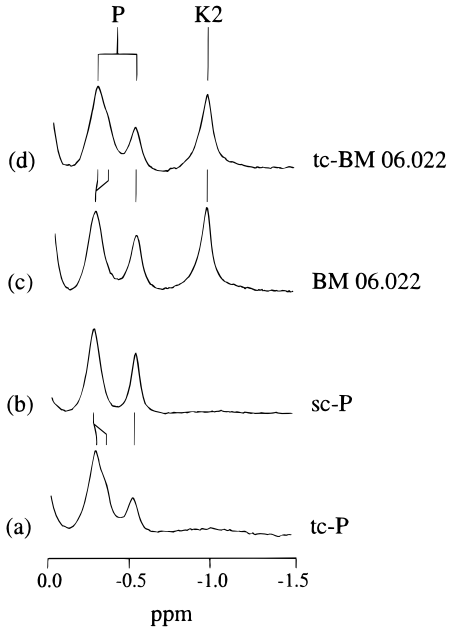


FIGURE 1: High-field ^{1}H NMR spectra of tc-protease (a), sc-protease (b), BM 06.022 (c), and tc-BM 06.022 (d). K2 and P label resonances from kringle 2 (Leu226 δ-methyl) and protease (unassigned) modules, respectively. Resonance shifts induced upon sc to tc conversion are indicated by kinked vertical lines. Spectra were recorded at pH* 4.6, 310 K.

evidence suggesting both high and low affinity binding of various bbzi's at, or neighboring, the protease catalytic pocket. Specifically, evidence is presented indicative of domain−domain interactions between the kringle 2 and protease modules in BM 06.022. The results fit the paradigm that the activation process relaxes the conformational state of the protease while leaving the kringle 2 essentially unaffected and structurally decoupling one from the other.

MATERIALS AND METHODS

Recombinant protease and BM 06.022 proteins were expressed in *E. coli* and converted to tc forms via specific hydrolysis with immobilized plasmin (Kohnert *et al.*, 1992; Hu *et al.*, 1994). EGRck was purchased from Calbiochem, San Diego, CA. Benzamidine and 7-aminoheptanoic acid (7AHA) originated from Aldrich Chemical Co., Milwaukee, WI. The bisbenzamidines were synthesized according to Wagner (1982).

For the NMR studies, the protein samples were preexchanged with ^2H_2O, lyophilized, and dissolved into 0.4 mL of ^2H_2O (99.996 atom % ^{2}H, Isotec Inc., Miamisburg, OH). Final protein concentrations were about 0.4−0.6 mM. The pH* was adjusted by additions of dilute ^{2}HOAc or NaO^{2}H. Quoted pH* values are glass electrode pH meter readings, uncorrected for the ^{2}H isotope effect. Chemical shifts are referred to the sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]-propionate signal, using *p*-dioxane as an internal standard (De Marco, 1977). ^{1}H NMR spectra were recorded at 500 MHz on a Bruker AM-500 spectrometer equipped with an Aspect 3000 minicomputer. The residual solvent $^1H^2HO$ signal was suppressed by gated low-power irradiation during the relaxation delay of 1.2−3.0 s introduced between scans. Data were collected in quadrature detection mode with a spectral width of 6500 Hz. A total of 16K data points were acquired for each spectrum. Between ~4000 and 8200 scans were combined for acceptable signal-to-noise ratios. Data

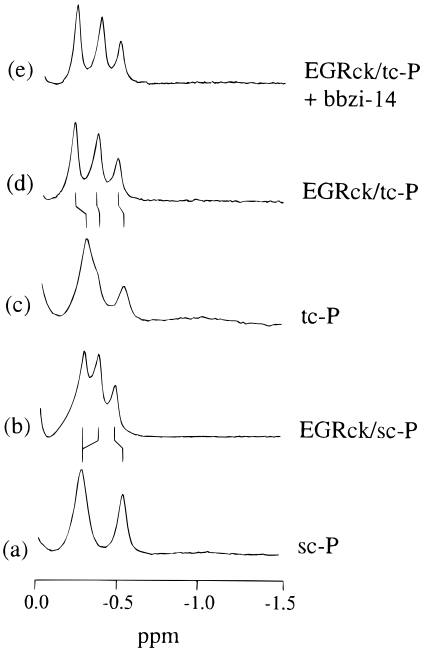


FIGURE 2: High-field ^{1}H NMR spectra of protease (a, c), EGRck-modified protease (b, d, e), sc form (a, b), tc form (c, d), and tc form in the presence of 7-fold excess bbzi-14 (e). Same experimental conditions as for Figure 1.

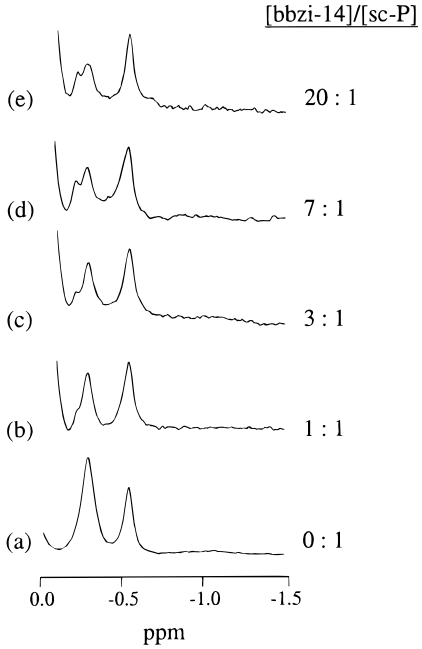


FIGURE 3: Effect of bbzi-14 on the high-field ^{1}H NMR spectrum of the sc-protease. Numbers indicate the [inhibitor]/[protease] molar concentration ratio. Same experimental conditions as for Figure 1.

were processed using the program FELIX, version 2.3 (BIOSYM, California). Prior to Fourier transformation, the data were zero-filled to 32K and subjected to slight digital filtering.

RESULTS

Reaction with EGRck causes the sc-protease resonances at ~−0.54 and −0.29 ppm to shift to ~−0.48, −0.37, and −0.28 ppm (Figure 2a,b); a similar, albeit more marked, effect is observed for the tc-protease (Figure 2c,d). This is consistent with a structural perturbation resulting from inhibitor binding which amplifies the one arising from activation cleavage (Hu *et al.*, 1994).

The effects of the bisbenzamidine inhibitor-14 (bbzi-14) on the high-field ^{1}H NMR spectra of the sc-protease are illustrated in Figure 3. In analogy with what is observed for the covalent binding of EGRck (Figure 2a,b), the high-field signals respond monotonically to increasing levels of bbzi-14, a noncovalent, reversible inhibitor (Figure 3b−e). From inspection of Figure 3, it is apparent that the spectra at intermediate inhibitor concentration (traces b and c) are basically scaled superpositions of spectra a and d, a reflection of slow exchange between inhibitor-free and inhibitor-bound states, at equilibrium. Upon raising the pH to 7.2, spectrum e remains essentially unperturbed (not shown), indicating that the binding interaction is preserved at physiological pH.

Figure 4 illustrates the effects of bbzi-14 on the high-field spectrum of the tc-protease. By comparison to what is observed for the sc-protease (Figure 3a−d), qualitatively similar spectral perturbations result from bbzi-14 binding to the tc-protease (Figure 4a−d). However, in contrast to what is the case for the sc form, where saturation is approached at an [inhibitor]/[protease] molar concentration ratio ~20, inhibitor binding to the tc form nears saturation at [inhibitor]/[protease] ~5 (Figure 4d,e), suggesting that access of bbzi-14 to its binding site is relatively more facile for the tc-protease than for its sc form. This would be consistent with a more open, relaxed conformation for the tc form.

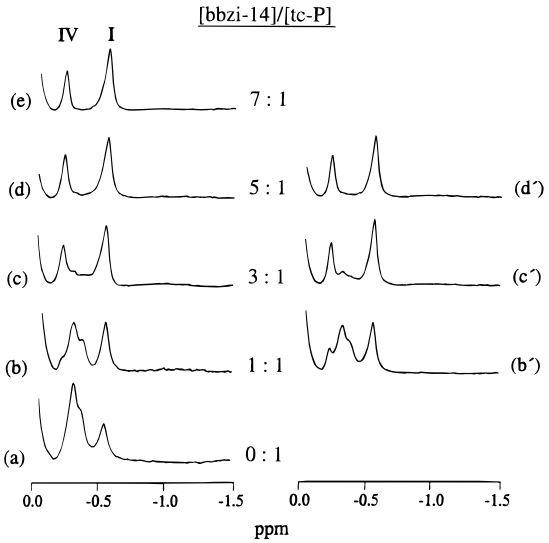


FIGURE 4: Effect of bbzi-14 on the high-field ^{1}H NMR spectrum of the tc-protease. (a−e) experimental; (b′−d′) computed, generated by superimposing experimental spectra traces a (inhibitor-free) and e (inhibitor-saturated). Signals labeled I and IV arise from inhibitor binding. No effort was made to compensate for line width differences in the computed spectra (b′−d′). Numbers indicate the [inhibitor]/[protease] molar concentration ratio. Same experimental conditions as for Figure 1.

Although the NMR spectral perturbations resulting from bbzi-14 binding to the protease are consistent with those detected upon irreversible modification of the protease active site with EGRck (Figure 2b,d), it remains uncertain as to whether the effects shown in Figures 3 and 4 (a−e) relate to specific binding of bbzi-14 to the catalytic site or to nonspecific interactions elsewhere on the surface of the protein. To address this question, the EGRck modified tc-protease was titrated with bbzi-14. Figure 2 illustrates spectra of the EGRck reacted tc-protease in the absence (trace

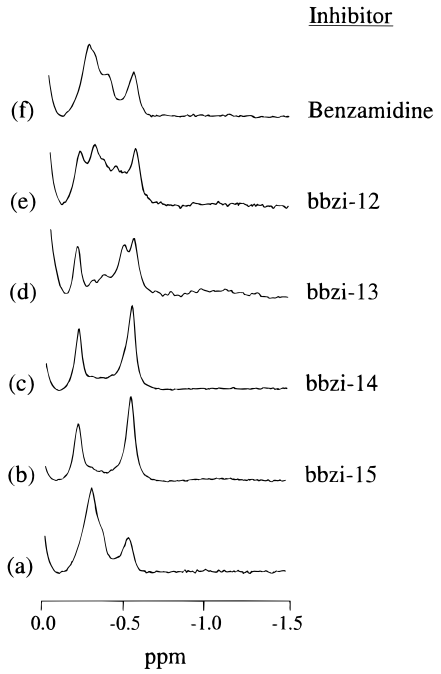


FIGURE 5: High-field ^{1}H NMR spectra of the tc-protease module: effect of benzamidine-type inhibitors. (a) Reference spectrum, inhibitor-free; (b−f) spectra of the protease in the presence of bbzi-15, bbzi-14, bbzi-13, bbzi-12, and benzamidine, respectively. Same experimental conditions as for Figure 1; [inhibitor]/[protease] ∼7.

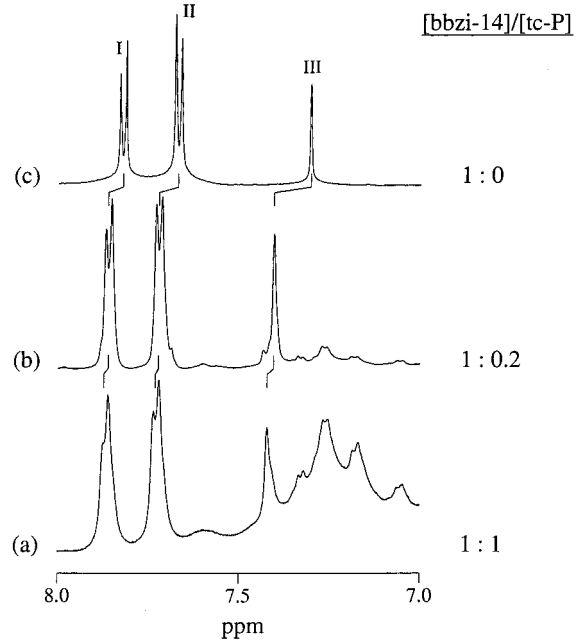


FIGURE 6: ^{1}H NMR response of bbzi-14 to tc-protease. Spectra a and b illustrate the inhibitor low-field resonances in the presence of equimolar (a) and 20% (b) presence of tc-protease: protein concentration 0.58 mM, pH* 4.6, 310 K. (c) Spectrum of bbzi-14 (20 mM) in the absence of protein: doublets I and II stem from the aromatic protons while singlet III originates from the vinyl group.

d) and in the presence (trace e) of 7-fold excess bbzi-14. In contrast to what is observed for the unmodified tc-protease (Figure 4a,e), in the case of the EGRck/tc-protease adduct (Figure 2d,e) excess bbzi-14 leaves the spectrum unperturbed. The same effect of EGRck was noticed for the sc-protease and for sc- and tc-BM 06.022 (not shown). This demonstrates that occupancy of the catalytic site by EGRck interferes with binding of bbzi-14, implying specificity of the interaction for the same site that reacts with EGRck.

Similar to what is observed for spectra of the bbzi-14/sc-protease complex, spectra of the bbzi-14/tc-protease appear as scaled superpositions of ligand-free (Figure 4a) and ligand-complexed (Figure 4e) spectra. Figure 4 (b′−d′) shows spectra that result of superposing experimental spectra (a) (ligand-free) and (e) (ligand-saturated): satisfactory agreement between computed (b′−d′) and experimental spectra (b−d) is apparent. This affords extra support to the hypothesis of slow exchange between the ligand-free and ligand-complexed forms. On this basis, it was found feasible to estimate relative concentrations of free and bound proteins, thus of the equilibrium dissociation constant (K_d) value for bbzi-14, the strongest inhibitor among the investigated bisbenzamidines (Stürzebecher *et al.*, 1992). After proper normalization, analysis (De Marco *et al.*, 1987) of the binding data yields K_d ∼910 μM for the bbzi-14/tc-protease interaction (pH* 4.6).

The spectral response of the tc-protease to the various benzamidine-type inhibitors (Chart 1) is illustrated in Figure 5 for [inhibitor]/[protease] ∼7:1. Although the different inhibitors affect basically the same set of protease resonances—consistent with interactions with the same protease site—they yield qualitatively different spectral perturbations. On the basis of the analysis given above (Figure 4) and from comparison of traces a and f in Figure 5, it is apparent that benzamidine is the ligand that causes the lesser spectral response on the tc-protease. The moderate effects caused by benzamidine correlate with its lower inhibition constant (K_i ∼690 μM at pH 8.0) for BM 06.022 relative to the bbzi's (Stürzebecher *et al.*, 1992). Similarly, spectra e and d corresponding bbzi-12 and bbzi-13 complexes (K_i ∼9.6 and 2.9 μM, respectively) exhibit a pattern different from that of spectra c and b corresponding to fully saturated forms of bbzi-14 and bbzi-15 (K_i ∼0.45 and 0.67 μM, respectively). Indeed, for benzamidine, bbzi-12, and bbzi-13, the spectral patterns are consistent with scaled superpositions of traces a (ligand-free) and b or c (ligand-saturated), suggestive of only partial inhibitor saturation of the protein. Thus, relative to bbzi-14, the detected interactions of benzamidine, bbzi-12 and bbzi-13 with the tc-protease are weak.

Titration data at low bbzi-14 concentration ([inhibitor]/[protein] ≤ 1) suggest that ligand binding to the tc-protease is a biphasic process. Figure 6 illustrates low-field spectra of bbzi-14 (trace c) and of the same in the presence of equimolar (trace a) and substoiquiometric (trace b) amounts of tc-protease. While, as expected, binding to the protease broadens the ligand signals, at the same time it shifts them to low fields. The chemical shift profile of the bbzi-14 resonances resulting from titration with tc-protease is illustrated in Figure 7. At [bbzi-14]/[protease] < 1 the bbzi-14 signals monotonically drift away from the intrinsic chemical shifts of the (protein-free) inhibitor: this we attribute to the higher affinity binding component that would be consistent with the low K_i's determined from the kinetic inhibition experiments (Stürzebecher *et al.*, 1992). Once first-phase saturation of the protease binding site is achieved at [inhibitor]/[protease] ∼1 (about 100% occupancy of the active site), a second inhibitor molecule still appears to affect the binding site as it perturbs the high-field ^{1}H NMR spectrum (Figure 4b−e). This relatively weaker secondary

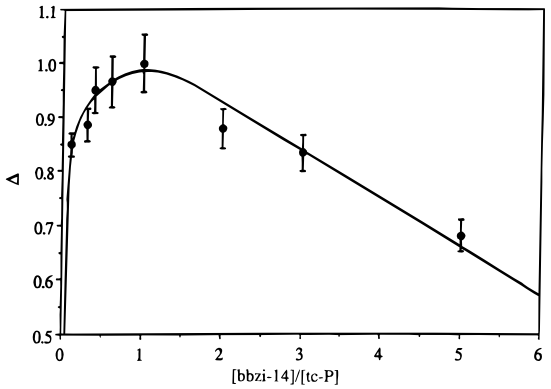


FIGURE 7: ^{1}H NMR response of bbzi-14 to tc-protease: chemical shift profile. The normalized, ligand-induced chemical shift change is denoted by $\Delta = (\delta - \delta_{free})/(\delta_{bound} - \delta_{free})$. Data extracted from experimental spectra as illustrated in Figure 6. Error bars represent the deviations from the average data of various monitored inhibitor proton signals. The continuous curve is a third-order polynomial fit.

binding gradually reverses the shifted (mostly bound) inhibitor signals toward to the chemical shifts of protein-free inhibitor, consistent with efficient free ↔ bound ligand exchange. The latter corresponds to $K_d \sim 910$ μM estimated above for the same ligand. For the high-affinity interaction, inspection of Figure 7 suggests $K_d < 23$ μM (pH* 4.6), in line with the values determined from the kinetic inhibition experiments at pH 8.0 (Stürzebecher *et al.*, 1992).[2]

Supporting evidence for the above interpretation is afforded by difference spectra in Figure 8. Spectra were generated by subtracting the inhibitor-free tc-protease spectrum (Figure 4 a) from bbzi-14 perturbed spectra (e.g., Figure 4b,d). Difference spectra signals II and III (Figure 8), which stem from the primary binding, can be distinguished from signals I and IV which are interpreted to result from the postulated secondary, weak binding. At [inhibitor]/[protease] ~1 (Figure 8c), the binding site of the tc-protease is close to saturation due to the primary (high affinity) interaction, while an incipient secondary binding, barely noticeable, eventually dominates the spectral perturbation at the higher ligand levels (Figure 8d,e). Thus, both primary and secondary interactions are suggested by the NMR spectra.

The effects of bbzi-14 on the sc and tc forms of BM 06.022 are shown in Figure 9. For the native, uncleaved BM 06.022, neither the kringle 2 nor the high-field protease signals are perturbed during titration with bbzi-14 (Figure 9A). This contrasts what is the case for the isolated sc-protease whose response to bbzi-14 presence is readily detected (Figure 3). Interestingly, the lack of response of (sc) BM-06.022 contrasts what is observed for tc-BM 06.022 (Figure 9B), whose protease signals shift, in agreement with the response elicited by the same ligand on the isolated tc-protease module (Figure 4a−c,e). When excess 7AHA—a high-affinity ligand for the kringle 2 (Byeon *et al.*, 1995)—was added in the presence of 20-fold excess of bbzi-14, no significant changes were observed in the BM 06.022 spectrum, indicating that a vacant ω-amino acid binding locus in the kringle 2 domain does not play a role in mediating interdomain interactions in the sc form. Essentially the same differences between sc

[2] For a simple, second order inhibitor (I) enzyme (E) complex (EI) formation, E + I ⇌ EI, $K_d = [E][I]/[EI] = K_i$ [see, e.g., Lehninger (1975)].

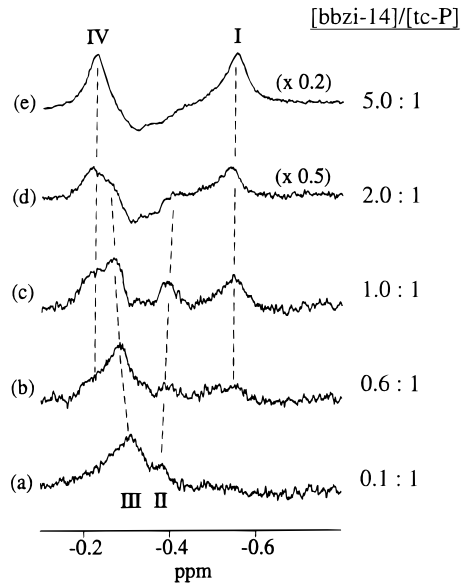


FIGURE 8: High-field ^{1}H NMR region of tc-protease: difference spectra. Each trace was generated by subtracting the inhibitor-perturbed tc-protease spectrum (Figure 4b−d) from the reference, inhibitor-free, tc-protease spectrum (Figure 4a). Dashed lines indicate shifts induced by bbzi-14 on selected signals. Signals II and III relate to the primary (high affinity) binding and signals I and IV to the secondary (low affinity) binding. Signal amplitudes scaling factors are denoted in parenthesis. Numbers indicate the [inhibitor]/[protease] molar concentration ratio.

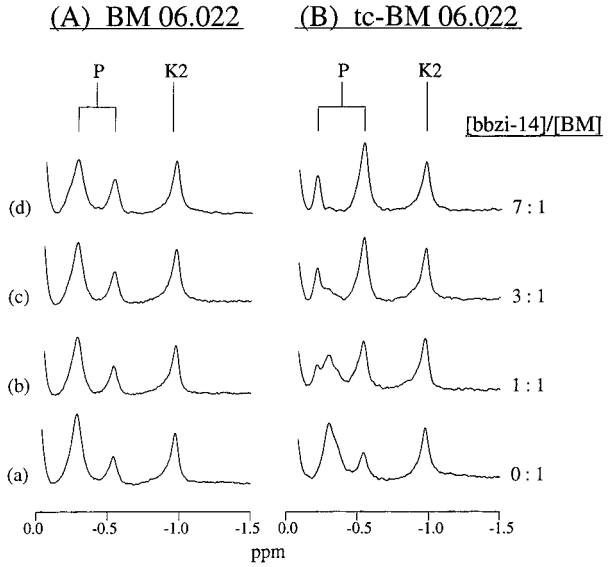


FIGURE 9: Effect of bbzi-14 on the high-field ^{1}H NMR spectrum of BM 06.022. (A) sc form; (B) tc form. The [inhibitor]/[BM 06.022] molar concentration ratios are indicated. P and K2 label methyl resonances from the protease modules and kringle 2 Leu226 δ-methyl group, respectively. Same experimental conditions as for Figure 1.

and tc forms of BM 06.022 toward ligand access detected for bbzi-14 were also observed for the weaker inhibitors bbzi-13 and benzamidine (not shown).

However, while the isolated sc-protease interacts with bbzi-14 (Figure 3), comparison of traces e in Figures 3 and 4 reveals that, relative to the tc form, bbzi access to its binding site on the sc-protease is measurably hindered, so that presence of the kringle 2 in BM 06.022 just aggravates a situation that is already manifest for the intact sc-protease. Thus two effects of the sc-to-tc conversion on the protease active site are concomitant: (a) intrinsic structural relaxation

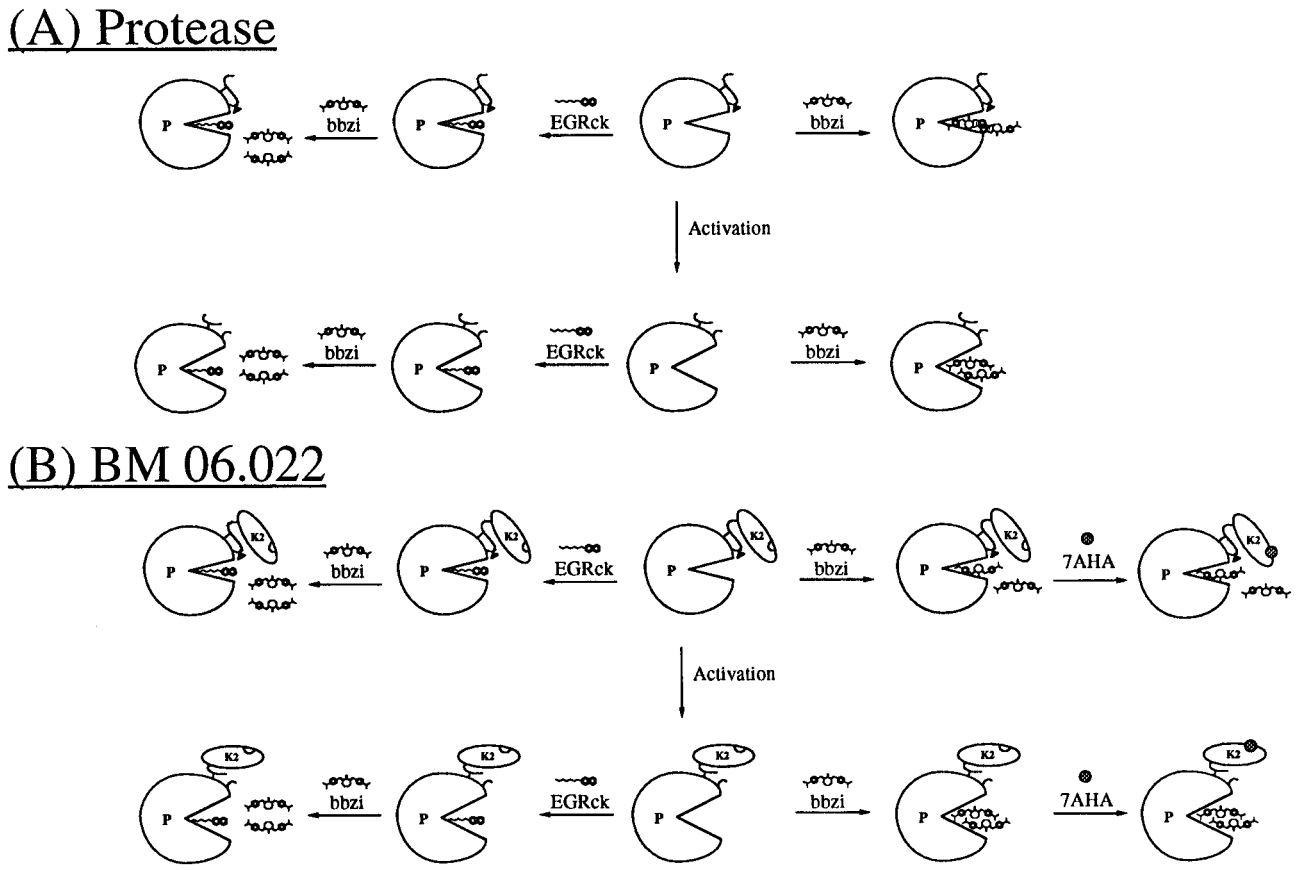


FIGURE 10: Cartoon summarizing inhibitor effects on the tPA protease (A) and BM 06.022 (B). K2 and P represent the kringle 2 and protease modules, respectively. The disulfide bond (Cys264−Cys393) joined linking peptide is indicated by continuous trace. ▽ denotes the activation cleavage site (Arg275−Ile276 bond). See Discussion for interpretation.

of the protease conformation surrounding the bbzi-binding locus, *and* (b) removal of hindrance to binding stemming from the covalently attached kringle 2 module.

DISCUSSION

The NMR-detected binding of benzamidine to the tPA protease is weak at pH* 4.6, in agreement with K_i values determined from kinetic studies on sc-, tc-BM 06.022 (K_i > 690 μM) and sc-, tc-CHO-tPA (K_i > 910 μM) at pH 8.0 (Stürzebecher *et al.*, 1992). The stronger association of the bbzi's relative to benzamidine suggests that their bindings involve interactions other than electrostatic forces holding the benzamidyl imino group proximal to the Asp371 anionic locus at the catalytic site.

The stoichiometric, primary binding detected by NMR is in line with the kinetic experiments (Stürzebecher *et al.*, 1992) which have further indicated that the bbzi's are effective inhibitors of tc-BM 06.022 and tc-CHO-tPA ($K_i \leq$ 9.6 μM). Our study also uncovers a definite difference between sc and tc forms of the protease in their inhibitor binding properties with the tc-protease exhibitting a more efficient complexation to the bbzi's than does the sc form. In case of the bbzi-14/BM 06.022 complex, the K_i values for the tc form result to be about 14 times smaller than for the sc form (Stürzebecher *et al.*, 1992). This may relate to the observations of Andreasen and co-workers (1984) showing that, *in the absence of fibrin*, the activity of the sc-tPA is at least one order of magnitude lower than that of the tc-tPA.

Several structural studies aimed at distinguishing the conformational states of sc and tc forms of tPA have been reported (Loscalzo, 1988; Higgins & Lamb, 1986; Nienaber *et al.*, 1992). The common approach of these experiments is to probe the active site with covalently attached fluorescent tags or spin labels. As demonstrated by our EGRck binding studies on BM 06.022 (Hu *et al.*, 1994), such modifications can affect the protease active site conformation in both the sc and tc forms. Under irreversible EGRck modification, the active site perturbations of the sc and tc forms are rather similar (Figure 2b,d). Covalent labels can hence be less than suitable to differentiate sc from tc forms. In contrast, when NMR is used to monitor *equilibrium* binding, benzamidine-type reversible inhibitors can distinguish conformational and ligand-binding differences between sc and tc forms which would not be apparent through the covalent probes.

The NMR experiments indicate that while at pH 4.6 the sc-protease binds bbzi's, the interaction of the latter with (sc) BM 06.022 is not observable. Hence, in (sc) BM 06.022 the (secondary) binding to the protease is blocked by the kringle 2 module. Thermal unfolding experiments have shown that the kringle 2 stabilizes the protease against thermal denaturation in the sc form of BM 06.022, but not in the tc form (Hu *et al.*, 1994). This concurs with the bbzi binding studies in suggesting interaction between kringle 2 and protease modules in the sc, but not in the tc, form. The cooperativity between the kringle 2 and the protease modules in the intact (sc) BM 06.022 may have a bearing on the physiological regulation of tPA via sc-to-tc conversion.

The sensitivity of protease NMR high-field resonances to inhibitor complexation, in particular to EGRck, may be interpreted to stem from inhibitor perturbations of the protease catalytic site that mimic substrate binding effects.

Thus, such signals would afford sensitive spectroscopic probes for monitoring conformational effects at the active site. Although several explanations may be proposed for the shift patterns of the high-field signals, the latter are consistent with both high- and low-affinity binding processes affecting a single site, namely, the protease catalytic locus. This would concur with the observed blockage of bbzi-14 binding when the active site is occupied with the covalently attached EGRck inhibitor, an effect that is quite general as it is observed for sc and tc forms of both the protease and BM 06.022 suggesting that secondary bbzi binding to an unspecified remote site is unlikely.

Figure 10 summarizes a plausible interpretation of the main results from the present study. Panel A refers to the isolated protease module while panel B applies to BM 06.022. The activation process converts the sc to tc forms via hydrolysis the Arg275−Ile276 peptide bond. A change in the conformational state of the protease is indicated. The irreversible inhibitor EGRck reacts with sc and tc forms of BM 06.022 and of the isolated protease and, in both cases, affects the NMR spectrum of the protease. Bbzi's fail to bind to sc- and tc- EGRck-modified BM 06.022, revealing that the covalently attached inhibitor prevents the bbzi's from interacting with the protease catalytic site. Occurrence of both weak (secondary) and stronger (primary) bindings of bbzi's to the protease catalytic site are suggested. At pH 8.0 the high affinity binding interaction of bbzi-14 with the protease is not significantly interfered by the presence of kringle 2 as it is characterized by similar (small) K_i values for BM 06.022 and for the isolated protease (Stürzebecher *et al*., 1992). On the other hand, the weak, secondary binding, evident for the tc-proteins, is only partial for the sc-protease and not apparent for (sc) BM 06.022. This demonstrates that (a) sc → tc conversion facilitates inhibitor access to the catalytic site, and (b) kringle 2 interferes with bbzi accessing the catalytic site in the intact BM 06.022. The addition of the kringle 2-specific ligand 7AHA does not affect the binding of bbzi's to either the sc or the tc forms of BM 06.002, suggesting that the vacant lysine binding site of kringle 2 does not significantly contribute to interdomain interactions.

Thus, the activation process both relaxes the active site conformation of the protease module and decouples the interdomain interactions in BM 06.022 resulting in the protease catalytic site becoming more accessible to inhibitors. It is tempting to extrapolate these results to the different reactivities of sc- and tc-tPA toward small chromogenic peptide substrates and, in the absence of fibrin, toward its natural partner, plasminogen.

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