p-Aminobenzamidine as a Fluorescent Probe for the Active Site of Serine Proteases

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p-Aminobenzamidine is weakly fluorescent in neutral aqueous buffer, with excitation and emission maxima at 293 and 376 nm, respectively. Binding to trypsin results in a blue shift of the emission peak to 362 nm, and 50-fold fluorescence enhancement, while binding to thrombin causes a shift to 368 nm and a 230-fold fluorescence enhancement. This phenomenon is due to hydrophobic interactions, as evidenced by the similar reaction kinetic techniques. The absorbance spectrum of p-aminobenzamidine is red-shifted by formation of a complex with proteases, with the major difference peak appearing at 317 nm and 323 nm for trypsin and thrombin, respectively. The difference extinction coefficients were 6000 M⁻¹ cm⁻¹ for trypsin complex and 13,300 M⁻¹ cm⁻¹ for thrombin complex at the peak wavelengths. Titration of trypsin and thrombin with the probe indicated one binding site per molecule, with dissociation constants equal to the kinetically determined inhibition constants. The Kᵦ values for trypsin and thrombin were 6.1 and 65 μM, respectively. An important potential use of this probe is in studies of inhibitor and substrate binding by rapid reaction kinetic techniques. Using this probe to study the interaction of thrombin with antithrombin III yielded a bimolecular rate constant of 8.0 × 10⁷ M⁻¹ s⁻¹, which compares favorably with the value of 8.7 × 10⁸ M⁻¹ s⁻¹ obtained from discontinuous assays of the rate of thrombin neutralization.

The use of probe molecules, which bind to the active center of enzymes with a concomitant spectral or fluorescence change, has resulted in much mechanistic information. Proflavin was first reported as a spectral probe of serine proteases in 1965 and used to study the transient kinetics of trypsin and chymotrypsin (1). Subsequent studies enabled characterization of the intermediate with various substrates as well as conformational changes involved in catalysis (2-5). Using relaxation kinetics and proflavin binding as a probe, it was possible to determine the rate constants for the formation and dissociation of the benzamidine-trypsin complex (6). Proflavin has also provided useful insights regarding the mechanism of inhibition by protein trypsin inhibitors (7).

There are certain limitations to the use of proflavin as a probe. The spectral method is not sensitive enough for study-

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DISCUSSION

p-Aminobenzamidine, a competitive inhibitor of serine proteases (8, 9), is highly fluorescent when bound to either trypsin or thrombin. The blue shift of its emission spectrum when bound to proteases is similar to that observed when it is dissolved in nonpolar solvents, indicating that hydrophobic interactions contribute significantly to the fluorescence properties of the bound probe. The substantial red shift of the absorbance spectrum due to binding is compatible with this interpretation (16). However, other interactions at the active center of proteases also affect the spectral and fluorescence properties of bound probe, as evidenced by a 50-fold enhancement and 14-nm blue emission shift with trypsin, compared with a 230-fold enhancement but smaller, 8-nm blue shift with thrombin. The greater hyperchemicity and red shift of the absorbance spectrum of the probe-thrombin complex compared with the probe-trypsin complex indicates that the spectral properties are also sensitive to the specific structural features of the enzyme active center.

Monitoring the increase in emission intensity associated with the binding of p-aminobenzamidine to trypsin or thrombin permits direct determination of the equilibrium constants for the formation of these complexes. The Kᵦ of 6.1 μM for binding to trypsin by fluorescence titration is in good agreement with the Kᵦ of 7 ± 2 μM, determined kinetically. Similarly, the average Kᵦ of 65 μM for the thrombin-p-aminobenzamidine complex is in direct agreement with the Kᵦ of 65 μM. These values are similar to reported values of the Kᵦ for p-aminobenzamidine possessing reactions at micromolar and submicromolar enzyme concentrations, and proflavin binds to other macromolecules. Consequently, we investigated the development of fluorescent probes which are specific for the active center of serine proteases.

Benzamidine and its derivatives are specific competitive inhibitors of trypsin (8), thrombin and plasmin (9), and should bind to all arginine-specific serine proteases. In the course of studying inhibition by p-aminobenzamidine, we found that the inhibitor displayed enhanced fluorescence in the presence of trypsin and thrombin. Consequently, we investigated the characteristics of this inhibitor as a fluorescent probe for arginine-specific serine proteases.

MATERIALS AND METHODS AND RESULTS

p-Aminobenzamidine, a competitive inhibitor of serine proteases (8, 9), is highly fluorescent when bound to either trypsin or thrombin. The blue shift of its emission spectrum when bound to proteases is similar to that observed when it is dissolved in nonpolar solvents, indicating that hydrophobic interactions contribute significantly to the fluorescence properties of the bound probe. The substantial red shift of the absorbance spectrum due to binding is compatible with this interpretation (16). However, other interactions at the active center of proteases also affect the spectral and fluorescence properties of bound probe, as evidenced by a 50-fold enhancement and 14-nm blue emission shift with trypsin, compared with a 230-fold enhancement but smaller, 8-nm blue shift with thrombin. The greater hyperchemicity and red shift of the absorbance spectrum of the probe-thrombin complex compared with the probe-trypsin complex indicates that the spectral properties are also sensitive to the specific structural features of the enzyme active center.

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benzamidine which are 8.25 μM (8) and 12.0 μM (9) for trypsin and 80 μM (9) for thrombin. The decrease in emission intensity due to dissociation of p-aminobenzamidine from binary complexes with trypsin and thrombin was used to determine the equilibrium binding constants for the interaction between benzamidine, another well established inhibitor of serine proteases (6), and these proteins. Benzamidine was able to completely displace p-aminobenzamidine from the active site of both proteins. A K_d value of 19.8 μM was determined for the benzamidine-trypsin complex which compares well with the value of 18.4 μM (8) obtained by inhibition kinetics. A value of 220 μM was obtained for the complex with thrombin, which is in direct agreement with the literature value (9) determined by enzyme kinetics. Benzamidine, as compared with p-aminobenzamidine, showed no enhancement of emission intensity when dissolved in the solvents used in Fig. 2. There was also no change in its fluorescence properties when bound to either trypsin or thrombin.

Several lines of evidence demonstrate that the fluorescence enhancement of p-aminobenzamidine bound to serine proteases results from a specific interaction at the active center. Identical values for the K_d by fluorescence titration and the K_d as a competitive inhibitor with both trypsin and thrombin is a compelling indication of specific binding, which is corroborated by the ability of benzamidine to totally displace the probe. Although our studies involved only two serine proteases, it seems likely that p-aminobenzamidine will bind to all enzymes for which benzamidine is a competitive inhibitor, with a concomitant increase in fluorescence yield.

Several probes already exist for the active site of serine proteases, but each has limitations. Proflavin has been used frequently and provided much useful information (1, 2) but lacks specificity, which complicates the interpretation of results. For example, in a study of heparin effects on the thrombin-antithrombin reaction, it was reported that proflavin bound to heparin and antithrombin as well as to thrombin, with different spectral properties for each complex (17). An additional limitation of proflavin is that the spectral change precludes studying reactions in the submicromolar range of enzyme concentration. Recently, dansylarginine N-(3-ethyl-1,5-pentanediyl)amine has been described as a specific fluorescent probe inhibitor for thrombin (18). Its specificity for thrombin and tight binding make this probe extremely useful for certain studies, such as thrombin generation in complex systems. However, the relatively low fluorescence yield, requiring high concentrations of enzyme and probe, combined with the high affinity, limits the value of this compound in kinetic studies. At the concentrations which must be used to observe substrate or inhibitor binding by probe displacement, the magnitude of the competitive effect of probe binding overwhelming influences the kinetic parameter being observed. Dansyl-arginine has been reported to bind to trypsin and trypsinogen with increased fluorescence yield (19, 20). The weak binding, lack of specificity for the active protease, and relatively low fluorescence yield limit its use. However, this compound has potential as a probe and is ideal for studies utilizing resonance energy transfer from tryptophan residues of the enzyme.

The large number of serine proteases with which p-aminobenzamidine could interact, and its specificity for the active site of these enzymes, indicate numerous potential applications. Several properties make this molecule ideal as a probe. Its large fluorescence change and dissociation constants in the range of 10^{-9} M should permit studies of tightly and weakly bound inhibitors, as well as irreversible inactivators. Although its affinity for proteases is weak enough that it will interfere minimally with the ligand being bound, it binds tightly enough that high concentrations, with substantial inner filter quenching effects, are not necessary.

The substantial red shift in absorbance of bound probe is fortuitous, since it enables excitation at wavelengths where inner filter quenching due to free probe is substantially lower. Furthermore, the difference extinction coefficients are large enough to permit use of p-aminobenzamidine as a spectral probe when sensitivity is not a limiting factor.

The most significant potential application of this probe is in rapid reaction kinetics of proteases using stopped flow fluorimetry. The natural protein substrates are frequently cleaved at several sites, but by following probe displacement rates and amplitudes it may be possible to determine the K_d, binding rate constant, and turnover number of the most reactive substrate site. In a similar manner, the binding rates of protein inhibitors such as antithrombin III can be determined, with resolution of multi-step binding processes.

The results in Fig. 7 demonstrate the feasibility of this approach in studying protease inhibition rates. An additional application would be in studies of the rate of formation of serine proteases from their precursorzymogens, since a large fluorescence increase would occur in the presence of p-aminobenzamidine. A sensitive probe specific for the active site should be particularly useful for coagulation enzymes, which are glycoproteins and may bind probes such as proflavin at additional sites.

Acknowledgment—Thanks are due to Dr. Paul Bock for an idea which led to the discovery of p-aminobenzamidine as a fluorescent probe of serine proteases.

REFERENCES
**RESULTS**

Panelled by a blue shift of the emission intensity, as the solvents decreased in polarity, a significant enhancement of emission intensity was observed, accompanied by a blue shift of the emission maximum of about 30 nm in ethanol and an increase of about 30 nm in ethylene glycol.

The emission maximum for the emission maximum increased by a factor of 20 in methanol, 60 in cyclohexane, and 200 in ethylene glycol.

The fluorescence excitation and emission spectra of p-aminobenzamidine were determined in distilled water and in 2-propanol using a Hitachi F-4010 spectrophotometer. The excitation spectrum was identical to the absorption spectrum of the probe, with an absorption maximum at 330 nm and an emission maximum at 375 nm. A significant enhancement of emission intensity was observed, accompanied by a blue shift of the emission maximum of about 30 nm in ethanol and an increase of about 30 nm in ethylene glycol.

**REFERENCES**

The increase in the fluorescence intensity of p-aminobenzamidine on interaction with the immobilized enzyme was used to determine the dissociation constants of the inhibitor to both proteins. By fitting the data (Figure 5), the value of the binding constant was determined from the maximum emission intensity in the presence of the enzyme.

**Figure 1:** Emission and excitation spectra of p-aminobenzamidine - HCl in distilled water. 375 nm excitation for excitation spectrum, 295 nm excitation for emission spectrum. 20°C.

**Figure 2:** Emission spectra of p-aminobenzamidine - HCl in different solvents, showing the effect of solvent polarity on the emission maximum.

**Figure 3:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 4:** Fluorescence spectra of p-aminobenzamidine - HCl in different solvents, showing the blue shift of the emission maximum upon increasing solvent polarity.

**Figure 5:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 6:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 7:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 8:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 9:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 10:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 11:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 12:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 13:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 14:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 15:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 16:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 17:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 18:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 19:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 20:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 21:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

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**Figure 24:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 25:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 26:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 27:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

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**Figure 35:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 36:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

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**Figure 38:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 39:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.

**Figure 40:** Difference absorption spectra of p-aminobenzamidine binding to trypsin and chymotrypsin, showing the increase in absorption at 365 nm upon binding.
To demonstrate that the fluorescence enhancement was due to an interaction at the enzyme active center, Kᵢ values were determined for inhibition of trypsin and thrombin by p-aminobenzamidine. For trypsin, using S-2251 as substrate, the Kᵢ was 7 ± 2 μM, in good agreement with the value of 6.1 μM determined by direct fluorimetric titration. Figure 6 shows a Dixon plot (1) of the data for thrombin, using S-2251 as substrate. The intersection of the three lines at V₀ demonstrates that inhibition is competitive and yields a Kᵢ of 65 μM, in good agreement with the average titration value of 65 μM.

An important use of p-aminobenzamidine as a probe is in rapid reaction studies, where its displacement can be used to determine the rate constant for binding of a substrate or inhibitor to a protein. Figure 7 shows the reaction between thrombin and antithrombin III followed by displacement of the probe, using probe and antithrombin concentrations much less than the thrombin concentration and assuming an irreversible reaction, the observed rate constant is related to the antithrombin binding rate constant, k₆, by the following equation:

\[ k_{obs} = \frac{k_{b}}{K_2} \]  

in which K₂ is the dissociation constant of the probe. The hemolytic rate constant for inhibition of thrombin by A-I II, calculated from Figure 7, was 0.05 ± 0.02 × 10⁻⁴ M⁻¹ sec⁻¹. This compared favorably with the average value 0.05 ± 0.02 × 10⁻⁴ M⁻¹ sec⁻¹ obtained by discontinuous assay of residual thrombin activity at antithrombin concentrations of 0.5, 0.5, and 1.03 μg/mL employing 25 μM thrombin.

Figure 6. Dixon plot of p-aminobenzamidine inhibition of S-2251 hydrolysis by p-aminobenzamidine. The initial rates of hydrolysis of S-2251 by p-aminobenzamidine-reduced A-I II (0.7 M) were measured at varying concentrations of p-aminobenzamidine in the absence of inhibitor. Using a nonlinear least squares algorithm.

Figure 7. Rate of irreversible complex formation of antithrombin III with p-aminobenzamidine monitored by displacement of p-aminobenzamidine from the thrombin antistate. Thrombin-p-aminobenzamidine was mixed in the stopped-flow fluorimeter with A-I II at final concentrations of 3 μM thrombin, 50 μM p-aminobenzamidine, and 20 μg A-I II.
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