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 properties observed when p-aminobenzamidine is dissolved in solvents of decreasing polarity. 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 × 10⁴ M⁻¹ s⁻¹, which compares favorably with the value of 8.7 × 10⁴ 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 also 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-
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 \( K_r \) 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)amide 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 overwhelmingly 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 multiple potential applications. Several properties make this molecule ideal as a probe. Its large fluorescence change and dissociation constants in the range of 10^{-10} 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 in 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 \( K_a \), 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.

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**REFERENCES**

p-Aminobenzamidine Fluorescent Probe for Proteases

RESULTS

Pan and by a blue shift of the emission maximum intensity, as the solvents decreased in polarity, a significant enhancement of emission intensity was observed, accompanied by a blue shift of the emission maximum wavelength. The emission peak shifted from 370 nm in distilled water to 375 nm in ethyl acetate with intermediate values of 365 nm in a 3:2 mixture of ethanol and distilled water, 360 nm in ethanol, 348 nm in ethyl acetate, and 316 nm in distilled water. The emission intensity of the emission maximum increased by a factor of 20 in ethyl acetate and 62 in ethyl acetate. The results obtained using tandem couplings, 0.1 absorbance scale, by mixing of various solvents and p-aminobenzamidine solutions in the two sample cuvets after attaining a base line. The spectra that represent bound p-aminobenzamidine were free from protamine and free from trypsin. In addition, the difference extinction coefficients, %, of 316 nm for trypsin and 65 nm for tryptophan were used to calculate the concentration of proteins.

The increase in the fluorescence intensity of p-aminobenzamidine on interaction with tryptic or trypsin enzymes was used to obtain the optimum binding constants of the inhibitor to both probes (Figure 3). Average k values for the binding of p-aminobenzamidine to trypsin and trypsin in solution were 8.1 and 7.6 µM, respectively, at a non-linear least-squares computer fit to the data. The curves represent the observed range of values over several experiments. In the case of trypsin, the binding constant was derived from the linear relationship of the molar fraction of bound p-aminobenzamidine to the ratio of the observed fluorescence intensity to the fluorescence intensity of the unbound p-aminobenzamidine. The binding constant of trypsin and p-aminobenzamidine was determined by the method of Hagedorn and colleagues [10], thus identifying a binding stoichiometry of 1:1. Benzamidine completely displaces p-aminobenzamidine from trypsin and trypsin. The increase in the fluorescence emission association with the p-aminobenzamidine-protein complexes was monitored and yielded a k value of 79.4 µM for the binding of benzamidine to trypsin and 205 µM for trypsin binding.

REFS

The fluorescence excitation and emission spectra of p-aminobenzamidine-3H3 dissolved in distilled water are shown in Figure 1. The excitation and emission spectra were identical with the absorption spectrum of 33.0 nm at the excitation wavelength of 315 nm. A significant absorbance spectrum was observed at 530 nm in chloroform and 540 nm in benzene. The fluorescence intensity of p-aminobenzamidine in solvents of varying polarity. The solvents used were distilled water, ethanol, methanol, and acetic acid. The excitation wavelength was 315 nm and the emission wavelength was 370 nm. The fluorescence intensity of the excitation maximum increased by a factor of 20 in ethyl acetate and 62 in ethyl acetate. The difference extinction coefficients % of 316 nm for trypsin and 65 nm for tryptophan were used to calculate the concentration of proteins.

The spectra of Figure 2 and 3 indicate that hypochromic interactions are involved. If this is the case, the fluorescence and absorption coefficients that change should be given by the linear sum of the corresponding coefficients. In Figure 2, the excitation and emission spectra were recorded after the addition of p-aminobenzamidine to the enzyme solutions. The fluorescence spectra were recorded after the addition of p-aminobenzamidine to the enzyme solutions. The fluorescence spectra were recorded after the addition of p-aminobenzamidine to the enzyme solutions. The fluorescence spectra were recorded after the addition of p-aminobenzamidine to the enzyme solutions. The fluorescence spectra were recorded after the addition of p-aminobenzamidine to the enzyme solutions.
To demonstrate that the fluorescence enhancement was due to an interaction at the enzyme active center, \( K_d \) values were determined for inhibition of trypsin and thrombins by \( p \)-aminobenzamidine. For thrombins, using S-2251 as substrate, the \( K_d \) was \( 7 \pm 2 \mu M \), in good agreement with the value of \( 6.1 \mu M \) determined by direct fluorimetric titration. Figure 6 shows a Scatchard plot (II) of the data for thrombins, using S-2251 as substrate. The intersection of the three lines at \( v_{\infty} \) demonstrates that inhibition is competitive and yields a \( K_d \) of 65 \( \mu M \), in direct agreement with the average titration value of 65 \( \mu M \).

![Graph showing competitive inhibition by \( p \)-aminobenzamidine of thrombin activity.](image)

Figure 6. Scatchard plot of \( p \)-aminobenzamidine inhibition of S-2251 hydrolysis by thrombin. The initial rates of hydrolysis of S-2251 by thrombin (in the absence of inhibitor) were measured at varying \( p \)-aminobenzamidine concentrations with a constant level of 25.0 \( \mu M \) (A), 17.5 \( \mu M \) (B) and 8.75 \( \mu M \) (C). The best fit of the data was determined by varying the S-2251 concentration and the absence of inhibitor, using a non-linear least squares algorithm.

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 probe. Figure 7 shows the reaction between thrombin and antithrombin III, followed by displacement of the probe. Using probe and antithrombin concentrations much greater than the thrombin concentration and assuming an irreversible reaction, the observed rate constant is related to the antithrombin binding rate constant, \( k_b \), by the following equation:

\[
k_b = \frac{k_{obs}}{\frac{1}{K_a} + \frac{1}{[E]_0}}
\]

In which \( k_b \) is the dissociation constant of the probe. The hemolysis rate constant for inhibition of thrombin by AT III, calculated from Figure 7, was 2.0 \( 10^{-7} M^{-1} sec^{-1} \). This compared favorably with the average value \( 2.7 \pm 0.1 \times 10^{-7} M^{-1} sec^{-1} \) obtained by discontinuous assay of residual thrombin activity as antithrombin concentrations of 25, 50, and 100 \( \mu M \) employing 25 \( \mu M \) thrombin.

![Graph showing rate of irreversible complex formation of antithrombin III with thrombin monitored by displacement of \( p \)-aminobenzamidine from the thrombin active site.](image)

Figure 7. Rate of irreversible complex formation of antithrombin III with thrombin monitored by displacement of \( p \)-aminobenzamidine from the thrombin active site. Thrombin-\( p \)-aminobenzamidine was mixed in the stopped-flow fluorimeter with AT III at final concentrations of 5 \( \mu M \) thrombin, 30 \( \mu M \) \( p \)-aminobenzamidine, and 20 \( \mu M \) AT III.
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