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 366 nm and a 230-fold fluorescence enhancement. This phenomenon is due to hydrophobic interactions, as evidenced by the similar reaction kinetic techniques. Using this probe to study the interaction of thrombin with antithrombin III yielded a bimolecular rate constant of 8.0 × 10⁹ M⁻¹ s⁻¹, which compares favorably with the value of 8.7 × 10⁹ 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 tryptsin inhibitors (7).

There are certain limitations to the use of proflavin as a probe. The spectral method is not sensitive enough for studying 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 hyperchomicity 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 Kₐ of 6.1 µM for binding to trypsin by fluorescence titration is in good agreement with the Kₐ of 7.2 ± 2 µM, determined kinetically. Similarly, the average Kₐ of 65 µM for the thrombin-p-aminobenzamidine complex is in direct agreement with the Kₐ of 65 µM. These values are similar to reported values of the Kₐ for p-aminobenzamidine.

DISCUSSION

Portions of this paper (including "Materials and Methods," "Results," and Figs. 1-7) are presented in miniprint at end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-1985, cite authors, and include a check or money order for $4.00 per set of photocopies. Full sized photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
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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_i 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 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 numerous 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 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

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RESULTS

Panelled by a blue shift of the maximum emission intensity, as the solvents decreased in polarity, a significant enhancement of emission intensity was observed, accompanied by a blue shift of the maximum emission intensity, as the solvents decreased in polarity. The emission peak shifted from 376 nm in distilled water to 329 nm in methanol, with intermediate values of 350 and 335 nm in ethanol and cyclohexanol, respectively. A further blue shift was observed as the polarity decreased, with the emission peak shifting from 329 nm in methanol to 287 nm in ethyl acetate. The fluorescence spectrum obtained in cyclohexanol was essentially the same as that obtained in methanol.

A comparison of the emission spectra of the probe in different solvents revealed that the maximum emission intensity increased by a factor of 20 in ethanol, 200 in methanol, and 2500 in acetonitrile. This increase in fluorescence intensity was attributed to the formation of a protein-probe complex, as evidenced by the blue shift of the emission maximum, which was accompanied by an increase in the quantum yield of the probe.

The increase in fluorescence intensity in polar solvents is attributed to the formation of a protein-probe complex, which results in a decrease in the quantum yield of the probe. The magnitude of the blue shift and the increase in fluorescence intensity are proportional to the degree of protein-protein interaction. Thus, the fluorescence intensity of the probe in different solvents can be used as a measure of the degree of protein-protein interaction.

Supplementary Material

**SUPPORTING MATERIAL**

p-Aminobenzamidine

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**MATERIALS AND METHODS**

The human pepsinase used in this study was a gift of Dr. John A. Burtis (II, of the Division of Laboratories and Research, New York State Department of Health, Albany. p-Aminobenzamidine was obtained from Sigma Chemical Company as the non-hygroscopic or as a gift from Dr. John A. Burtis (II). The fluorescence spectra and excitation and emission spectra of the probe in different solvents were obtained by a Perkin-Elmer Spectrophotometer, Model 400, and the fluorescence intensities were measured as described above. The fluorescence intensity of the probe in different solvents was plotted against the inverse of the solvent viscosity, as shown in Figure 2. The emission spectra of the probe in different solvents were obtained by using a Perkin-Elmer Spectrophotometer, Model 400, and the excitation spectra of the probe in different solvents were obtained by using a Perkin-Elmer Spectrophotometer, Model 400.
To demonstrate that the fluorescence enhancement was due to an interaction of the enzyme active center, IC values were determined for inhibition of trypsin and thrombin by p-aminobenzamidine. For trypsin, using B-280 as substrate, the IC was 2 ± 2 μM, in good agreement with the value of 8.1 μM determined by direct fluorimetric titration. Figure 6 shows a Dixon plot (III) of the data for thrombin, using B-280 as substrate. The intersection of the three lines at Vmax demonstrates that inhibition is competitive and yields an IC 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 protease. 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:

\[ k = \frac{1}{t} - \frac{1}{t_0} \]

where τ is the dissociation constant of the probe. The influenza rate constant for inhibition of trypsin by B-280, calculated from Figure 7, was \( 0.9 \pm 0.2 \text{ M}^{-1} \text{s}^{-1} \). This compares favorably with the average value \( (9.7 \pm 0.11) \times 10^8 \text{ M}^{-1} \text{s}^{-1} \), obtained by displacement of residual thrombin activity in an antithrombin concentration of 0.26, 0.52, and 1.03 μM employing 35 μM thrombin.

Figure 6. Dixon plot of p-aminobenzamidine inhibition of 280 hydrolysis by p-aminobenzamidine. The initial rates of hydrolysis of B-280 (50 μM) in the presence of various p-aminobenzamidine concentrations were measured under non-inhibitory conditions with a constant level of 25 μM (41.5 ± 3.2 μM) and 50 μM (28.3 ± 1 μM) B-280. A k' of 0.19 ± 0.02 s^{-1} was determined by varying the B-280 concentration in the absence of inhibitor, using a non-linear 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 active site. Thrombin-p-aminobenzamidine was mixed in the stopped-flow fluorimeter with AT III at final concentrations of 5 μM thrombin, 10 μM p-aminobenzamidine, and 20 μM AT III.
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