4,4′-Bis[8-(phenylamino)naphthalene-1-sulfonate] Binding to Human Thrombins: A Sensitive Exo Site Fluorescent Affinity Probe†

Giovanni Musci, George D. Metz,‡ Hideaki Tsunematsu,§ and Lawrence J. Berliner*

Department of Chemistry, The Ohio State University, Columbus, Ohio 43210

Received August 10, 1984

ABSTRACT: The binding of the fluorescent probe 4,4′-bis[8-(phenylamino)naphthalene-1-sulfonate] (bis-ANS) to human α- and γ-thrombins was investigated. Bis-ANS binds in a 1:1 complex to both forms of the enzyme, with K_d = 14.8 ± 2.2 μM and 5.8 ± 1.0 μM for α- and γ-thrombin, respectively, at pH 7.0 [25 mM tris(hydroxymethyl)aminomethane, 0.15 M NaCl]. Fluorescence changes upon complexation included a considerable (~30-nm) blue shift in the fluorescence emission maximum as well as a dramatic increase in the fluorescence emission intensity: a 70-fold enhancement was observed with α-thrombin vs. a ~220-fold enhancement with γ-thrombin. Proflavin was not displaced upon bis-ANS binding. The unknown thrombin effectors ATP, Ca(II)ATP, Co(III)ATP, phosphate, and pyrophosphate bound with enhancement of the fluorescence of the bis-ANS–α-thrombin complex. The two inhibitors benzamidine and *p*-chlorobenzylamine as well as heparin caused decreases in bis-ANS–thrombin fluorescence: valerylamidine had no effect on the fluorescence of the bis-ANS–thrombin complex. Kinetic measurements with two chromogenic substrates, S-2238 and S-2160, indicated that bis-ANS acts as a partial noncompetitive inhibitor of thrombin amidase activity. The kinetic evidence combined with the ligand binding results suggests that bis-ANS does not overlap the catalytic site. The fluorophore ANS complexed with equal affinity to both α- and γ-thrombins (K_d = 24 ± 4 μM); however, the γ-thrombin–ANS complex emission at 470 nm was enhanced 26% more than that for the α form.

Coagulant human α-thrombin (EC 3.4.21.5) plays a crucial role in the blood coagulation pathway and has many important regulatory functions in hemostasis (Fenton, 1981). In vitro it displays esterase and amidase activity toward a variety of synthetic substrates. However, retainment of the esterolytic/amidolytic function does not parallel structural changes in the two-chain protein structure: the γ derivative, generated by autolytic or tryptic fragmentation of the B chain of the native α form, loses all clotting activity yet retains the ability to hydrolyze synthetic substrates as does the α form (Lottenberg et al., 1982). The relationship between structure and function in the α-thrombin to γ-thrombin conversion has been the subject of several recent studies, but a full understanding has not yet been achieved (Fenton, 1981; Berliner, 1984). To date, no three-dimensional structure of any thrombin species has been obtained, although the crystallization of both the human and the bovine enzyme was reported several years ago (McKay et al., 1977; Tsernoglou & Petsko, 1977). Information about the molecular structure of the active site has thus far been inferred from conformational studies with spin-labels and fluorophores (Berliner et al., 1981; Berliner & Shen, 1977b).

The use of ligands that affect the spectroscopic features and/or the functional behavior of a protein in solution is a useful approach in elucidating functionally important regions of enzymes. Successful attempts have been made in the case of α- and γ-thrombin in this and other laboratories and a tentative description of the active site structure involving the main catalytic residues has been drawn (Berliner et al., 1981; Berliner, 1984).

The fluorescent probe ANS[1] and its dimeric analogue, bis-ANS, have been shown to be sensitive probes of protein conformation (Brand & Gohlke, 1972; Rosen & Weber, 1969). Stryer (1965) has shown that 1,8-ANS is very sensitive to the polarity of its environment; e.g., as solvent polarity decreases, the relative fluorescence quantum yield increases with a blue-shifted λ_{em}^{max}. Bis-ANS displays the same behavior and can also be used to probe the hydrophobicity of binding site(s) on proteins. We found that bis-ANS binds to α- and γ-thrombins with a higher affinity than that for the monomeric dye and that it reports subtle structural differences between the two enzyme forms.

In this work, bis-ANS has been used to elucidate the spatial relationships between the catalytic site and the apolar binding and other binding sites on thrombin. Several ligands were investigated and their interactions with the α-thrombin–bis-ANS complex were analyzed. A kinetic study of bis-ANS inhibition of amidase activity of α-thrombin is also presented.

Experimental Procedures

Enzymes. Electrophoretically pure human α- and γ-thrombins (~3000 NIH units/mg, 95%–98% NPGB active sites) were generous gifts from Dr. John W. Fenton II, New York State Department of Health, Albany. Thrombin (M_r

† This work was supported in part by grants from the U.S. Public Health Service (HL 24549). A preliminary report of this work was reported at the 8th International Congress on Thrombosis and Haemostasis, Toronto, Ontario, Canada, 1981.

‡ Present address: Mead Paper Co., Chillicothe, OH.

§ Present address: Research Institute for Diseases of the Chest, Faculty of Medicine, Kyushu University 3-1-1, Higashi-ku, Fukuoka 812, Japan.

[1] Abbreviations: 1,8-ANS or ANS, 8-anilinonaphthalene-1-sulfonic acid; bis-ANS, 4,4′-bis[8-(phenylamino)naphthalene-1-sulfonate]; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; Tos-Arg-OMe, tosylarginine methyl ester; S-2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide hydrochloride; PEG, poly(ethylene glycol); DAPA, dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/85/0424-2034$01.50/0

36 600) concentration was estimated by its absorbance at 280 nm, with a coefficient $\epsilon = 1.83$ mL mg^{-1} cm^{-1} in 0.1 M NaOH (Fenton et al., 1977).

Chemicals. Bis-ANS (4,4'-bis[8-(phenylamino)-naphthalene-1-sulfonate] dipotassium salt) was from Molecular Probes, Junction City, OR. PEG 6000 [poly(ethylene glycol), lot TB 10205] was from Dow Chemical Co. Tos-Arg-OMe (lot 77C-0415), ANS (lot 33C-18989), ATP (lot 121F-7110), AMP (lot 39C-7690), adenosine (lot 122F-0303), tryptamine (lot 93F-0146), 5-hydroxytryptamine (lot 43F-2045), and heparin (lot 95E-0275) were from Sigma Chemical Co. Proflavin sulfate (lot W3716) was purchased from Schwarz/Mann, while sodium phosphate and pyrophosphate were from Mallinckrodt Chemical Corp. $Co(NH_3)_4HATP$ was prepared according to Cornelius et al. (1977). The chromogenic substrates S-2238 and S-2160 were generous gifts from Kabi Diagnostics, Sweden. Benzamidine hydrochloride hydrate (lot 022567), *p*-chlorobenzylamine hydrochloride and dioxane (lot PC 091387) were from Aldrich Chemical Co. Valerylamidine was a gift from Dr. W. B. Lawson, New York State Department of Health. Dioxane was purified by passage over an alumina column to remove peroxides. All other reagents were of analytical grade and were used without further purification.

Methods. Thrombin amidase and esterase activity was monitored on a Unicam SP 1800 spectrophotometer at 25 °C with S-2238 or S-2160 (Lottenberg et al., 1982). Tos-Arg-OMe kinetics were measured by the method of Hummel (1959). Proflavin dye binding was measured by UV–vis difference spectroscopy after Kohler & Magnusson (1974). Clotting activity was measured with a BBL Fibrometer by the method of Fenton et al. (1977); some assays were performed in the presence (0.66% v/v) and absence of PEG 6000 for reasons explained under Results. All fluorescence measurements were made with a Perkin-Elmer MPF44A spectrophotometer. The data were fit by nonlinear regression analysis on a Hewlett-Packard 9835 computer (Conery & Berliner, 1983).

The following iterative method was used to calculate the dissociation constants $K_{d(true)}$ for the α- or γ-thrombin–bis-ANS complex: after fitting $[\text{bis-ANS}]_{total}$ vs. intensity (I_{obsd}) by nonlinear regression analysis, I_{max} and $K_{d(app)}$ were obtained. A parameter $R = I_{obsd}/I_{max}$ (fraction of enzyme saturated) was calculated for each observation from which the free ligand concentration was estimated, according to eq 1, where [E] is

$$[\text{bis-ANS}]_{free} = [\text{bis-ANS}]_{total} - R[\text{E}]_{total} \qquad (1)$$

α- or γ-thrombin. The observed intensities were then fit to the calculated $[\text{bis-ANS}]_{free}$ to obtain an estimate for $K_{d(true)}$. This same procedure also applies when titrating ligand with increasing concentrations of protein: in fact, this latter titration yields the most accurate values for $K_{d(true)}$, since no inner filter effects occur.

The number of binding sites was estimated by the method of Christian & Janetzko (1971):

$$\frac{1}{d} = \frac{1}{nK_a(1-x)[\text{bis-ANS}]_{total}} + \frac{1}{n}$$

where d is the number of moles of bis-ANS bound per mole of protein, n is the number of binding sites per protein molecule, K_a is the association constant, and x is the fraction of bis-ANS bound. Since the fluorescence of bis-ANS in water is much lower than that in the thrombin complex, the fraction of bound ligand can be written as $x = I_p/I_{max}$, where I_p is the fluorescence of bis-ANS in the presence of protein and I_{max} is the fluorescence intensity at a protein concentration where

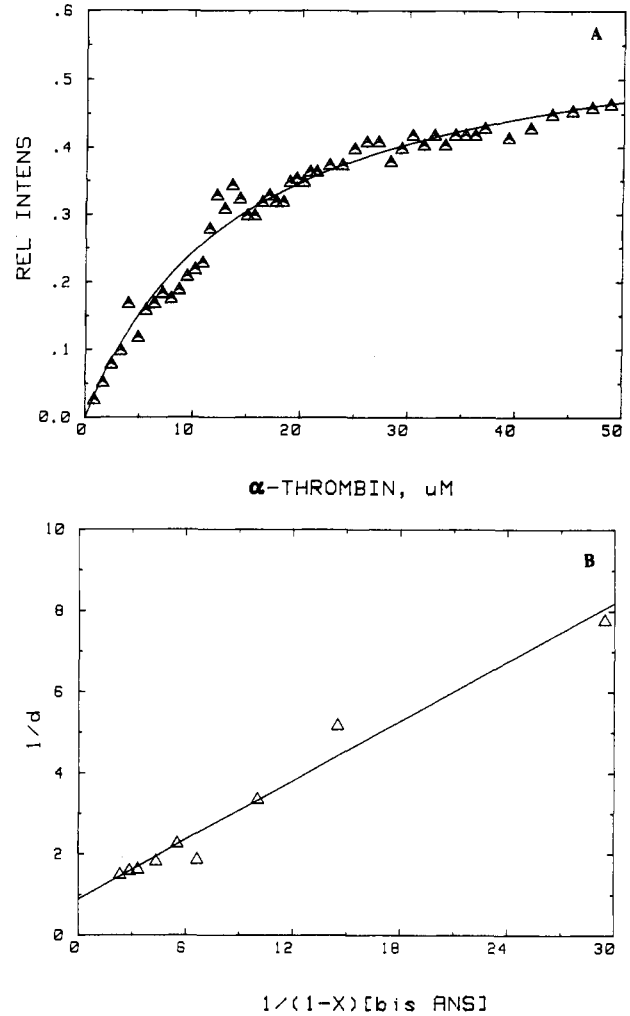

FIGURE 1: (A) α-Thrombin fluorescence titration of 1.0 μM bis-ANS at pH 7.0 (25 mM Tris-HCl, 0.75 M NaCl). Experimental conditions were $\lambda_{ex} = 375$ nm, $\lambda_{em} = 485$ nm, and 25 °C. (b) Plot of $1/d$ vs. $1/(1-x)[\text{bis-ANS}]_{total}$ for α-thrombin–bis-ANS complex.

all dye is bound. (It is assumed that the fluorescence is a linear function of ligand–protein complex concentration.) I_{max} can be measured from the titration of a fixed amount of bis-ANS with increasing concentration of protein. A plot of $1/d$ vs. $1/(1-x)[\text{bis-ANS}]_{total}$ gives a straight line intercepting the y axis at $1/n$.

The enhancement factor is defined as

$$\text{EF} = \frac{I_{max} \text{ of the thrombin–bis-ANS complex}}{I_{obsd} \text{ of free bis-ANS}}$$

for the same concentration of bis-ANS.

RESULTS

Titrations of human α- or γ-thrombin with bis-ANS resulted in a large enhancement in fluorescence intensity and a large blue shift in the emission maximum. The excitation spectrum (not shown) showed two maxima at 290 and 375 nm, which originated from a protein (tryptophan) energy-transfer band and the fluorophore excitation band (blue shifted from a λ_{ex}^{max} of 385 nm in H_2O). The emission band for this fluorophore was also blue shifted when complexed with thrombin to 485 nm from 515 nm in H_2O. In Figure 1A, a typical titration curve is shown for an α-thrombin titration of a fixed amount of bis-ANS at pH 7.0, 25 mM Tris–0.75 M NaCl. The $K_{d(true)}$ calculated from these data was 14.8 ± 2.2 μM. It was impossible to obtain accurate $K_{d(true)}$ values by titrating

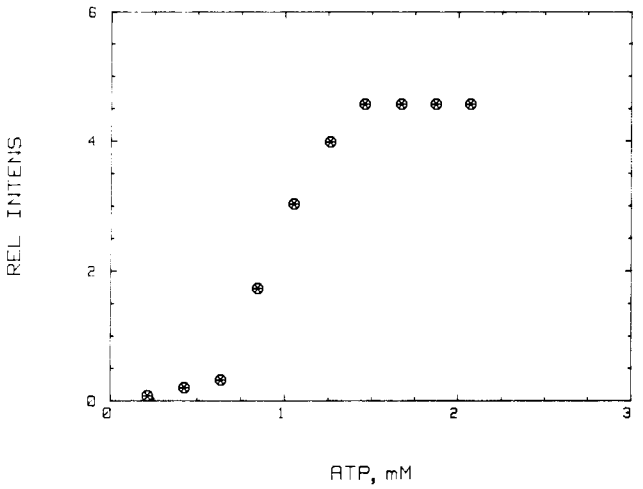

FIGURE 2: ATP titration of α-thrombin–bis-ANS complex at pH 7.0. α-Thrombin concentration was 0.54 μM; bis-ANS concentration was 1.83 μM. Experimental conditions were identical with those of Figure 1.

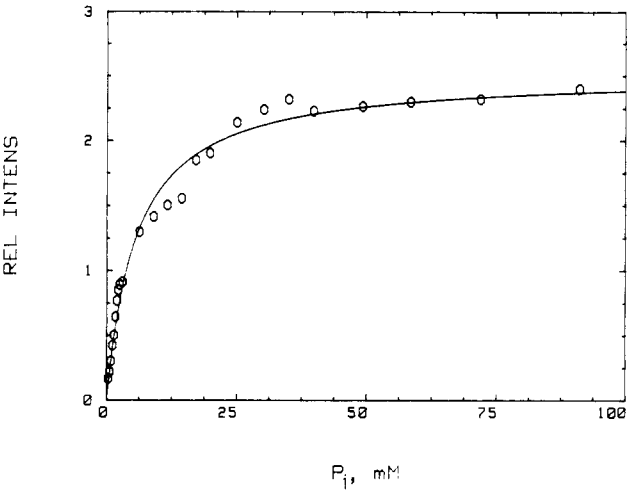

FIGURE 3: NaH_2PO_4 titration of α-thrombin–bis-ANS complex at pH 7.0. α-Thrombin concentration was 0.54 μM; bis-ANS concentration was 3.58 μM. Experimental conditions were identical with those of Figure 1.

bis-ANS vs. a fixed protein concentration since inner filter effects became severe above 15 μM and protein precipitation occurred above 15–20 μM. The precipitation problem occurred at either 0.15 or 0.75 M NaCl.

In the case of γ-thrombin, a $K_{d(true)} = 5.8 \pm 1.0$ μM was obtained, which was about 3 times lower than that for the α form. Here we were able to vary either protein or bis-ANS without any precipitation or inner filter problems, due to the higher affinity of the dye for this form. The K_d values for either α- or γ-thrombin were unchanged with salt concentration over the range 0.15–0.75 M NaCl.

The difference in affinity for the ligand was not the only difference between α- and γ-thrombins. The enhancement factor, EF, defined as the ratio of the intensity of the bound bis-ANS to that of the free-ANS was ~70 for α-thrombin vs. ~220 for γ-thrombin.

In order to calculate the exact number of bis-ANS binding sites to each form, we plotted these data according to the method of Christian & Janetzko (1971). Figure 1B shows such a plot for α-thrombin of $1/d$ vs. $1/(1-x)[\text{bis-ANS}]_{\text{total}}$. From the y intercept a value of 1.13 ± 0.37 sites was obtained; for γ-thrombin (plot not shown) a value of 0.93 ± 0.07 was measured. The stoichiometry may be assumed to be 1:1 in both cases, within experimental error. Measurement of the energy-transfer efficiency between protein tryptophan residue(s) and the bound fluorophore yielded a value of ca. 70% for both α- and γ-thrombins. This is very similar to the results obtained by Berliner & Shen (1977b) with two active serine fluorescent labels. Titrations carried out on bovine thrombin (gift from Dr. K. G. Mann, Mayo Foundation) gave qualitatively similar K_d and EF values.

In an attempt to localize the bis-ANS binding site on α-thrombin, we examined several previously studied exogenous thrombin ligands for their interaction with the bis-ANS–enzyme complex (Conery & Berliner, 1983). We found essentially three classes of behavior with regard to the bis-ANS–thrombin fluorescence: (1) ligands that enhance the intensity of the bound fluorophore; (2) ligands that decrease this intensity; (3) ligands that have no effect. The first group (effectors that enhance bis-ANS–α-thrombin fluorescence) comprises nucleotide derivatives and phosphate. ATP, Ca(II)ATP, and AMP were the most effective in enhancing the fluorescence, increasing the emission intensity up to ~80 times that of bound fluorophore.[2] Relative to free bis-ANS fluorescence, this could in theory amount to a 5600-fold enhancement! Figure 2 shows a typical titration of a bis-ANS–α-thrombin complex with increasing ATP up to 2 mM (at higher ATP concentrations, some protein precipitation occurred). The sigmoidal nature of this curve indicates that at least two ATP sites are involved and that these sites are cooperative. Ca(II)ATP and AMP gave identical enhancements as for ATP (Figure 2). The substitution-inert Co$(NH_3)_4$HATP also enhanced the fluorescence intensity of the bound bis-ANS with a sigmoidal behavior; however, the quantum yield was lower than that in the case of ATP, Ca(II)ATP, or AMP, even after correction for inner filter effects. This latter result may involve specific energy-transfer quenching of the bis-ANS fluorescence by Co(III).

Adenosine, inorganic phosphate, inorganic pyrophosphate, and dioxane were also found to enhance bound bis-ANS fluorescence. However, in contrast to the nucleotides above, these latter compounds showed a normal hyperbolic titration. Figure 3 depicts a titration of bis-ANS–α-thrombin by inorganic phosphate at constant pH. The enhancement factor for this complex approached a maximum of ca. 25. Dioxane was an exception in that, at the concentrations needed to see an enhancement (1–2 M), solvent effects were significant and deviation from hyperbolicity was observed.

Three ligands were found to *decrease* the fluorescence of a bis-ANS–thrombin mixture. Titration with the active site inhibitor benzamidine caused a reduction in emission intensity with an apparent $K_d = 6$ mM. Control experiments in the *absence* of thrombin indicated that benzamidine also complexed with the free fluorophore alone; however, at the same concentrations, free bis-ANS fluorescence was reduced to a much lesser extent than was bound bis-ANS–thrombin. That is, the evidence from these steady-state fluorescence measurements indicates that ternary complex formation with concomitant quenching occurred even though a small contribution was due to dissociation to form the bis-ANS–benzamidine binary complex. A similar fluorescence "quenching" was observed when mixing bis-ANS–thrombin with *p*-chlorobenzylamine; however, the control experiment (i.e, *ab-*

[2] There was a noticeable time dependence (~10–20 min) for binding of ATP and ATP analogues to the bis-ANS complex before the emission intensity approached its maximum. While we did not quantitate these results kinetically, they were absolutely reproducible, suggesting a slow conformational change involved in the fluorescence enhancement of the bis-ANS–α-thrombin complex.

Table I: Summary of Effects of Various Ligands on the Bis-ANS–α-Thrombin Complex[a]

ligand	effect	$K_{d(app)}$ (mM)	EF
ATP	+	1.0 ± 0.1	~80
Ca(II)ATP	+	0.95	~80
$Co(NH_3)_4$HATP	+	1.0 ± 0.04	~50
AMP	+	0.98	~80
adenosine	+	0.13	~6
phosphate	+	6.6	~25
pyrophosphate	+	<2	~3
dioxane	+	*b*	
tryptamine	ne		
5-hydroxytryptamine	ne		
valerylamidine	ne		
benzamidine	– or ne[b]	6	
p-chlorobenzylamine	– or ne[b]		
heparin	–	<0.02	

[a] Conditions were 25 mM Tris-HCl, pH 7.0, 0.15 M NaCl, 25 °C, λ_{ex} = 375 nm, and λ_{em} = 485 nm. The symbols (+), (–), and ne reflect a fluorescence emission enhancement, decrease, or no effect, respectively. [b] See text.

sence of thrombin) revealed that the quenching of an equal concentration of "free" bis-ANS was identical. Thus in the case of *p*-chlorobenzylamine, it appeared that the effect was competition with thrombin for bis-ANS, i.e., reducing the concentration of bis-ANS–thrombin by complexing with free bis-ANS in solution as well as binding to the basic specificity pocket on thrombin. Heparin, which binds to α-thrombin with a stoichiometry of three to four molecules per protein molecule (Li et al., 1974; Jordan et al., 1980), decreased bis-ANS–α-thrombin fluorescence by ~23%. In this case, the effect was most likely due to quenching rather than physical displacement of the dye.

Valerylamidine was by far the most significant among the ligands that had no effect on fluorescence, since this inhibitor binds strongly to the basic specificity binding pocket. The lack of any interaction with bis-ANS, by virtue of the insensitivity of the fluorescence spectra, suggests that the fluorophore does not bind at the catalytic site. This also supports the hypothesis that benzamidine (which also binds to the basic specificity pocket) was either acting by some aspecific effect or binding at a second site on thrombin (which cannot be excluded a priori). Tryptamine and 5-hydroxytryptamine also showed no effect on the bis-ANS–α-thrombin complex. Table I summarizes the results of the studies discussed above.

The acridine dye, proflavin, binds strongly to several serine proteases, including thrombin. Its displacement by a variety of ligands aids in probing the active sites of these enzymes (Koehler & Magnusson, 1974; Berliner & Shen, 1977). Figure 4 shows difference spectra for the proflavin–α-thrombin complex with increasing concentrations of bis-ANS. A new band at 425 nm was found upon addition of bis-ANS, which was substantially higher in intensity than that for the "native" proflavin–α-thrombin complex (λ = 468 nm). This new 425-nm band was not due to free proflavin, which shows a maximum at ~440 nm; rather, the new peak represented evidence for a ternary bis-ANS–proflavin–α-thrombin complex.

Bis-ANS was also found to inhibit both α-thrombin clotting and amidase activity. However, precise clotting activity measurements were not possible since fibrinogen was found

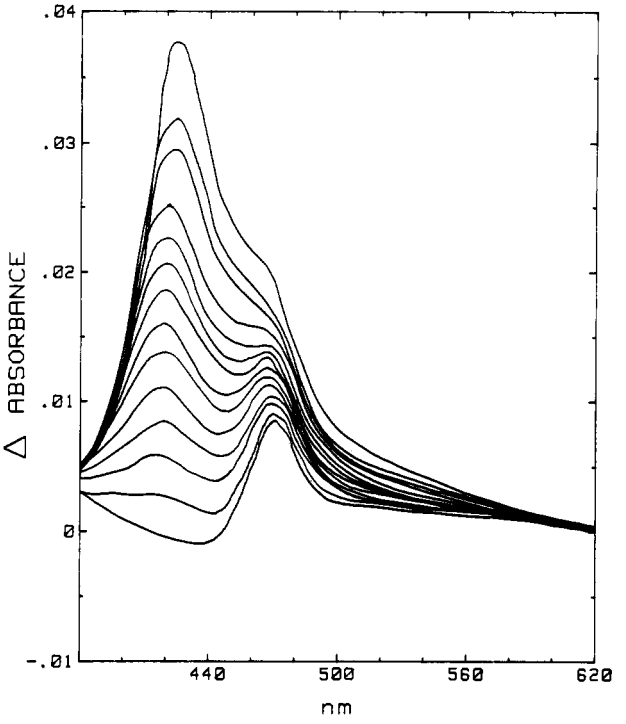

FIGURE 4: Difference absorbance spectra of 12.3 μM proflavin and 5.6 μM α-thrombin at pH 7.0 (25 mM Tris-HCl, 0.15 M NaCl, 25 °C) in the presence of increasing concentration of bis-ANS. Bis-ANS concentrations were (from bottom to top) 0, 4.1, 8.2, 12.3, 16.3, 20.3, 24.2, 28.1, 32.0, 36.0, 39.7, 52.6, 65.4, and 90.3 μM.

to enhance the fluorescence of bis-ANS alone. Furthermore, bis-ANS binding to α-thrombin was obliterated in the presence of PEG (i.e., fluorescence enhancement was observed) when PEG 6000 was present at the concentrations normally required for reliable clotting assays (Fenton & Fasco, 1974). We nonetheless measured the effects of bis-ANS in the absence of PEG 6000 and found inhibition of fibrinogen clotting. Unfortunately, it was not absolutely clear whether the inhibition was due only to thrombin inhibition, to fibrinogen–bis-ANS complexation, or (most likely) a combination of both mechanisms. More detailed kinetic information was obtained from studies of bis-ANS inhibition of amidase activity. Figure 5A shows the inhibition of α-thrombin-catalyzed hydrolysis of S-2238 with increasing concentrations of bis-ANS. It is important to point out here that even at infinite inhibitor concentration, about 20% residual activity remained. The apparent bis-ANS inhibition constant (K_i = 9.25 μM) agreed fairly well with its dissociation constant (Table II). Figure 5B shows double-reciprocal plots for several bis-ANS/S-2238 concentrations. The plots suggested that bis-ANS was a noncompetitive inhibitor; however, the fact that infinite inhibitor concentrations did not totally obliterate activity indicated that a partial rather than a pure noncompetitive inhibition model best described these results (Segel, 1975). A minor problem that we encountered with these kinetic experiments was apparent from a careful analysis of the slopes in the double-reciprocal plot (Figure 5B). In control experiments we found that bis-ANS bound weakly to S-2238 as monitored by a slightly enhanced bis-ANS fluorescence. Thus, the accuracy of the kinetic measurements at very high inhibitor

Table II: Fluorescence Parameters and Equilibrium Constants for Bis-ANS Complexes on α- and γ-Thrombins[a]

enzyme	λ_{ex}^{max} (nm)	λ_{em}^{max} (nm)	$K_{d(true)}$ (μM)	EF	*n*
α-thrombin	375	485	14.8 ± 2.2	70.7 ± 7.6	1.13 ± 0.37
γ-thrombin	375	485	5.8 ± 1.0	222.0 ± 27.9	0.93 ± 0.07

[a] Conditions were pH 7.0, 25 mM Tris-HCl, 0.15 M or 0.75 M NaCl, and 25 °C. Note for bis-ANS in water, λ_{ex}^{max} = 385 nm and λ_{em}^{max} = 515 nm.

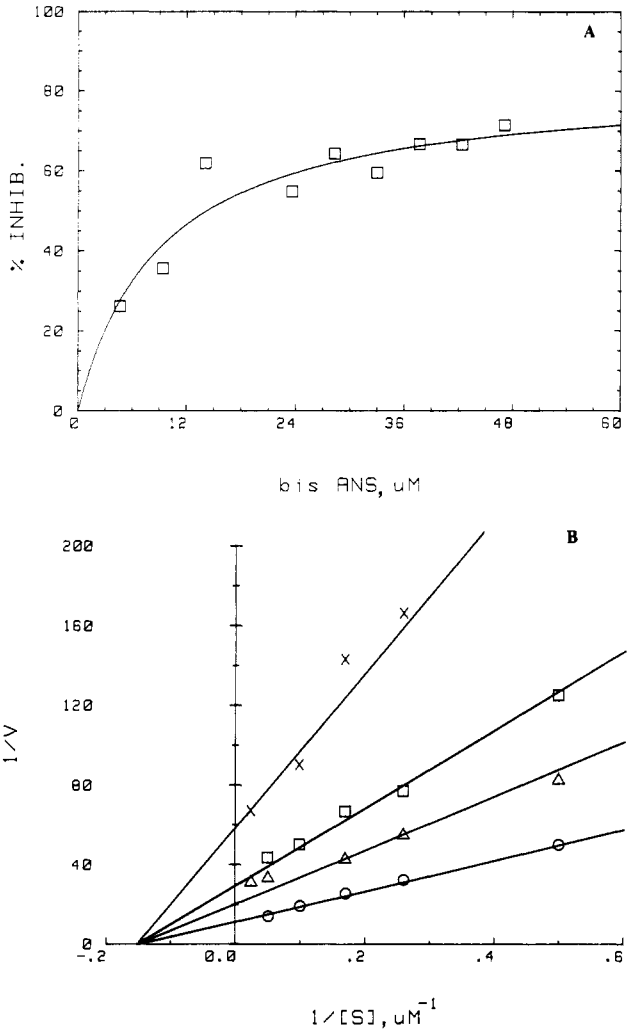

FIGURE 5: (A) Bis-ANS inhibition of α-thrombin-catalyzed hydrolysis of S-2238 at pH 8.3 (50 mM Tris-HCl, 0.1 M NaCl). Experimental conditions were as follows: α-thrombin, 10^{-9} M; S-2238, 10^{-5} M; 25 °C. (B) Double-reciprocal plots of bis-ANS inhibition of α-thrombin-catalyzed hydrolysis of S-2238. All conditions were identical with those in (A); bis-ANS concentrations were (○) 0, (△) 5, (□) 10, and (×) 15 μM.

concentration was less reliable, since it led to an overestimate of the degree of inhibition, which was emphasized in the low S-2238 concentration data. This effect shows up for the highest bis-ANS concentration in Figure 5B where the slope was a bit higher than expected. Another chromogenic substrate, S-2160, also showed about 20% residual activity at infinite bis-ANS concentration. Tos-Arg-OMe esterase activity, on the other hand, was not affected in the presence of bis-ANS.

The monomeric dye ANS bound with equal affinity (K_d = 24 ± 4 μM) to both thrombin forms; however, γ-thrombin ANS was 26% more fluorescent (at 470 nm) than the α form (pH 6.5, 0.05 M phosphate, 0.75 M NaCl).

Discussion

Several conclusions may be drawn from the results of these studies as to the efficacy of bis-ANS as a powerful exo site affinity probe of thrombin. The data of Table II clearly demonstrate the ability of this probe to discriminate between the subtle features of procoagulant human α-thrombin vs. noncoagulant γ-thrombin. The difference in the enhancement factor between α- and γ-thrombin strongly suggests a difference in the hydrophobicity of the binding site of the two forms of the enzyme. On the other hand, the blue shift in the emission maxima, which is usually indicative of apolar features of a binding site, was essentially the same (~30 nm) for both α- and γ-thrombins. While an identical λ_{em}^{max} = 485 nm was indicative of topographically similar apolar binding regions in each form, the 3-fold difference in emission enhancement between the two forms suggests subtle environmental differences, e.g., a higher degree of shielding from the solvent in the γ vs. α species. These features are probably reflected in the 3-fold higher affinity of γ-thrombin for bis-ANS (Table II).

As to the location of the bis-ANS site, the kinetic and spectroscopic data in this paper suggest that it does not overlap the catalytic center. The results presented here, particularly the kinetic studies with S-2238 and S-2160, are consistent with a model that requires that both substrate and inhibitor (bis-ANS) bind simultaneously to the enzyme; that is, the partial noncompetitive inhibition mechanism fits a model where bis-ANS does not overlap the chromogenic substrate binding locus. Furthermore, since the properties that distinguish α- from γ-thrombin include a severely decreased inability of the latter to bind or hydrolyze macromolecular substrates such as fibrinogen while retaining esterolytic/amidolytic function with small substrates (Fenton, 1981), the structural regions where these differences occur must be somewhat removed from the immediate catalytic site [see Berliner (1984)].

Several additional aspects of these results have been invaluable in attempting to localize the various thrombin effector sites as well as corroborating other evidence of their function. For example, our previous kinetic studies gave evidence for cooperative binding phenomena with ATP analogues (Conery & Berliner, 1983). The data of Figure 2, which monitored ATP binding to thrombin, clearly showed (sigmoidal) positive cooperative binding. The large fluorescence emission enhancement was possible only when ATP was bound to at least two sites that did not compete with bis-ANS. Moreover, the ~80-fold fluorescence enhancement of enzyme-bound bis-ANS upon ATP binding is remarkable; it will be interesting to see whether distance measurements between bis-ANS and Co(II)ATP fit a model where the nucleotide moiety shields the fluorophore from external solvent molecules.

The benzamidine binding data were consistent, in part, with a physical displacement of bis-ANS from the thrombin complex. That is, we could not rule out the possibility of a second (hydrophobic) site for benzamidine on thrombin. This is not without precedent, as Mares-Guia et al. (1981) found benzamidine activation of trypsin-catalyzed Tos-Arg-OMe hydrolysis that was quite analogous to the same rate activation found with hydrophobic ligands by Howard & Mehl (1965) and with Tos-Arg-OMe hydrolysis by human thrombin in our laboratory (Berliner & Shen, 1977a; Conery & Berliner, 1983).

The results with *p*-chlorobenzylamine and valerylamidine indicated that their binding at the basic specificity pocket neither overlapped nor was linked to the bis-ANS binding site; i.e., bis-ANS was distant from the catalytic center. The results with heparin suggest that the three to four molecules must be able to bind without sterically overlapping the bis-ANS site. Heparin-induced quenching of bis-ANS–α-thrombin fluorescence could be due to conformational changes, contact quenching (by sulfated saccharide moieties?), or increased solvent accessibility to the bis-ANS site. Some of these mechanisms may be ruled out by future lifetime and anisotropy measurements.

In summary, bis-ANS has several advantages over other thrombin-specific fluorophores such as DAPA (Okamoto et

al., 1976; Nesheim et al., 1979). The maximum enhancement in fluorescence emission observed with DAPA–thrombin complexes was about 3-fold over that in water, while the enhancements with bis-ANS ranged from 80 to a theoretical maximum of 5600. Bis-ANS is an exo site probe that can monitor interactions at the active site while DAPA is a tightly bound competitive inhibitor for most active site directed substrates and inhibitors.

ACKNOWLEDGMENTS

We are extremely grateful to Dr. Franklin Prendergast, Mayo Foundation, for several invaluable discussions. We are also indebted to Dr. John W. Fenton II, New York State Department of Health, Albany, for generous supplies of human thrombins and for many helpful discussions. We are pleased to acknowledge Dr. Y. Y. Shen for providing the preliminary experiments with ANS.

Registry No. Bis-ANS, 63741-13-9; ATP, 56-65-5; Ca(II)ATP, 15866-84-9; $Co(NH_3)_4HATP$, 63915-26-4; AMP, 61-19-8; adenosine, 58-61-7; phosphate, 7664-38-2; pyrophosphate, 2466-09-3; benzamidine, 618-39-3; heparin, 9005-49-6; thrombin, 9002-04-4.

REFERENCES

Berliner, L. J. (1984) *Mol. Cell Biochem. 61*, 159–172.

Berliner, L. J., & Shen, Y. Y. L. (1977a) *Biochemistry 16*, 4622–4626.

Berliner, L. J., & Shen, Y. Y. L. (1977b) *Thromb. Res. 12*, 15–25.

Berliner, L. J., Bauer, R. S., Chang, T.-L., Fenton, J. W., II, & Shen, Y. Y. L. (1981) *Biochemistry 20*, 1831–1837.

Brand, L., & Gohlke, J. R. (1972) *Annu. Rev. Biochem. 41*, 843–868.

Christian, S. T., & Janetzko, R. (1971) *Arch. Biochem. Biophys. 145*, 169.

Conery, B. G., & Berliner, L. J. (1983) *Biochemistry 22*, 369–375.

Cornelius, R. D., Hart, P. A., & Cleland, W. W. (1977) *Inorg. Chem. 16*, 2799–2805.

Fenton, J. W., II (1981) *Ann. N.Y. Acad. Sci. 370*, 468–495.

Fenton, J. W., II, & Fasco, M. J. (1974) *Thromb. Res. 4*, 809–817.

Fenton, J. W., II, Fasco, M. J., Stackrow, A. B., Aronson, D. L., Young, A. M., & Finlayson, J. S. (1977) *J. Biol. Chem. 252*, 3587–3598.

Howard, S. M., & Mehl, J. W. (1965) *Biochim. Biophys. Acta 105*, 596–599.

Hummel, B. C. W. (1959) *Can. J. Biochem. Physiol. 37*, 1393–1399.

Jordan, R. E., Oosta, G. M., Gardner, W. T., & Rosenberg, R. D. (1980) *J. Biol. Chem. 255*, 10073–10080.

Koehler, K., & Magnusson, S. (1974) *Arch. Biochem. Biophys. 160*, 175–184.

Li, E. H. H., Orton, C., & Feinman, R. D. (1974) *Biochemistry 13*, 5012–5017.

Lottenberg, R., Hall, J. A., Fenton, J. W., II, & Jackson, C. M. (1982) *Thromb. Res. 28*, 313–332.

Mares-Guia, M., Rogana, E., Amorim, A. F., & Magalhaes-Rocha, N. (1981) *J. Biol. Chem. 256*, 1661–1668.

McKay, D. B., Kay, L. M., & Stroud, R. M. (1977) in *Chemistry and Biology of Thrombin* (Lundblad, R. L., Fenton, J. W., II, & Mann, K. G., Eds.) pp 113–121, Ann Arbor Science Publishers, Ann Arbor, MI.

Nesheim, M. E., Prendergast, F. G., & Mann, K. G. (1979) *Biochemistry 18*, 996–1003.

Okamoto, S., Hijikata, A., Ikezawa, K., Kinjo, K., Kibumoto, R., Tonomura, S., & Tamao, Y. (1976) *Thromb. Res. 8*, 77–82.

Rosen, C. G., & Weber, G. (1969) *Biochemistry 8*, 3915–3920.

Segel, I. H. (1975) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 161–169, Wiley, New York.

Stryer, L. (1965) *J. Mol. Biol. 13*, 482–495.

Tsernoglou, D., & Petsko, G. A. (1977) in *Chemistry and Biology of Thrombin* (Lundblad, R. L., Fenton, J. W., II, & Mann, K. G., Eds.) pp 123–128, Ann Arbor Science Publishers, Ann Arbor, MI.