Progressive Development of a Thrombin Inhibitor Binding Site†

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ABSTRACT: The studies reported here were undertaken to determine whether the thrombin precursors prothrombin, prethrombin 1, prethrombin 2, and Meizo thrombin interact with the fluorescent, reversible thrombin inhibitor dansylarginine *N*,*N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) [Nesheim, M. E., Prendergast, F. G., & Mann, K. G. (1979) *Biochemistry 18*, 996–1003]. The results indicate that prothrombin and prethrombin 1, in which the cleavage sites at Arg_{274}-Thr_{275} and Arg_{323}-Ile_{324} both remain intact, do not bind DAPA, while prethrombin 2 or Meizo thrombin, which results respectively from a single cleavage of prothrombin at Arg_{274}-Thr_{275} or Arg_{323}-Ile_{324}, do bind the inhibitor. Since prethrombin 2 is a precursor of thrombin without measurable enzymatic activity, a thorough characterization of its interaction with DAPA was undertaken. The interaction of DAPA with bovine thrombin similarly was studied for comparative purposes. The binding of DAPA to either protein is accompanied by changes in the fluorescence properties of the dansyl moiety including increases in emission intensity, excited-state lifetime, polarization, and a slight blue shift in the wavelength of maximum emission intensity. Corrected excitation spectra indicate energy transfer to DAPA from one or more aromatic side chains of both proteins. Values of P_0 for both complexes were extrapolated from Perrin plots of polarization vs. temperature and suggest that the dansyl moiety is held more rigidly in thrombin than in prethrombin 2. With excitation at either 280 or 335 nm the emission intensity of DAPA–prethrombin 2 is substantially less than that of the DAPA–thrombin complex. In contrast, the intensity of the Meizo thrombin–DAPA complex is greater than that of the DAPA–thrombin complex. From measurements of intensity changes the dissociation constants and stoichiometry of DAPA binding to thrombin and prethrombin 2 were measured. Prethrombin 2 binds to DAPA with a $K_d = 5.9 \times 10^{-7}$ M ($n = 1$) while thrombin binds about 30 times more tightly with a $K_d = 2.0 \times 10^{-8}$ M ($n = 1$). The active site directed irreversible thrombin inhibitors diisopropyl phosphorofluoridate and D-phenylalanylprolylarginyl chloromethyl ketone displace DAPA from thrombin but not from prethrombin 2. The results of these studies indicate the binding of a presumed substrate analogue (DAPA) to an inactive zymogen, prethrombin 2. In addition, the lack of DAPA binding to prothrombin and prethrombin 1, under conditions in which it binds to prethrombin 2, implicates events that accompany cleavage at Arg_{274}-Arg_{275} in the "progressive" formation of an active site, even though further cleavage at Arg_{323}-Ile_{324} is required for expression of enzymatic activity.

The blood clotting enzyme thrombin (bovine) is formed from its precursor prothrombin through two proteolytic cleavages. These cleavages are accomplished physiologically by a complex enzyme known as the prothrombinase complex, comprising factor Xa (a serine protease), factor Va (a protein cofactor), negatively charged phospholipids (or platelets), and calcium ion (Mann, 1976; Lundblad et al., 1976; Suttie & Jackson, 1977). The first cleavage of prothrombin[1] (M_r = 72 100) occurs at Arg_{274}-Thr_{275} and yields prethrombin 2 from the COOH′ terminus (M_r = 37 400) plus prothrombin fragment 1·2 from the NH_2 terminus (M_r = 34 800). The second cleavage occurs at Arg_{323}-Ile_{324} of prethrombin 2, between a disulfide bond, to yield the two-chain serine protease thrombin (Heldebrant & Mann, 1973; Heldebrant et al., 1973; Owen et al., 1974; Esmon & Jackson, 1974). In addition, cleavage of prothrombin at Arg_{156}-Ser_{157} can be catalyzed by thrombin (Mann, 1976) to yield prothrombin fragment 1 (M_r = 22000) from the NH_2 terminus and prethrombin 1 (M_r = 50 200). Subsequent cleavage of prethrombin 1 at Arg_{274}-Thr_{275} by the prothrombinase complex yields prethrombin 2 plus fragment 2 (Owen et al., 1974; Mann, 1976). The prothrombinase complex catalyzes the two cleavages in the distinct order Arg_{274}-Thr_{275} followed by Arg_{323}-Ile_{324}. Cleavage in the opposite order, although searched for, has not been observed (Esmon & Jackson, 1974).

Since thrombin activity is observed only after the second cleavage, this cleavage appears essential in the formation of an active enzyme. In addition, studies with Meizo thrombin, a prothrombin derivative cleaved only at the second site (Arg_{323}-Thr_{324}) through use of a protease from *Echis carinatus* venom (ECV-P), indicate that the second cleavage is sufficient to yield activity. This activity is expressed, however, only toward small substrates. The activity toward the natural substrate fibrinogen is minimal. Cleavage of Meizo thrombin at Arg_{274}-Thr_{275}, however, yields full fibrinogen clotting activity (Morita et al., 1976; Morita & Iwanaga, 1978). This property of Meizo thrombin suggests that cleavage at Arg_{274}-Thr_{275}, although insufficient in itself to produce an active site, may contribute substantially toward the development of the substrate binding or catalytic properties of thrombin.

This paper constitutes a report of studies of the interaction (or lack thereof) of prothrombin, prethrombin 1, prethrombin 2, Meizo thrombin, and thrombin with the fluorescent, specific, thrombin inhibitor dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide. These studies were performed in attempts to deduce the relative contribution of cleavage of the bonds at Arg_{274}-Thr_{275} and Arg_{323}-Ile_{324} toward the development of the thrombin active site.

Experimental Procedures

Dansylarginine *N*,*N*-(3-ethyl-1,5-pentanediyl)amide (DAPA)[2] was prepared by the method of Nesheim et al.

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[1] Figure 9 is a schematic representation of the cleavages involved in prothrombin conversion. Amino acid residues are numbered according to the bovine sequence.

[2] Abbreviations: DFP, diisopropyl phosphorofluoridate; DAPA, dansylarginine *N*,*N*-(3-ethyl-1,5-pentanediyl)amide; PPACK, D-phenylalanylprolylarginyl chloromethyl ketone; $NaDodSO_4$, sodium dodecyl sulfate; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; Pre-2, prethrombin 2.

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(1979a). Concentrations of DAPA solutions were checked by measurements of the absorbance at 330 nm and use of the extinction coefficient $E^{1mM}_{330nm} = 4.01$ (Nesheim et al., 1979a). Diisopropyl phosphorofluoridate (DFP) and Tris base (Trizma) were obtained from Sigma Chemical Co. The thrombin active site histidine alkylating agent D-phenylalanylprolylarginyl chloromethyl ketone (PPACK) was a gift of Dr. Charles Kettner, Brookhaven National Laboratory (Kettner & Shaw, 1979). Sodium dodecyl sulfate ($NaDodSO_4$) was from Pierce Chemical Co.; acrylamide, *N,N'*-methylenebis(acrylamide), and TEMED were from Eastman, and ammonium persulfate was from Baker. The water used throughout this study was purified by a Milli-Q four-place cartridge filtration system (Millipore Corp.), and it, as well as buffers made with it, contained no detectable fluorescent impurities.

Proteins. Bovine α-thrombin was purified from Parke-Davis thrombin on sulfopropyl-Sephadex C-50 by a modification (Mann et al., 1971) of the method of Lundblad (Lundblad, 1971). Bovine prothrombin and factor X were purified by the method of Bajaj & Mann (1973); factor X was activated to factor Xa by Russell's viper venom covalently coupled to Sepharose (Downing et al., 1975). Bovine prethrombin 1 was prepared by incubating prothrombin with thrombin, followed by chromatography on DEAE-cellulose (Heldebrant et al., 1973). Bovine prethrombin 2 and prothrombin fragment 2 were prepared by factor Xa cleavage of prethrombin 1 and separated by chromatography on DEAE-cellulose or on sulfoethyl-Sephadex C-50 (Heldebrant & Mann, 1973).

The thrombin activity present in the prethrombin 2 and thrombin preparations was assayed by a modification (Lundblad et al., 1976) of the standard NIH assay procedure, *Minimum Requirements for Dried Thrombin* (1946). The molecular weights (including carbohydrate, where applicable) and extinction coefficients ($E^{1\%}_{280nm}$) used are as follows (Mann & Elion, 1978): prethrombin 1, 5.02×10^4 and 16.4; prethrombin 2 and thrombin, 3.74×10^4 and 19.5; prothrombin fragment 2, 1.28×10^4 and 12.5; and prothrombin, 7.21×10^4 and 14.4. The prothrombin activator of *Echis carinatus* venom (obtained from Sigma) was partially purified by chromatography on DEAE-cellulose as described by Morita et al. (1976), followed by preparative electrophoresis with an apparatus described by Nesheim et al. (1979a). Electrophoresis was performed in Tris–borate (0.05 M) and $CaCl_2$ (0.005 M), pH 8.3, at 4 °C with a gel 5 cm in length with a final concentration (w/v) of acrylamide and bis(acrylamide) of 8.0% and 0.1%, respectively. The activator was detected by adding samples to a solution of 1.4 μM bovine prothrombin, 5.0 μM DAPA, 2 mM $CaCl_2$, 0.02 M Tris-HCl, and 0.15 M NaCl, pH 7.4, and recording fluorescence intensity ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 545$ nm) as a function of time. In this assay conversion of prothrombin to Meizo thrombin is indicated by enhanced fluorescence. Rates of fluorescence enhancement were directly proportional to activator concentration.

Electrophoresis. Analyses of the proteins used in these experiments were carried out on polyacrylamide gels (10%) in the presence of $NaDodSO_4$ by the method of Weber & Osborne (1969). The gels were stained with Coomassie brilliant blue and destained electrophoretically.

Methods. (A) Fluorescence Measurements. The binding of DAPA to bovine thrombin and prethrombin 2 was examined by observing the increase in polarization and the increase in emission intensity upon addition of protein to solutions of DAPA. Fluorescence energy transfer has been observed (Straight et al., 1980) from the protein to DAPA, and this phenomenon was used presently to obtain binding data and to provide a means of comparison of the prethrombin 2 and thrombin binding sites.

PPACK was added to all the prethrombin 2 solutions in this study to provide for rigorous comparison of spectra and to remove any possible ambiguity from the prethrombin 2–DAPA titration results. Our prethrombin 2 preparations typically contained thrombin at 6–8 mol %, measured by the clotting assay. PPACK effectively displaces DAPA from the thrombin active site (see Results) at a concentration 1.25 times the thrombin concentration (typically about 5×10^{-8} M in the experiments reported below) with no apparent effect on the fluorescence properties of the prethrombin 2–DAPA complex. Therefore, PPACK was included in the prethrombin 2 solutions to remove any contribution to the observed fluorescence due to the thrombin–DAPA complex.

Emission and excitation spectra of DAPA and its complexes with prethrombin 2 and thrombin, corrected for instrumental factors, were measured with a Perkin-Elmer MPF-44a spectrofluorometer equipped with a DCSU-2 differential-corrected spectra unit. Correction data were measured and stored in memory each time the xenon lamp was ignited. Typically, spectra were measured with excitation and emission slits set at 10 and 15 nm, respectively, with a scanning rate of 120 nm/min and a 1.5-s response time. All spectra were recorded with a 430-nm sharp-cut filter between the sample and the detector to reduce Rayleigh scattering and to eliminate second-order transmission of scattered exciting light.

Polarization measurements of DAPA–thrombin binding were made with the Perkin-Elmer MPF-44A fluorometer equipped with an automatic polarization accessory and interface (Wood Manufacturing Co., Newtown, PA) with Polacoat polarizers. Monochromater *g* factors (Azumi & McGlynn, 1962) were calculated from measured values for the emission intensities with the excitation polarizer in the horizontal setting. A 430-nm filter was inserted before the emission monochromater for all measurements.

Polarization studies of DAPA binding to prethrombin 2 were done with an SLM 4800 subnanosecond lifetime fluorometer in T-format (Weber, 1956; Weber & Bablouzian, 1966). Polarization values were corrected for scatter according to Shinitzky et al. (1971).

Differential phase lifetime measurements were made with this instrument in single-end format (Spencer & Weber, 1969) with a glycogen reference solution. All the measurements were made with a modulation frequency of 18 MHz and with the excitation slits set at 0.5 nm.

(B) Binding Studies and Data Analysis. The association constants and stoichiometries of the DAPA–protein complexes were determined from titrations in which protein was added to DAPA solutions, and emission intensities were measured as a function of protein concentration. The data were analyzed by the equation (Nesheim et al., 1979a):

$$\frac{1}{(1-b)[L]_0} = \frac{n[P]_0 K_a}{b[L]_0} - K_a \qquad (1)$$

in which b is the fraction of total ligand concentration bound, K_a is the association constant, n is the number of independent, noninteracting ligand binding sites per protein molecule, and $[P]_0$ and $[L]_0$ are the total concentrations of protein and ligand, respectively. This equation is a variation of the Scatchard equation (Scatchard, 1949) and is similar to one derived by Gutfreund (Gutfreund, 1972) except that b is the fraction of bound ligand rather than the fraction of bound protein. Values for K_a and n were obtained with Fortran programs, following the method outlined previously (Nesheim et al., 1979a).

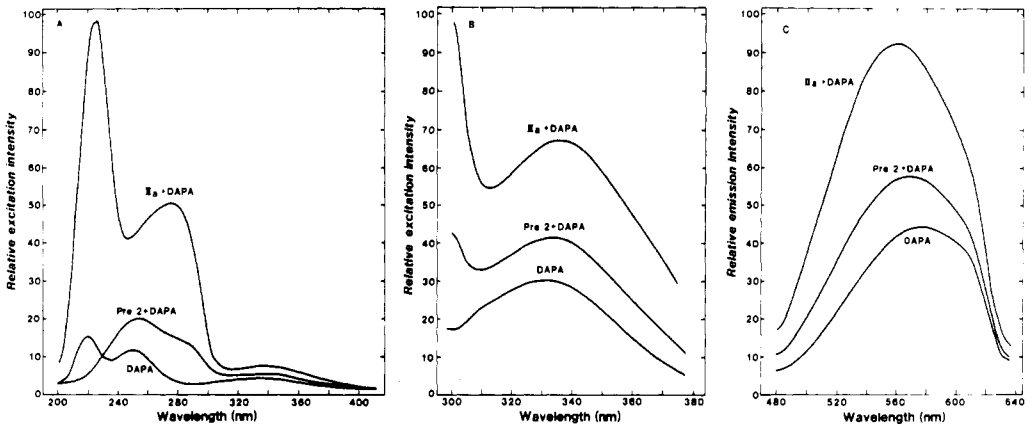


FIGURE 1: DAPA, prethrombin 2–DAPA, and α-thrombin–DAPA fluorescence spectra: (A) Corrected excitation spectra, λ_{em} = 565 nm for all spectra; (B) corrected excitation spectra showing detail in the range of λ_{ex} = 300–380 nm, λ_{em} = 565 nm for all three spectra; (C) corrected emission spectra, λ_{ex} = 335 nm for all three spectra. All spectra were obtained with one set of solutions: [DAPA] = 9.6×10^{-7} M, [factor IIa] = 9.7×10^{-7} M, and [Pre-2] = 4.8×10^{-6} M in 0.05 M Tris-HCl, 0.15 M NaCl, and 0.05 M NH_4Cl, pH 7.4. All spectra were recorded with a 430-nm sharp-cut filter in place between the sample and the emission monochromater.

Emission intensity titrations were conducted by recording the corrected emission spectrum for the sample after each addition of protein. Because the DAPA emission peak is blue shifted by 15–20 nm upon binding to the proteins, the maximum intensity in the range 560–580 nm was taken as the signal corresponding to bound ligand.

Qualitative estimations of the interaction of DAPA with Meizo thrombin were performed by adding the purified ECV-P (10 μg/mL) to a solution of prothrombin (0.1 mg/mL, 1.39 μM), DAPA (5 μM), $CaCl_2$ (2.0 mM), 0.02 M Tris-HCl, and 0.15 M NaCl, pH 7.4 at 22 °C. The progress of Meizo thrombin formation was followed by fluorescence (λ_{ex} = 280 or 335 nm; λ_{em} = 545 nm). At the completion of the reaction further conversion of Meizo thrombin to thrombin was accomplished by the addition of factor Xa, factor Va, and vesicles of phosphatidylcholine–phosphatidylserine (Nesheim et al., 1979b). The formation of thrombin from Meizo thrombin was indicated by a slight decrease in fluorescence intensity. The identity of products formed in the prothrombin activation mixture was assessed by acrylamide gel electrophoresis in $NaDodSO_4$ before and after reduction of disulfide bonds.

(C) Determination of P_0 for the DAPA–Protein Complexes. Values for P_0, the polarization observed in the absence of rotation of the fluorophore, were obtained from plots of observed polarization vs. temperature with the Perrin equation (Perrin, 1926)

$$\frac{1}{P} - \frac{1}{3} = \frac{1}{P_0} - \frac{1}{3}\left(1 + \frac{RT}{\eta V}\tau_0\right)$$

in which P is the observed polarization, R is the gas constant, T is the temperature, η is the solvent viscosity, V is the volume of the fluorescent group, and τ_0 is the lifetime of the excited state. Polarization measurements were made with the SLM 4800 lifetime fluorometer operated in T-format (Weber, 1956; Weber & Bablouzian, 1966) with the sample cell temperature controlled by a Brinkmann refrigerated constant temperature bath. Temperature measurements were made with a Newport digital thermometer.

Results

Fluorescence Properties of DAPA, DAPA–Prethrombin 2, and DAPA–Thrombin. Corrected excitation and emission spectra of solutions of DAPA, DAPA and thrombin, and DAPA and prethrombin 2 are shown in Figure 1. All spectra were obtained with a set of solutions in which the concentration of DAPA was constant, 9.6×10^{-7} M, and the concentrations of thrombin and prethrombin 2 used were 9.7×10^{-7} M and 4.8×10^{-6} M, respectively. In addition, the prethrombin 2–DAPA solution contained PPACK at a concentration of 1.2×10^{-6} M (see Methods).

Corrected excitation spectra from 200 to 400 nm are shown in Figure 1A. The DAPA spectrum consists of three excitation bands centered at about 330, 250, and 220 nm. The prethrombin 2–DAPA spectrum consists of a band centered at about 335 nm and a broad band from about 240 to 290 nm, with no band appearing at 220 nm. The broad shoulder appearing at 280–290 nm is evidence of energy transfer from the prethrombin 2 molecule to the DAPA molecule. This feature appears with greater intensity in the thrombin–DAPA spectrum as a peak with a maximum at 275 nm. The thrombin–DAPA spectrum also contains a band at about 226 nm with an intensity several times that of DAPA alone. The thrombin–DAPA excitation spectrum is similar to that of human dansylserine-254 thrombin obtained by Berliner & Shen (1977).

Figure 1B shows the corrected excitation spectra for the three solutions over the range λ_{ex} = 300–380 nm in which the dansyl group is excited directly. The excitation peak maximum is shifted slightly to longer wavelengths with binding: λ_{max} = 330 (DAPA), 335 (prethrombin 2–DAPA), and 340 nm (thrombin–DAPA). Figure 1C is the corrected emission spectra of the three samples with λ_{ex} = 335 nm. The emission peak maxima show a small blue shift upon binding of DAPA by the proteins with λ_{max} = 575 (DAPA), 565 (prethrombin 2–DAPA), and 560 nm (thrombin–DAPA).

In addition to spectra, differential phase lifetime measurements were obtained with solutions of DAPA, DAPA plus thrombin, and DAPA plus prethrombin 2. Whether bound to prethrombin 2 or thrombin, DAPA exhibited a 2.2-fold increase in lifetime, suggesting that in either case binding to the protein removes DAPA from the dynamic quenching influences of the bulk solution. The values obtained presently with the bovine proteins are similar to the values observed with DAPA and human thrombin (τ_ϕ = 9.61 ns, τ_m = 9.65 ns; Nesheim et al., 1979a). In addition, polarization measurements were performed at various temperatures, and the data were analyzed to determine P_0 (Perrin, 1926) in order to obtain information about the relative freedom of mobility of DAPA bound to prethrombin 2 compared to DAPA bound to thrombin. Perrin plots of the variation of polarization with temperature are indicated in Figure 2. The viscosity of the

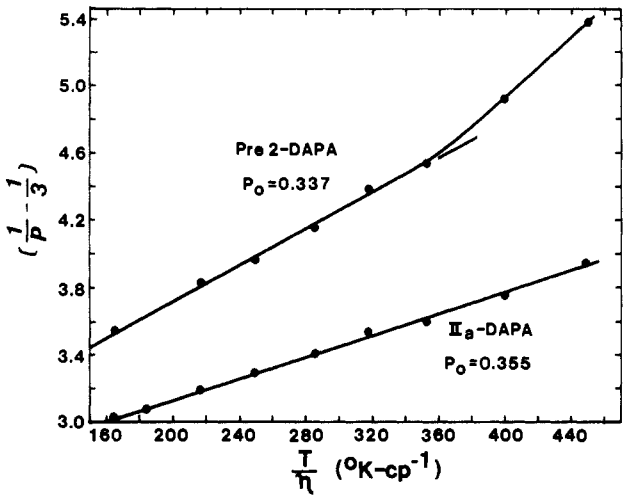


FIGURE 2: Perrin plot of observed polarization vs. the temperature–viscosity term T/η. Lines drawn through the data points (α-thrombin–DAPA, all points; prethrombin 2–DAPA, points in the interval 180–360 K cP^{-1} only) were placed by linear regression analysis. Values of P_0 were obtained by extrapolation of T/η to 0, with a value of 0.337 for prethrombin 2–DAPA and 0.355 for α-thrombin–DAPA.

Table I: Fluorescence Properties of DAPA, DAPA-Prethrombin 2, and DAPA-Thrombin

sample	excitation max[a]	emission max[b]	lifetime[c] τ_ϕ	lifetime[c] τ_m	P_0[d]
DAPA	220, 250, 330	575	4.31	4.42	
DAPA-Pre-2	260, 280, 335	565	9.50	9.30	0.337
DAPA-factor IIa	226, 275, 340	560	9.57	9.11	0.355

[a] λ_{em} = 565 nm. [b] λ_{ex} = 335 nm. [c] See text for details. [d] P_0 = polarization.

buffer was taken to be equal to that of water, and its value at various temperatures was taken from the *CRC Handbook of Chemistry and Physics* (1969). By extrapolation to infinite viscosity (or absolute zero temperature), values for P_0 were determined. The upper curved line in Figure 2 is that of the prethrombin 2–DAPA complex, and a linear least-squares line fit to the data in the T/η interval, 160–260 K cP^{-1} extrapolated to infinite viscosity produced a P_0 value of 0.337. The two points in this curve in the interval 380 $\leq T/\eta \leq$ 460 K cP^{-1} ($T \leq 30$ °C) indicate that DAPA is bound less tightly above 30 °C than below that temperature by prethrombin 2. The plot of the data for the thrombin–DAPA complex appears below that of the prethrombin 2–DAPA complex, and the linear least-squares line through all the data is shown. The value of P_0 obtained from these data is 0.355, slightly higher than that of the prethrombin 2–DAPA complex. Therefore, the DAPA dansyl group apparently is held in place slightly less firmly in the prethrombin 2 complex than in the thrombin complex. The fluorescence properties of DAPA, DAPA–prethrombin 2, and DAPA–thrombin are summarized in Table I.

Binding of DAPA to Bovine Prethrombin 2 and Thrombin. The formation of the complexes of DAPA and prethrombin 2 or thrombin was followed by observing the increase in emission intensity with the addition of prethrombin 2 or thrombin to solutions of DAPA with excitation at 280 nm. Figure 3A shows a typical titration of DAPA with prethrombin 2 with λ_{ex} = 280 nm. (See figure caption for experimental details.) The analysis of these data (shown in Figure 4B) using the method described previously (Nesheim et al., 1979a) gave a stoichiometry n = 1.05 mol of DAPA/mol of prethrombin 2 and a dissociation constant $K_d = 5.85 \times 10^{-7}$ M.

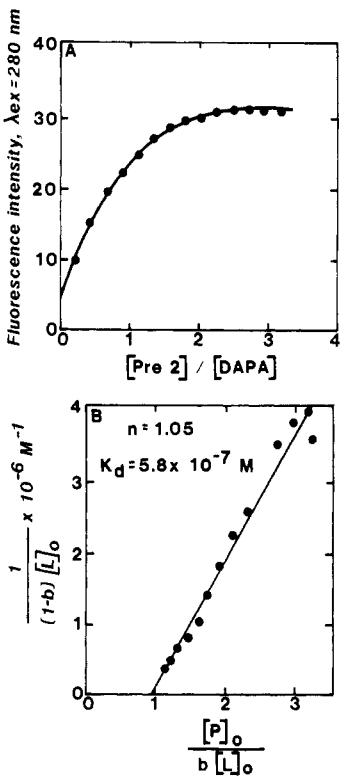


FIGURE 3: (A) Bovine prethrombin 2 and DAPA intensity titrations, λ_{ex} = 280 nm. Observed fluorescence emission intensity is plotted as a function of [Pre-2]/[DAPA]. [DAPA] = 3.0×10^{-6} M. (B) Analysis of data from part A gives a stoichiometry of 1.05 mol of DAPA/mol of prethrombin 2 with a dissociation constant $K_d = 5.8 \times 10^{-7}$ M.

Titrations with direct excitation of the dansyl moiety of DAPA at 335 nm were also performed and analyzed. With excitation at 335 nm intensity increases were quite small (about 10–20%) and were characterized by a slight linear increase observed upon the addition of excess protein. This linear increase was interpreted as a reflection of self-association of prethrombin 2 at relatively high protein concentrations. When titration data were corrected for this slight increase in intensity, five intensity titrations performed with λ_{ex} = 335 nm produced the following values for the stoichiometry and the dissociation constant: $n = 0.99 \pm 0.21$ and $K_d = (5.9 \pm 1.4) \times 10^{-7}$ M (see Table II).

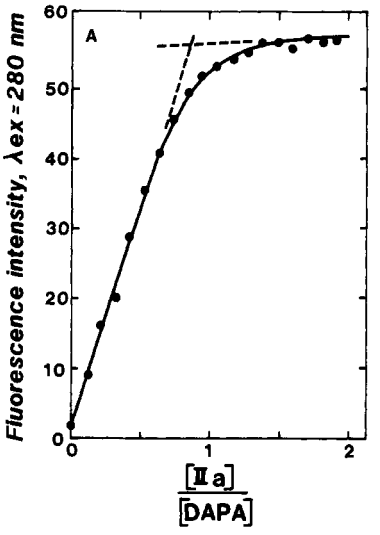
The properties of the (bovine) thrombin–DAPA complex were studied for comparison with those of the prethrombin 2–DAPA complex. The formation of the thrombin–DAPA complex was observed in emission intensity titrations with excitation at 280 nm. Figure 4A shows the data from an experiment in which thrombin was added to a solution of DAPA with λ_{ex} = 280 nm. Figure 4B shows the analysis resulting in a stoichiometry of 1.3 mol of DAPA/mol of thrombin and a dissociation constant $K_d = 1.9 \times 10^{-8}$ M. Four intensity titration experiments were performed, and the overall results are given in Table II: $n = 1.20 \pm 0.14$ mol of DAPA/mol of thrombin and $K_d = (2.0 \pm 0.6) \times 10^{-8}$ M. By comparison, a dissociation constant of 4.3×10^{-8} M was determined by Nesheim et al. (1979a) for the human thrombin–DAPA complex. These data indicate that both prethrombin 2 and thrombin bind DAPA with a 1:1 stoichiometry, and thrombin binds the probe more tightly than prethrombin 2 by a factor of about 30.

The interaction of DAPA with prethrombin 2 was also investigated by fluorescence polarization with λ_{ex} = 335 nm. Typical results are shown in Figure 5. Polarization values

Table II: Binding of Bovine Thrombin and Prethrombin 2 to DAPA[a]

complex	n	K_a (M^{-1})	K_d (M)	increased intensity[b] λ_{ex} = 280 nm	increased intensity[b] λ_{ex} = 335 nm
thrombin–DAPA	1.20 ± 0.14	(5.2 ± 1.4) × 10^7	(2.0 ± 0.6) × 10^{-8}	~30	2.4
Pre-2–DAPA	0.99 ± 0.21	(1.8 ± 0.4) × 10^6	(5.9 ± 1.4) × 10^{-7}	8.3	1.2

[a] All the data presented here were obtained by intensity titrations. The error values are the standard deviations of multiple determinations of each parameter. [b] These values are the observed increases in emission intensity for the complexes at saturating protein with respect to DAPA alone in solution. The value obtained for prethrombin 2–DAPA at λ_{ex} = 335 nm applies after correction for a slight linear increase that occurred beyond saturating levels of protein (see text).



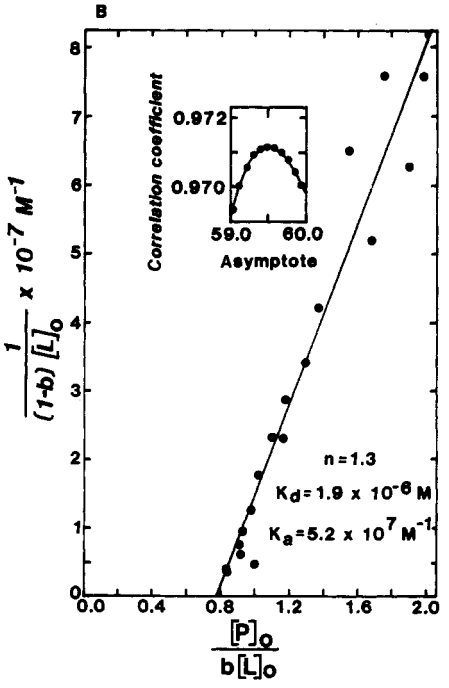


FIGURE 4: (A) Bovine α-thrombin and DAPA fluorescence emission intensity titration. The plot indicates the observed emission intensity of a solution of DAPA to which α-thrombin was added. λ_{ex} = 280 nm; [DAPA = 3.2 × 10^{-7} M in 0.05 M NH_4Cl, pH 7.4. (B) Analysis of the titration data of Figure 4A. Binding data analysis revealed a stoichiometry of 1.3 mol of DAPA/mol of α-thrombin and a dissociation constant of 1.9 × 10^{-8} M.

increased with increased ratios of prethrombin 2 to DAPA. Extrapolations of the two approximately linear portions of the polarization value intersected at a 1:1 molar ratio of prethrombin 2 to DAPA and a polarization value of about 0.165. The slight increase in polarization values obtained at high protein concentrations is suggestive of prethrombin 2 self-

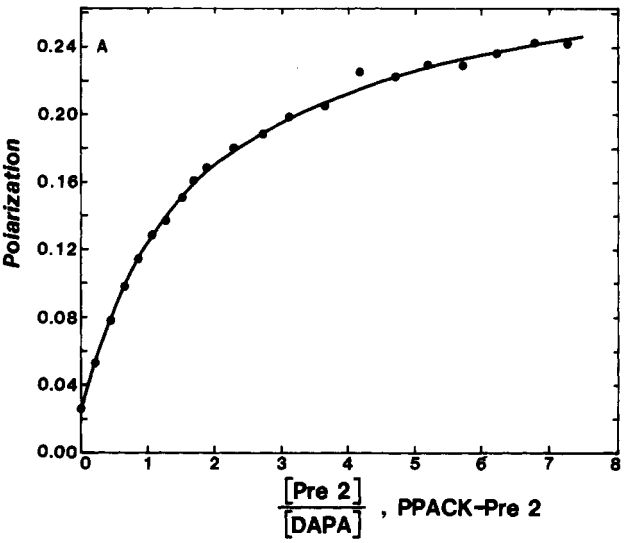


FIGURE 5: Polarization titration of prethrombin 2 and DAPA (corrected for scattered light artifacts).

association, as was inferred from intensity titrations with excitation at 335 nm.

Lack of DAPA Binding to Prethrombin 1 and Prothrombin. Similar experiments employing fluorescence polarization were performed to ascertain whether DAPA binds to prethrombin 1 or prothrombin at protein concentrations and solvent conditions similar to those used in the studies of prethrombin 2. Polarization measurements were made on a solution of DAPA (3 × 10^{-6} M) to which aliquots of a stock solution of prethrombin 1 were added. No increase in polarization was observed for concentrations of prethrombin 1 up to 8 × 10^{-6} M. Similarly, polarization measurements were made on a DAPA solution (1 × 10^{-6} M) to which bovine prothrombin was added to a final concentration of 3 × 10^{-6} M, and again no increase in polarization was detected. These observations, that DAPA does not bind prothrombin and prethrombin 1 but does bind prethrombin 2, suggest that when the A–B chain cleavage site (Arg_{323}-Ile_{324}) is intact, a minimal requirement for the expression of DAPA (and presumably substrate) binding properties is the cleavage at Arg_{274}-Thr_{275}, which defines the conversion of either prothrombin or prethrombin 1 to prethrombin 2.

Evidence for Binding of DAPA to Meizo Thrombin. Whether the cleavage of Arg_{274}-Thr_{275} is also necessary for substrate binding or catalysis when the A–B chain cleavage (Arg_{323}-Ile_{324}) already exists was investigated by binding experiments with Meizo thrombin prepared with ECV-P. This protein cleaves prothrombin at Arg_{323}-Ile_{324} to yield an otherwise intact prothrombin derivative, Meizo thrombin (Morita et al., 1976; Morita & Iwanaga, 1978), that possesses esterase activity comparable to that of thrombin but little fibrinogen clotting activity. The ability of Meizo thrombin to bind DAPA is indicated in Figure 6, which represents, in the presence of DAPA, conversion of prothrombin to Meizo thrombin by

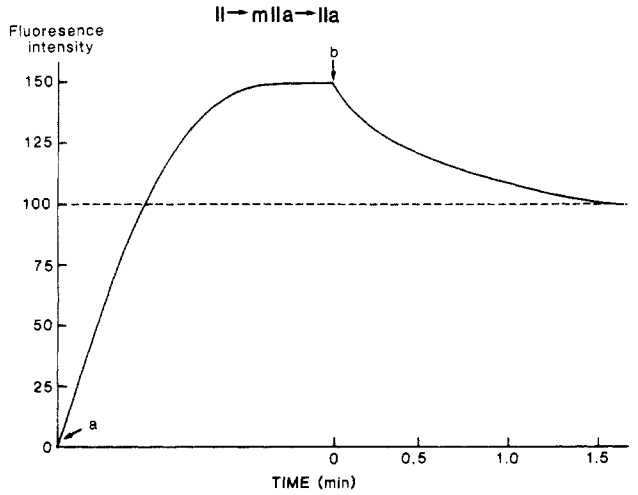


FIGURE 6: Time course of the conversion of prothrombin to Meizo thrombin and subsequent conversion to α-thrombin monitored by fluorescence intensity. At point a ECV-P (10 μg/mL final concentration) was added to a solution of prothrombin (1.4 μM) in 0.02 M Tris-HCl, 0.15 M NaCl, 2.0 nM $CaCl_2$, and 5 μM DAPA, pH 7.4. The progress of conversion of prothrombin to Meizo thrombin is indicated by the enhanced fluorescence (λ_{ex} = 280 nm, λ_{em} = 545 nm). At point b factor Va (5.0 nM), vesicles of phosphatidylcholine and phosphatidylserine (15 μM), and finally factor Xa (approximately 2.0 nM) were added to convert Meizo thrombin to α-thrombin. The dashed line represents the maximum intensity obtained by direct conversion of prothrombin (1.4 μM) to α-thrombin.

ECV-P, followed by conversion of Meizo thrombin to thrombin plus fragment 1·2 by factor Xa, factor Va, Ca^{2+}, and phospholipid. The reaction solution consisted of vesicles of phosphatidylserine–phosphatidylcholine, Ca^{2+}, DAPA (5 μM), and bovine prothrombin (1.4 μM). At point a, ECV-P was added and the reaction was followed to completion. A substantial increase in fluorescence intensity (λ_{ex} = 280 nm) marked the reaction. The intensity obtained at completion exceeded that which could be obtained by direct activation of the prothrombin to thrombin plus fragment 1·2 by factor Xa, factor Va, Ca^{2+}, and phospholipid (dashed line, Figure 6). At point b in Figure 6, factor Xa and factor Va were added to convert Meizo thrombin to thrombin and fragment 1·2. This conversion was indicated by a decrease in fluorescence that, at the completion of the reaction, was equivalent to that obtained by direct conversion of prothrombin to thrombin plus fragment 1·2. The identity of Meizo thrombin, fragment 1·2, and thrombin at various stages of this and similar reactions was established by gel electrophoresis in $NaDodSO_4$ with and without reduction of disulfide bonds. The product of the incubation with ECV-P comigrated with prothrombin in the absence of reducing agents. After reduction of disulfide bonds, two components were seen, one comigrating with the B chain of thrombin and the other migrating to a position consistent with the identification of the component as fragment 1·2 in linkage with the A chain of thrombin. Over the brief interval in which prothrombin was exposed to ECV-P in the presence of DAPA, relatively little (<5%) fragment 1 and Meizo thrombin (des fragment 1), resulting from autolysis at Arg_{156}-Ser_{157} (Morita & Iwanaga, 1978), were observed. Subsequent addition of the combination of factor Xa, factor Va, and phosphatidylcholine–phosphatidylserine gave products that comigrated with thrombin (thrombin B chain after reduction) and fragment 1·2. These results show that when the bond at Arg_{274}-Thr_{275} is intact but the bond at Arg_{323}-Ile_{324} is not, DAPA binding occurs with a fluorescence intensity that exceeds that of the α-thrombin–DAPA complex. An effect on the DAPA binding site expressed through the bond

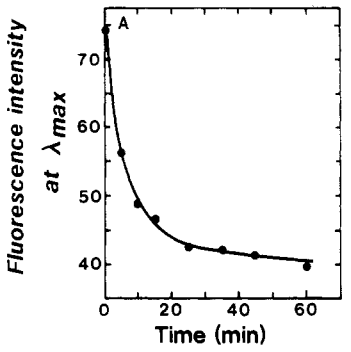


FIGURE 7: Interaction of the α-thrombin–DAPA complex with DFP. Time course of emission intensity of the α-thrombin–DAPA complex incubated (1×10^{-6} M) with DFP (4.7×10^{-3} M) is shown. Intensities are from corrected emission spectra (λ_{ex} = 335 nm), and maxima changed from 560 to ≤580 nm during the incubation.

Arg_{274}-Thr_{275} is also indicated, as when this bond is cleaved in Meizo thrombin to yield fragment 1·2 plus thrombin, sufficient alterations in the environment of bound DAPA occur to decrease the fluorescence yield by about one-third of the value observed prior to cleavage. The lack of DAPA binding when both bonds are intact (prothrombin or prethrombin 1), the appearance of binding following cleavage only at Arg_{274}-Thr_{275} (prethrombin 2), and the decrease in fluorescence intensity of bound DAPA observed when Arg_{274}-Thr_{275} is cleaved after cleavage at Arg_{323}-Ile_{324} suggest that cleavage at Arg_{274}-Thr_{275} measurably influences the structure of the domain that ultimately constitutes the active site of thrombin.

Interaction of DAPA–Thrombin and DAPA–Prethrombin 2 with Other Thrombin Inhibitors. Experiments were performed in order to determine whether reagents that interact covalently with active site residues in thrombin would displace DAPA from its complexes with thrombin or prethrombin 2. Figure 7 shows the time course of incubation of DAPA–thrombin at about 1 μM concentration with 4.7 mM DFP. Corrected emission spectra (λ_{ex} = 335 nm) were obtained at each time point indicated, and the intensity at the emission maximum is plotted in Figure 7 vs. time in minutes. In addition to the decrease in intensity, the wavelength of the emission maximum went from 560 nm for the DAPA–thrombin to 575 nm at 60 min of incubation. Thus, under the experimental conditions shown, DFP effectively diplaces the bound DAPA from the complex with thrombin. Displacement by DFP of DAPA from prethrombin 2 under similar conditions was not observed.

The effect of the histidine alkylating reagent D-phenylalanylprolylarginyl chloromethyl ketone (PPACK) on a mixture of DAPA, thrombin, and prethrombin 2 was also examined, and the results are shown in Figure 8. In this experiment, a solution of prethrombin 2 (4.3×10^{-6} M) containing about 8 mol % thrombin by clotting assays was made 1×10^{-6} M in DAPA, and a titration with PPACK (in a stock solution at 1.0×10^{-3} M) was performed with polarization measurements performed throughout the titration. A decrease in polarization was obtained during the titration up to a PPACK concentration equal to 0.08 of the prethrombin 2 concentration. At higher concentrations of PPACK, polarization values remained constant at about the value observed previously for a 1:1 complex of DAPA and prethrombin 2. Two conclusions are suggested by these data. The initial sharp decline in polarization over the range 0.0–0.08 ([PPACK]/[Pre-2]) most likely represents the displacement, by the chloromethyl ketone, of DAPA from the thrombin present in the prethrombin 2 preparation. In addition, since the polarization does not significantly decrease with further additions

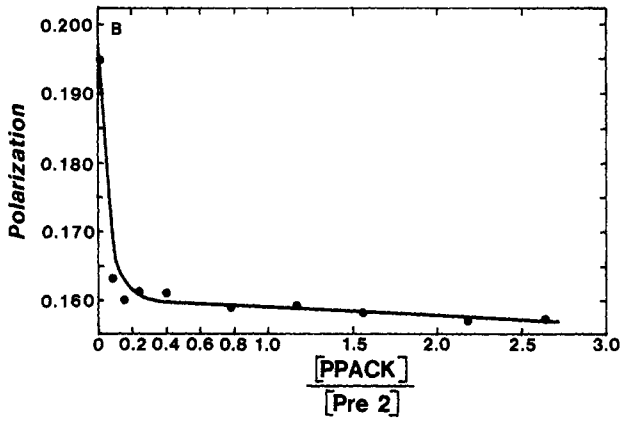


FIGURE 8: Polarization titrations of a mixture of DAPA (1×10^{-6} M) and prethrombin 2 (4.3×10^{-6} M) to which aliquots of PPACK were added.

of PPACK, the chloromethyl ketone does not displace DAPA from its binding site on prethrombin 2 under these experimental conditions.

Discussion

Conversion of the zymogen prothrombin to thrombin by the prothrombinase complex is accomplished by two cleavages that occur in sequence at Arg_{274}-Thr_{275} and Arg_{323}-Ile_{324}, respectively. These cleavages are designated "1st" and "2nd" cleavages in Figure 9. Their occurrence produces initially prothrombin fragment 1·2 plus prethrombin 2 and finally α-thrombin. Since prethrombin 2 is not active, cleavage at the second site to produce α-thrombin appears essential in the development of the active site. The data of this paper suggest that cleavage at the first site, although insufficient in itself to yield an active enzyme, also contributes to the development of the active site beyond the need for it to precede the second cleavage. This conclusion follows from the observation that when the bonds at Arg_{274}-Thr_{275} and Arg_{323}-Ile_{324} are both intact, as in prothrombin and prethrombin 1, no DAPA binding occurs while with cleavage at Arg_{274}-Thr_{275} to yield prethrombin 2, DAPA binding properties are evident. Even when cleavage at Arg_{324}-Ile_{325} precedes cleavage at Arg_{274}-Thr_{275} such that an active site is present (Meizo thrombin), alterations in this active site result upon further cleavage at the fragment 1·2–prethrombin 2 bond (Arg_{274}-Thr_{275}). Cleavage at this bond converts Meizo thrombin from an enzyme with activity only toward small substrates to α-thrombin with its capacity to catalyze proteolysis of its natural macromolecular substrate fibrinogen (Morita et al., 1976; Morita & Iwanaga, 1978). In addition, as indicated by the present work, cleavage of Meizo thrombin at Arg_{274}-Thr_{275} is marked by a decrease in the fluorescence intensity of bound DAPA, an effect that further suggests that cleavage of this bond influences the environment of the active site.

Inferences concerning local changes in the active site domain of thrombin and its precursor prethrombin 2 drawn from investigations of DAPA binding require two assumptions: (1) that the DAPA binding site on prethrombin 2 is analogous to the binding site on α-thrombin and (2) that the DAPA binding site on thrombin is identical with the substrate binding site. The first assumption is supported by the observation that both protein–DAPA complexes are composed of 1 mol of DAPA/mol of protein, and DAPA binds with similar (but not identical) avidity to both proteins. In addition, the changes observed in the spectral properties of DAPA upon complex formation are similar with each of the proteins and include increases in emission intensity (with excitation at either 335 or 280 nm), blue shifts in the emission maxima, and fluorescence polarization and lifetime of similar values at saturating protein concentrations. The second assumption is supported by the results presented above in which DAPA is displaced from thrombin by both DFP and PPACK, each of which produce covalent modifications of active site residues. In addition, the action of DAPA as a competitive inhibitor of human α-thrombin (Nesheim et al., 1979a; Prendergast et al., 1977) has been demonstrated. Thus the environment experienced by DAPA bound to thrombin appears representative of that available to substrate.

The differences in the fluorescence properties of α-thrombin–DAPA and prethrombin 2–DAPA undoubtedly reflect the differences between the configuration of the thrombin active site and its analogue in the zymogen prethrombin 2. Such structural differences are most likely of limited extent, however, as previous studies from this laboratory using circular dichroism spectroscopy (Bloom & Mann, 1979) indicate that no gross conformational changes occur upon cleavage of prothrombin to its activation peptides, intermediates, or

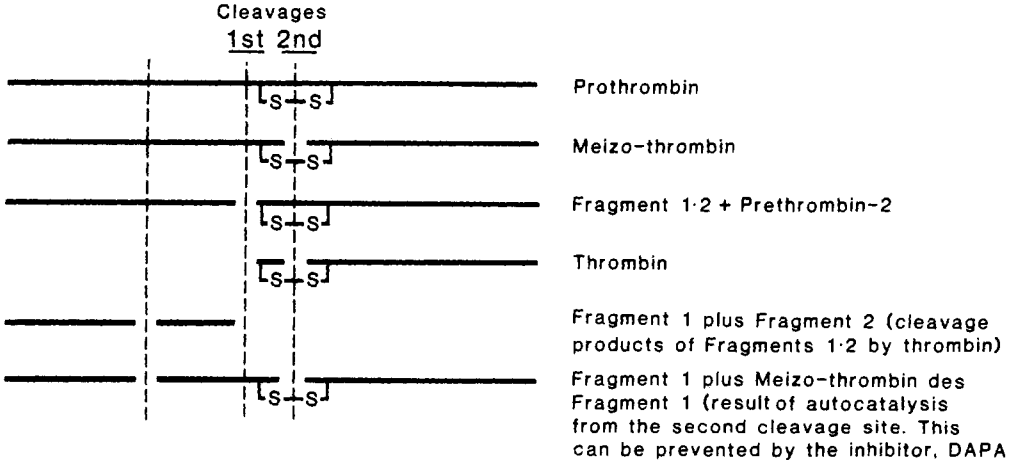


FIGURE 9: Prothrombin and its cleavage products. The first cleavage occurs at Arg_{274}-Thr_{275} and is typically catalyzed by factor Xa. The second cleavage is at Arg_{323}-Ile_{324} and is catalyzed by either factor Xa or ECV-P. The cleavage indicated to the left of these is at Arg_{156}-Ser_{157} and is typically catalyzed by α-thrombin, Meizo thrombin, or Meizo thrombin (des fragment 1). When both cleavage sites (Arg_{274}-Thr_{275} and Arg_{323}-Ile_{324}) are intact, no DAPA binding or enzymatic properties are evident. Cleavage at either yields DAPA binding properties. Enzymatic activity, however, appears only after cleavage at Arg_{323}-Ile_{324}. Further cleavage of either of these bonds in derivatives in which the other cleavage preexists (prethrombin 2 or Meizo thrombin) causes pronounced changes in DAPA binding and either appearance (prethrombin 2) or change (Meizo thrombin) in enzymatic properties.

thrombin.

All of the fluorescence properties of the prethrombin 2–DAPA complex examined are intermediate in value between those of thrombin–DAPA and DAPA alone. The binding parameters (Table II) and the value of the polarization in the limit of infinite viscosity, P_0 (Figure 2), indicate that DAPA is less tightly bound by prethrombin 2 than by α-thrombin and that the motion of DAPA bound to prethrombin 2 is somewhat less restricted than that bound to α-thrombin. The emission maximum of prethrombin 2–DAPA is at 565 nm, blue shifted by about 10 nm from the emission maximum of DAPA alone. For α-thrombin–DAPA a 15-nm blue shift is observed. Regardless of whether the blue shift is due to a less polar environment in the complex than in solution or to some other phenomenon, it occurs in both complexes and to a slightly greater extent in α-thrombin–DAPA than in prethrombin 2–DAPA. Finally, the data show clearly that energy transfer occurs from a group (or groups) on the protein to the DAPA dansyl moiety. The efficiency of transfer is less, however, for the prethrombin 2–DAPA complex than for the DAPA–thrombin complex (assuming that the same donor groups are responsible for transfer in both complexes). The efficiency of energy transfer depends on the distance separating the donor and acceptor groups and the relative orientations of the donor and acceptor transition dipoles (Fairclough & Cantor, 1978). A quantitative treatment of energy transfer in these two complexes was not attempted presently as the precise identity of the donor group(s) is not known. Nonetheless, with the assumption of identical donor(s) in both complexes, the difference in the efficiency of energy transfer for the two complexes suggests the probe associates more intimately with the binding site of thrombin than that of prethrombin 2.

The influence of the initial cleavage of prothrombin at Arg_{274}-Thr_{275} to yield prothrombin fragment 1·2 and prethrombin 2 with the attendant appearance of DAPA (and presumably substrate) binding properties may have relevance in rationalizing the sequence of bond cleavage involved in conversion of prothrombin to thrombin by physiologic activator prothrombinase. The observed sequence of cleavage is distinctly Arg_{274}-Thr_{275} followed by Arg_{323}-Ile_{324}; cleavage in the reverse order, although searched for, has not been observed. This preferred order could be the result of intrinsic differences in the properties of the two cleavage sites as factor Xa substrates, such that the kinetics of cleavage of the second bond are distinctly rate limiting. Alternatively, the alterations (suggested by changes in DAPA fluorescence) of the environment of the developing active site affected by the first cleavage may imply that the second cleavage site does not exist until structural changes occur subsequent to the first cleavage. In this latter case the sequential order of bond cleavage would not be the result in intrinsic differences in the kinetics of cleavage at the individual sites but rather the consequence of progressive structural alterations in the substrate that occur during its conversion to product.

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