Collman, J. P., Brauman, J. I., Halbert, T. R., & Suslick, K. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3333–3337.
Collman, J. P., Brauman, J. I., & Doxsee, K. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6035–6039.
Felton, R. H., & Yu, N.-T. (1978) in *The Porphyrins* (Dolphin, D., Ed.) Vol. 3, pp 347–393, Academic Press, New York.
Heidner, E. J., Ladner, R. C., & Perutz, M. F. (1976) *J. Mol. Biol. 104*, 707–722.
Hoard, J. L. (1975) in *Porphyrins and Metalloporphyrins* (Smith, K. M., Ed.) p 351, Elsevier, New York.
Huber, R., Epp, O., & Formanek, H. (1970) *J. Mol. Biol. 52*, 349–354.
Kerr, E. A., Mackin, H. C., & Yu, N.-T. (1982) *Biophys. J. 37*, 371a.
Kerr, E. A., Mackin, H. C., & Yu, N.-T. (1983) *Biochemistry* (in press).
Kim, J. B., Adler, A. D., & Longo, F. R. (1978) in *The Porphyrins* (Dolphin, D., Ed.) Vol. 1, Chapter III, Academic Press, New York.
Kroeker, R. M., Hansma, P. K., & Kaska, W. C. (1980) *J. Chem. Phys. 72*, 4845–4852.
Mackin, H. C., Tsubaki, M., & Yu, N.-T. (1983) *Biophys. J. 41*, 349–357.
Makinen, M. W., Houtchens, R. A., & Caughey, W. S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6042–6046.
Moffat, K., Deatherage, J. F., & Seybert, D. W. (1970) *Science (Washington, D.C.) 206*, 1035–1042.
Norvell, J. C., Nunes, A. C., & Schoenborn, B. P. (1975) *Science (Washington, D.C.) 190*, 568–570.
Padlan, E. A., & Love, W. E. (1975) *J. Biol. Chem. 249*, 4067–4078.
Peng, S. M., & Ibers, J. A. (1976) *J. Am. Chem. Soc. 98*, 8032–8036.
Perutz, M. F. (1976) *Br. Med. Bull. 32*, 195–208.
Phillips, S. E. V. (1978) *Nature (London) 273*, 247–248.
Phillips, S. E. V. (1980) *J. Mol. Biol. 142*, 531–554.
Rousseau, D. L., & Ondrias, M. R. (1983) *Annu. Rev. Biophys. Bioeng.* (in press).
Rousseau, D. L., Ondrias, M. R., LaMar, G. N., Kong, S. B., & Smith, K. M. (1983) *J. Biol. Chem. 258*, 1740–1746.
Shaanan, B. (1982) *Nature (London) 296*, 683–684.
Spaulding, L. D., Chang, C. C., Yu, N.-T., & Felton, R. H. (1975) *J. Am. Chem. Soc. 97*, 2517–2525.
Spiro, T. G., Stong, J. D., & Stein, P. (1979) *J. Am. Chem. Soc. 101*, 2648–2655.
Steigemann, W., & Weber, E. (1979) *J. Mol. Biol. 127*, 309–338.
Szabo, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2108–2111.
Traylor, T. G. (1981) *Acc. Chem. Res. 14*, 102–109.
Tsubaki, M., Srivastava, R. B., & Yu, N.-T. (1982) *Biochemistry 21*, 1132–1140.
Ward, B., Wang, C.-B., & Chang, C. K. (1981) *J. Am. Chem. Soc. 103*, 5236–5238.
Wilson, E. (1939) *J. Chem. Phys. 7*, 1047–1052.
Yu, N.-T., & Srivastava, R. B. (1981) *J. Raman Spectrosc. 9*, 166–171.

Differentiation of Enzyme and Substrate Binding in the Prothrombinase Complex†

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ABSTRACT: The Ca^{2+} dependence of factor Xa binding to phospholipid vesicles was measured in the presence and absence of factor Va. The increase in polarization of a fluorescently labeled derivative of factor Xa, [5-(dimethylamino)-1-naphthalenesulfonyl]glutamylglycylarginyl factor Xa (Dns-EGR-Xa), was used as a probe to measure the interaction of factor Xa with phospholipid. The Ca^{2+} concentration required for half-maximal binding of Dns-EGR-Xa to phospholipid vesicles was 3.5×10^{-4} M in the presence of factor Va and 9.5×10^{-4} M in the absence of factor Va. At a Ca^{2+} concentration of 5×10^{-4} M, the binding of Dns-EGR-Xa to phospholipid-bound factor Va was near maximal, whereas there was no detectable interaction of Dns-EGR-Xa with phospholipid alone at this Ca^{2+} concentration as detected by fluorescence polarization. These results were qualitatively confirmed by high-performance liquid chromatography. The rate of hydrolysis of the factor Xa synthetic substrate, benzoylisoleucylglutamylglycylarginine *p*-nitroanilide, by factor Xa in the presence of factor Va and phospholipid decreased in a Ca^{2+}-dependent manner. These data were analyzed as fraction of factor Xa bound to the phospholipid. A Ca^{2+} concentration of 2.7×10^{-4} M resulted in half-maximal binding by this technique. The relationship observed between rates of prothrombin activation and Ca^{2+} concentration could be predicted quantitatively from calculations of local enzyme and substrate concentrations.

The maximum rate of conversion of prothrombin to thrombin by factor Xa requires factor Va, phospholipid, and Ca^{2+} ions. The activity is dependent on the condensation of substrate (prothrombin), enzyme (factor Xa), and cofactor (factor Va) on the surface of the phospholipid. The integrity of this prothrombinase complex is dependent on Ca^{2+} ions, as was initially shown by Papahadjopoulos & Hanahan (1964). The binding of prothrombin to phospholipid is also dependent on Ca^{2+} ions (Nelsestuen et al., 1976; Nelsestuen & Lim, 1977; Prendergast & Mann, 1977; Nelsestuen & Broderius, 1977; Lim et al., 1977; Dombrose et al., 1979). The binding of the vitamin K dependent proteins to phospholipid is thought to

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0006-2960/83/0422-4540$01.50/0

occur through Ca^{2+} bridges between γ-carboxyglutamic acid residues of the proteins and acidic groups on the phospholipid surface (Papahadjopoulos & Hanahan, 1964; Bull et al., 1972; Stenflo & Suttie, 1977; Nelsestuen et al., 1974; Howard & Nelsestuen, 1975). Both the γ-carboxyglutamic acid residue containing region of thrombin fragment 1 and factor X bind Ca^{2+} ions with dissociation constants of 6.3×10^{-4} M (Bajaj et al., 1975; Henriksen & Jackson, 1975).

Although the binding of factor Va to phospholipid is not Ca^{2+} dependent (Bloom et al., 1979; Papahadjopoulos & Hanahan, 1964), the association of factor Va peptide chains is dependent upon Ca^{2+} (Esmon, 1979; Hibbard & Mann, 1980). Factor V has two classes of binding sites, one site containing a single Ca^{2+} with a dissociation constant of less than 10^{-8} M and a second site that binds 2 mol of Ca^{2+}/mol of factor V with a dissociation constant of 5.9×10^{-5} M (Hibbard & Mann, 1980). Evidence has been presented indicating that factor Va binds a single Ca^{2+} ion with a dissociation constant of 2.4×10^{-5} M (Guinto & Esmon, 1982).

Factor Va serves as a factor Xa receptor on both phospholipid vesicles (Nesheim et al., 1979a, 1981a,b) and platelets (Tracy et al., 1979, 1981; Miletich et al., 1978; Kane et al., 1980; Dählback & Stenflo, 1978). In the present study, we examined the Ca^{2+} dependence of factor Xa binding to phospholipid vesicles both in the presence and absence of factor Va. Experiments of Nesheim et al. (1981a) suggested that the Ca^{2+} dependence of these two processes is different. Thus, we anticipated that by selecting an appropriate Ca^{2+} concentration, we could distinguish between the binding of factor Xa to phospholipid and the binding of factor Xa to phospholipid–factor Va and relate this to the Ca^{2+} dependence of prothrombin conversion to thrombin. The Ca^{2+} dependence of prothrombinase activity was rationalized from knowledge of the Ca^{2+} dependence of the condensation of enzyme, factor Xa, and substrate, prothrombin, onto the phospholipid surface.

Materials and Methods

Materials. The factor Xa chromogenic substrate, S2222,[1] was obtained from Ortho Pharmaceutical. The Dns-glutamylglycylarginine chloromethyl ketone (Dns-EGR-CK) was the generous gift of Drs. Charles Kettner and Elliott Shaw. The phosphatidylcholine–phosphatidylserine (PCPS) vesicles (25% phosphatidylserine by weight) were prepared by a modification of the method of Barenholz et al. (1977) as previously described (Bloom et al., 1979). Egg L-α-phosphatidylcholine and bovine brain L-α-phosphatidyl-L-serine were purchased from Sigma.

Proteins. Bovine prothrombin and factor X were prepared as described by Bajaj & Mann (1973). Factor X was activated to factor Xa by a modification of the procedure of Downing et al. (1975), with factor X activator from Russell's viper venom immobilized on agarose. The electrophoretically homogeneous factor X activator was the generous gift of Dr. Walter Kisiel. The Dns-glutamylglycylarginyl factor Xa (Dns-EGR-Xa) was prepared as described by Nesheim et al. (1981a). Bovine factor V was isolated and assayed as described by Nesheim et al. (1982). Factor Va was prepared by incubating factor V (approximately 0.6 mg/mL in 0.02 M Tris-HCl, pH 7.4, containing 0.15 M NaCl) with 2 NIH units/mL of thrombin at 37 °C for 4 min. The molecular weights and extinction coefficients ($\epsilon^{1\%}_{1cm,280nm}$) of the proteins were taken to be as follows: prothrombin, 72000, 14.4 (Heldebrant et al., 1973; Owen et al., 1974); factor Xa, 55100, 12.4 (Jackson et al., 1968; Fujikawa et al., 1972); factor V, 330000, 9.6 (Nesheim et al., 1979b).

Polarization Measurements. Fluorescence polarization measurements were made in a Perkin-Elmer Model MPF-44A fluorescence spectrophotometer equipped with a DSU-2 unit and a Wood automated polarizer. An excitation wavelength of 335 nm and an emission wavelength of 545 nm were used with band-passes of 10 and 15 nm, respectively. A wavelength cutoff of 430 nm was used. Measurements were made at 22 °C in a 1-cm cuvette. The initial volume for the titrations was 1.5 mL of 0.02 M Tris-HCl, pH 7.4, containing 0.15 M NaCl, 30 μM PCPS vesicles, 0.3 μM Dns-EGR-Xa, and 1% poly(ethylene glycol) 6000. Where stated in the text, solutions contained 0.4 μM factor Va. The Ca^{2+} concentrations were varied as described in the text. All proteins used in the polarization experiments were dialyzed for 2 h vs. 0.02 M Tris-HCl, pH 7.4, containing 0.15 M NaCl, prior to use.

Kinetic Data of S2222 Hydrolysis. The spectrophotometric measurements were made on a Beckman Acta III recording spectrophotometer. Measurements were made at 22 °C in a 1-cm cuvette. The initial volume for the measurements was 0.6 mL of 0.02 M Tris-HCl, pH 7.4, containing 0.15 M NaCl and 25 μM S2222. The Ca^{2+}, PCPS, and factor Va concentrations were varied as described in the text. The reaction was initiated by the addition of microliter amounts of factor Xa, and the reaction was monitored continuously at 405 nm. The reactions performed with 2.9 nM factor Xa also contained 1% poly(ethylene glycol) 6000. Reaction velocities were determined by measuring the slopes of the recorded data. An extinction coefficient ($\epsilon^{1mM}_{1cm,405nm}$) of 14.6 was used for the release of PNA from S2222.

The decrease in rate of hydrolysis observed at increasing Ca^{2+} concentrations was converted to the fraction of factor Xa bound to PCPS vesicles by using the following equation described by Nesheim et al. (1981b), where b is the fraction of factor Xa bound, v_0 and v_∞ are the limiting velocities when no factor Xa is bound and all the factor Xa is bound, respectively, and v is the velocity at any point between these extremes:

$$b = \frac{1}{2}\left(1 - \frac{v}{v_0}\right)\left(1 + \left[\frac{4v_0v_\infty}{(v_0 - v_\infty)(v_0 - v)} + 1\right]^{1/2}\right) \quad (1)$$

High-Performance Liquid Chromatography. A high-performance liquid chromatograph equipped with an Altex TSK 3000 SW 0.75 × 30 cm column was used in the gel-filtration experiments. The column was equilibrated with 0.02 M Tris-HCl, pH 7.4, containing 0.15 M NaCl and the Ca^{2+} concentrations stated in the text. All experiments were performed at 22 °C with a flow rate of 1.2 mL/min. The column was monitored with a Varian fluorescence detector with the excitation wavelength centered at 360 nm and the emission wavelength cutoff at 390 nm. The fluorescence data were collected on a Bascom-Turner Series 8000 recorder and corrected for lipid autofluorescence.

Prothrombinase Activity. The rate of formation of thrombin from prothrombin was measured by following the increase in fluorescence intensity of DAPA (DAPA–thrombin) with time as described by Nesheim et al. (1982). Fluorescence intensity was measured on a Perkin-Elmer MPF-44A fluorescence spectrophotometer. Measurements were made at 22 °C in 0.02 M Tris-HCl, pH 7.4, containing 0.15 M NaCl, 20 μM PCPS,

[1] Abbreviations: S2222, benzoylisoleucylglutamylglycylarginine *p*-nitroanilide; PNA, *p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane; Dns, 5-(dimethylamino)-1-naphthalenesulfonyl; Dns-EGR-Xa, Dns-glutamylglycylarginyl factor Xa; Dns-EGR-CK, Dns-glutamylglycylarginine chloromethyl ketone; PCPS, phosphatidylcholine–phosphatidylserine; DAPA, Dns-arginine *N*-(3-ethyl-1,5-pentanediyl)amide.

1.6 μM prothrombin, and 5.5 nM factor Va. The Ca^{2+} concentrations were varied as described in the text. The reaction was started by the addition of microliter quantities of factor Xa, resulting in a final concentration of 6.0 nM. The prothrombin used in these assays was dialyzed for 2 h vs. 0.02 M Tris-HCl, pH 7.4, containing 0.15 M NaCl prior to use. The prothrombin was allowed to incubate at the appropriate Ca^{2+} concentration for at least 20 min prior to the start of the assay.

Light Scattering Measurements. Light scattering measurements were made at 90° with a Perkin-Elmer Model MPF-44A. The excitation and emission wavelengths used were 320 nm with band-passes of 5 nm. Measurements were performed at 22 °C in 1-cm cuvettes. The initial volume for titrations was 1.5 mL of 0.02 M Tris-HCl, pH 7.4, containing 0.15 M NaCl, 30 μM PCPS, and 1.6 μM prothrombin. Additions of $CaCl_2$ were made with a microsyringe. The increase in light scattering intensity was converted to increase in relative molecular weight by a method developed by Nelsestuen & Lim (1977). At the highest Ca^{2+} concentration used, 3.7 mM, the relative molecular weight of the vesicle had increased 1.45-fold.

Calculated Rate of Prothrombinase Activity. When factor Xa and prothrombin bind to PCPS in the presence of Ca^{2+} and factor Va, the enzyme and substrate presumably coconcentrate in the vicinity of the PCPS vesicles. The binding of these proteins to the phospholipid surface, as measured by light scattering, results in an increase in the hydrodynamic radius of the scattering particle (Lim et al., 1977), which can be defined as a "shell" around the phospholipid vesicle (Nesheim et al., 1981b). When factor Va, the largest of the proteins in the prothrombinase complex, binds to the vesicle, the hydrodynamic radius of scatter increases from 163 to 283 Å (Higgins & Mann, 1982). This increase in radius describes a shell with a volume of 7.68×10^{-20} L/vesicle. A PCPS concentration of 20 μM was used in these experiments, and this corresponds to a vesicle concentration of 1.54 nM (vesicle $M_r = 7.0 \times 10^6$; Higgins & Mann, 1982). At this concentration, the volume in which bound protein is found (the shells) comprises approximately 0.01% of the total volume of the reaction mixture. The amounts of prothrombin and factor Xa bound to the PCPS at varying Ca^{2+} concentrations were obtained from measurements described earlier in this paper, allowing for the calculation of the protein concentration in this 120-Å shell. Knowing the concentration of enzyme, factor Xa, and substrate, prothrombin, in this region around the phospholipid vesicle allows the calculation of the velocity of prothrombin conversion to thrombin according to the Michaelis–Menten rate equation

$$v = \frac{k[E][S]}{K_m + [S]} \quad (2)$$

where v is the velocity of prothrombin conversion, k is the turnover number for the reaction, [E] is the concentration of factor Xa on the vesicles, [S] is the concentration of prothrombin on the vesicles, and K_m is the Michaelis constant. A K_m value of 131 μM was used in the calculations (Rosing et al., 1980).

Results

Ca^{2+}-Dependent Factor Xa Interactions with PCPS and Factor Va, Studied by Fluorescence Polarization. Factor Xa binds to phospholipid surfaces alone and to PCPS–factor Va. The dissociation constant for the latter interaction is 3 orders of magnitude lower than the former (Nesheim et al., 1979a, 1981b). Both of these interactions are dependent on Ca^{2+} ions. Initial studies by Nesheim et al. (1981a) suggested that the

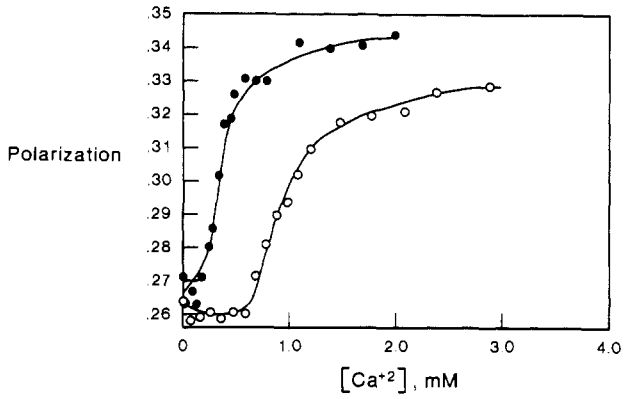

FIGURE 1: Ca^{2+} dependence of interaction of Dns-EGR-Xa with PCPS and PCPS–factor Va. The fluorescence polarization of Dns-EGR-Xa in the presence of PCPS vesicles and factor Va (●) or PCPS vesicles (○) is shown at increasing Ca^{2+} concentrations.

binding of factor Xa to PCPS–factor Va occurs at lower Ca^{2+} concentrations than those required for the binding of the vitamin K dependent proteins to phospholipid (Dombrose et al., 1979). Thus, the Ca^{2+} dependence of the binding of factor Xa to phospholipid alone and to PCPS–factor Va was studied in further detail in efforts to distinguish these interactions.

The interaction of factor Xa with phospholipid plus factor Va was studied by using a fluorescently labeled derivative of factor Xa, Dns-EGR-Xa. Dns-EGR-Xa is a factor Xa molecule that has been modified at its active site with Dns-glutamylglycylarginine chloromethyl ketone (Nesheim et al., 1981a). As illustrated in Figure 1, increasing Ca^{2+} concentrations result in an increase in the fluorescence polarization of Dns-EGR-Xa in the presence of phospholipid alone or in the presence of phospholipid plus factor Va. This increase in polarization has been interpreted as resulting from the immobilization of the fluorescent probe on the phospholipid surface (Nesheim et al., 1981a). The transition midpoint obtained for the phospholipid–factor Xa interaction, 0.95 mM Ca^{2+}, is similar to the calcium concentration required for half-maximal binding of other vitamin K dependent proteins to phospholipid vesicles (Dombrose et al., 1979). The Ca^{2+} dependence of this curve was shifted in the presence of factor Va with the transition midpoint occurring at 0.35 mM Ca^{2+}. At concentrations of Ca^{2+} of between 0.5 and 0.6 mM, nearly maximal binding of factor Xa to the phospholipid surface occurred in the presence of factor Va; but in the absence of factor Va (at these Ca^{2+} concentrations), no detectable interaction of factor Xa with phospholipid was observed. Thus, if one chooses the appropriate Ca^{2+} concentration, the binding of factor Xa to PCPS–factor Va and the binding of factor Xa to phospholipid itself can be distinguished.

Ca^{2+}-Dependent Factor Xa, PCPS, and Factor Va Interactions Studied by High-Performance Liquid Chromatography. The results obtained from the fluorescence polarization measurements were verified by high-performance liquid chromatography. Figure 2 shows the elution position of Dns-EGR-Xa in the presence of PCPS at increasing concentrations of Ca^{2+}. The elution position of Dns-EGR-Xa alone is shown in panel A. In the presence of PCPS vesicles and 0.5 mM Ca^{2+}, panel B, the majority of the fluorescent material eluted in the position of free Dns-EGR-Xa. The material eluting at approximately 300 s elutes at the position of the PCPS vesicles. Only a small amount of Dns-EGR-Xa was found eluting with the PCPS vesicles at this Ca^{2+} concentration. The elution position of Dns-EGR-Xa in the presence of PCPS at 2 mM Ca^{2+} is shown in panel C. The peak of fluorescence eluting at the position of free Dns-EGR-Xa was

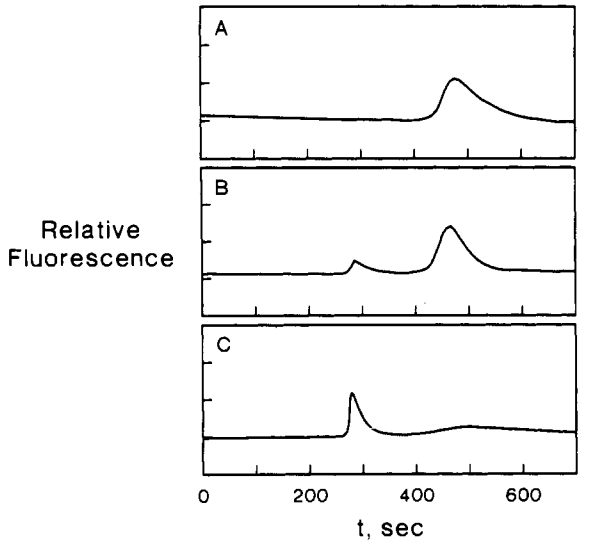

FIGURE 2: High-performance liquid chromatography of Dns-EGR-Xa and PCPS vesicles at increasing Ca^{2+} concentrations. The panels correspond to (panel A) Dns-EGR-Xa, (panel B) Dns-EGR-Xa and PCPS at 0.5 mM Ca^{2+}, and (panel C) Dns-EGR-Xa and PCPS at 2.0 mM Ca^{2+}. The amounts of Dns-EGR-Xa and PCPS applied were 7.3×10^{-11} and 3.8×10^{-8} mol, respectively. All samples were injected in volumes of 0.042 mL or less at zero time. The elution position of Dns-EGR-Xa was followed by measuring relative fluorescence.

markedly diminished at this Ca^{2+} concentration, and a significant increase in the fluorescent material eluting with the PCPS vesicles was observed. Since these are not equilibrium measurements, some broadening of the peaks occurred as the phospholipid-bound Dns-EGR-Xa separated from the free Dns-EGR-Xa. These results are in qualitative agreement with the results obtained with fluorescence polarization.

The elution position of Dns-EGR-Xa in the presence of PCPS and factor Va at increasing concentrations of Ca^{2+} is shown in Figure 3. The elution position of Dns-EGR-Xa is again shown in panel A. The inclusion of factor Va and PCPS had no influence on the elution of Dns-EGR-Xa when no Ca^{2+} was added (panel B). At 0.5 mM Ca^{2+}, the peak of fluorescence eluting at the position of free Dns-EGR-Xa had markedly diminished, and a significant increase occurred in the fluorescent material eluting with the PCPS vesicles (panel C). This is in contrast to the results obtained in the absence of factor Va (Figure 2, panel B) where the majority of the fluorescent material eluted as free Dns-EGR-Xa at this Ca^{2+} concentration. The elution position of Dns-EGR-Xa in the presence of 2 mM Ca^{2+}, PCPS, and factor Va is shown in Figure 3, panel D. At this Ca^{2+} concentration, no fluorescent material eluted at the position of free Dns-EGR-Xa. It was all found migrating with the PCPS vesicles. These results again illustrate that the binding of factor Xa to PCPS vesicles alone and to PCPS–factor Va can be differentiated by selecting an appropriate Ca^{2+} concentration. These data, although qualitative, are in excellent agreement with the data obtained from fluorescence polarization.

Kinetics of S2222 Hydrolysis. Having determined that the binding of factor Xa to phospholipid alone or to PCPS–factor Va can be differentiated by varying the Ca^{2+} concentration, we then ascertained whether the enzymatic activity of factor Xa would show Ca^{2+}-dependent changes that reflected its binding to phospholipid or its binding to PCPS–factor Va. Nesheim et al. (1981b) have shown that the rate of hydrolysis of the factor Xa synthetic substrate, S2222, decreases when factor Xa forms a complex with factor Va and phospholipid when the substrate is at less than saturating concentrations.

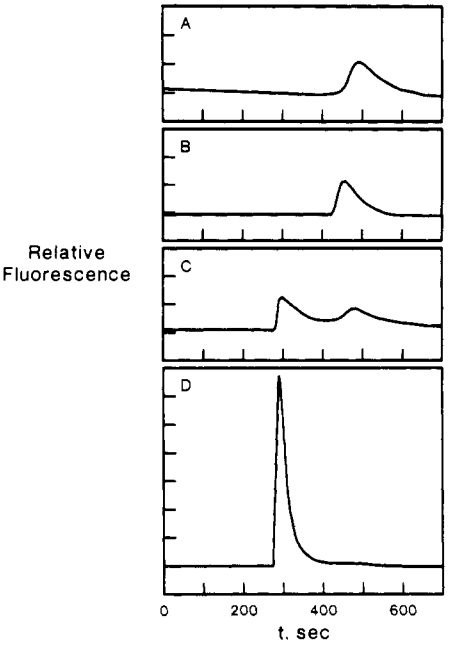

FIGURE 3: High-performance liquid chromatography of Dns-EGR-Xa, PCPS vesicles, and factor Va at increasing Ca^{2+} concentrations. The panels correspond to (panel A) Dns-EGR-Xa, (panel B) Dns-EGR-Xa, factor Va, and PCPS with no added Ca^{2+}, (panel C) Dns-EGR-Xa, factor Va, and PCPS at 0.5 mM Ca^{2+}, and (panel D) Dns-EGR-Xa, factor Va, and PCPS at 2 mM Ca^{2+}. The amounts of Dns-EGR-Xa, factor Va, and PCPS applied were 7.3×10^{-11}, 2.18×10^{-10}, and 3.8×10^{-8} mol, respectively. Samples were injected in volumes of 0.16 mL or less at zero time. The elution position of Dns-EGR-Xa was followed by measuring relative fluorescence.

The Ca^{2+} dependence of this decrease in rate was examined. The data are shown in Figure 4A. Maximal inhibition was obtained at concentrations of Ca^{2+} exceeding 1.0 mM. This decrease in hydrolysis rate most likely reflects the immobilization of factor Xa on the phospholipid surface (Nesheim et al., 1981b). The fraction of bound factor Xa was calculated from these measurements, as described under Materials and Methods, and is shown in the insert in Figure 4A. The concentration of Ca^{2+} required for half-maximal binding was 0.28 mM. This value is comparable to that of 0.35 mM Ca^{2+} required for half-maximal binding of Dns-EGR-Xa to PCPS–factor Va obtained from the fluorescence polarization studies (Figure 1). These observations suggest that the Ca^{2+}-dependent profile obtained by measuring an enzymatic property of factor Xa reflects the binding of factor Xa to factor Va on the phospholipid surface and not the binding of factor Xa to the phospholipid surface alone.

The Ca^{2+} dependence of the factor Xa–PCPS–factor Va interaction was determined at two factor Va concentrations, as shown in Figure 4B. An identical Ca^{2+} dependence was obtained with either 6 or 132 nM factor Va. Although a systematic study of the protein concentration dependence of the reaction has not been undertaken, a number of experiments have been performed with different preparations of factor Va and factor Xa at different, randomly selected concentrations, and these results are summarized in Table I. These data illustrate that the Ca^{2+} dependence of the factor Xa–PCPS–factor Va interaction is independent of protein concentration as factor Xa is varied from 1.36 to 300 nM and as factor Va is varied from 6 to 400 nM.

Ca^{2+} Dependence of both Prothrombinase Activity and Binding of Prothrombin to PCPS. The conversion of prothrombin to thrombin by factor Xa in the presence of phospholipid and factor Va is dependent on Ca^{2+}, and the maximal

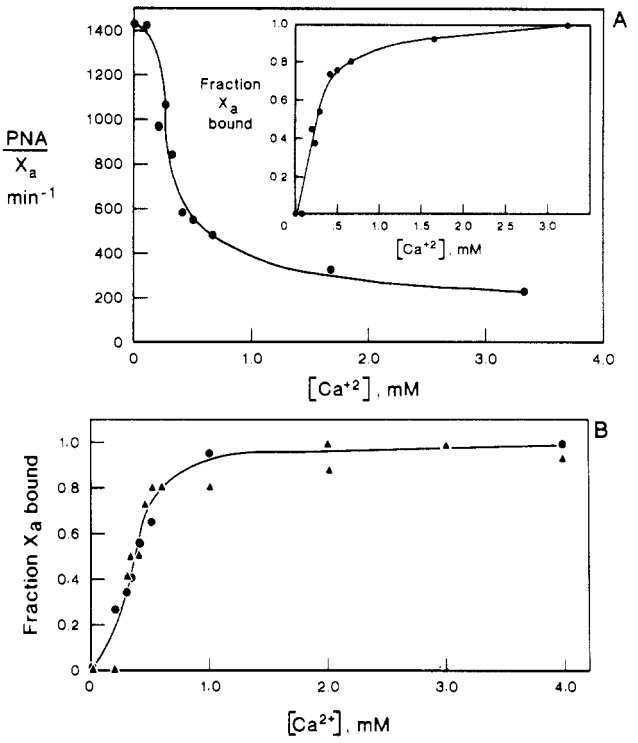

FIGURE 4: Ca^{2+} dependence of hydrolysis of S2222. The rate of hydrolysis of S2222 by factor Xa was measured at increasing concentrations of Ca^{2+} in the presence of PCPS and factor Va. (A) The rate of hydrolysis is expressed as moles of PNA released per mole of factor Xa per minute. The insert shows a replot of these data expressed as fraction of factor Xa bound to the PCPS vesicles. The reaction mixture contained factor Xa (2.9 nM), factor Va (400 nM), and PCPS (30 μM). (B) The fraction of factor Xa bound to the PCPS vesicle was determined at 6 (▲) or 132 nM factor Va (●). Factor Xa was 6 nM, and PCPS was 20 μM for both titration curves.

Table I: Ca^{2+} Dependence of the Factor Xa–PCPS–Factor Va Interaction

method[a]	[PCPS] (μM)	[Va] (nM)	[Xa] (nM)	[Ca^{2+}] at transition midpoint (mM)
kinetic	30	10.6	1.36	0.36
kinetic	26	7.3	1.65	0.27
kinetic	30	400	3.0	0.27
kinetic	20	6.0	6.0	0.35
kinetic	20	132	6.0	0.35
Dns-EGR-Xa	16	100	70.0	0.25[b]
Dns-EGR-Xa	30	400	300	0.35

[a] The kinetic measurements were made by measuring the decrease in the rate of S2222 hydrolysis as described under Materials and Methods. The increase in polarization of Dns-EGR-Xa was measured at increasing concentrations of Ca^{2+} as described under Materials and Methods. [b] Nesheim et al., 1981a.

rate of the reaction occurs at approximately 2 mM Ca^{2+} (Nesheim et al., 1979a). We wished to study the Ca^{2+} dependence of prothrombinase activity in more detail, especially at the lower Ca^{2+} concentrations, to determine whether the expression of prothrombinase activity reflects (1) the formation of the enzymatic complex (the condensation of factor Xa on PCPS–factor Va), (2) the binding of factor Xa to phospholipid, (3) the binding of the substrate, prothrombin, to phospholipid, or (4) a combination of these phenomena.

The rate of prothrombin activation as a function of concentration of Ca^{2+} is shown in Figure 5. In agreement with others, maximal activity was obtained at Ca^{2+} concentrations of 2.0 mM. The activity profile is complex and is reminiscent

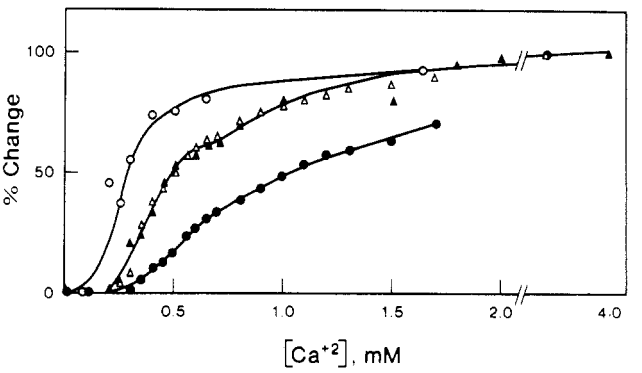

FIGURE 5: Ca^{2+} dependence of prothrombinase activity. Prothrombin conversion by factor Xa was measured at increasing concentrations of Ca^{2+} in the presence of PCPS and factor Va. The observed prothrombinase activity (▲) and the calculated prothrombinase activity (△) are expressed as percent of maximal activity. The binding of factor Xa to factor Va–PCPS (○) is expressed as percent of maximal binding as determined in Figure 4A. The binding of prothrombin to PCPS (●) is expressed as percent of maximal binding.

of the data obtained by Prendergast & Mann (1977) from studies in which prothrombin activation was studied at increasing Ca^{2+} concentrations in the presence of 1 mM Mg^{2+} ion. For comparison, the Ca^{2+} dependence of binding of prothrombin to PCPS and the Ca^{2+} dependence of binding of factor Xa to PCPS–factor Va are also shown in Figure 5. Detectable prothrombinase activity was observed at Ca^{2+} concentrations less than those required for maximum binding of prothrombin to the PCPS vesicles. Half-maximal prothrombinase activity occurred at approximately 0.5 mM Ca^{2+}, while half-maximal prothrombin binding did not occur until approximately 1 mM Ca^{2+}. Thus, the initial rise in prothrombinase activity seems to reflect, primarily, the formation of the enzyme complex on the surface of the phospholipid. As Ca^{2+} concentrations were increased beyond those required for maximal binding of factor Xa to PCPS–factor Va, a further increase in prothrombinase activity was observed. This further increase in activity appears to reflect the increase in prothrombin binding to the phospholipid vesicles. From knowledge of the Ca^{2+} dependence of the discrete events of factor Xa–factor Va–PCPS binding and prothrombin–PCPS binding, a value for prothrombinase activity at any given Ca^{2+} concentration was calculated. The calculated values assume that the rate of prothrombin conversion is predictable from the local concentrations of factor Xa and prothrombin bound at the phospholipid vesicle surface. These calculated values are also shown in Figure 5 and accurately predict the observed increase in prothrombinase activity. These results support the idea that the Ca^{2+} dependence of prothrombinase activity is determined by the resulting effect on local concentrations of prothrombin and factor Xa on the surface of the phospholipid vesicle.

Discussion

The interaction of the vitamin K dependent proteins with phospholipid is Ca^{2+} dependent (Papahadjopoulos & Hanahan, 1964; Bull et al., 1972; Stenflo & Suttie, 1977; Nelsestuen et al., 1974; Howard & Nelsestuen, 1975). The interaction of factor Xa with phospholipid-bound factor Va also is Ca^{2+} dependent (Nesheim et al., 1981b). In this study we examined the Ca^{2+} dependence of these interactions and have found that the two processes can be distinguished by selecting an appropriate Ca^{2+} concentration. At a Ca^{2+} concentration of 0.5 mM, the binding of factor Xa to phospholipid-bound factor Va is nearly complete, whereas the interaction of factor Xa with phospholipid per se could not be detected. This infor-

mation permits differentiation of the direct binding of factor Xa to phospholipid and the factor Xa–factor Va–PCPS interaction.

One can imagine a variety of Ca^{2+}-dependent interactions that could be important in the assembly and function of the prothrombinase complex. A few of the processes that we know require Ca^{2+} are outlined below:

$$\text{II} + \text{PCPS} \overset{Ca^{2+}}{\rightleftharpoons} \text{II–PCPS} \quad (3)$$

$$\text{Xa} + \text{PCPS} \overset{Ca^{2+}}{\rightleftharpoons} \text{Xa–PCPS} \quad (4)$$

$$\text{Xa} + \text{Va} + \text{PCPS} \overset{Ca^{2+}}{\rightleftharpoons} \text{Xa–Va–PCPS} \quad (5)$$

$$\text{II} + \text{Xa–Va–PCPS} \overset{Ca^{2+}}{\rightleftharpoons} \text{IIa} + \text{F1·2} + \text{Xa–Va–PCPS} \quad (6)$$

The Ca^{2+} concentration at which half-maximal saturation of each of these processes occurs is approximately 1, 1, 0.3, and 0.5 mM for processes 3–6, respectively. The nature of the factor Xa–factor Va–PCPS interaction is unknown. Factor Xa may bind directly to factor Va on the PCPS surface or it might interact in a bidentate manner with both factor Va and PCPS. Either or both of these interactions may require Ca^{2+}.

Although factor Xa binds to factor Va–phospholipid at Ca^{2+} concentrations lower than those required for binding to phospholipid alone, higher concentrations of Ca^{2+} conceivably might further influence the catalytic properties of factor Xa. Studies with the factor Xa synthetic substrate (S2222), however, suggest that the enzymatic activity of factor Xa shows a similar Ca^{2+} dependence to the binding of factor Xa to phospholipid-bound factor Va. This result would be expected if the binding of factor Xa to phospholipid-bound factor Va is the primary interaction essential for the function of factor Xa in the prothrombinase complex.

In spite of the obvious complexity of the Ca^{2+} dependence of prothrombinase assembly and function, the Ca^{2+} dependence of prothrombinase activity can be rationalized quantitatively on the basis of the coconcentration of enzyme (factor Xa) and substrate (prothrombin) on the surface of the phospholipid (Nesheim et al., 1981b). The increase in prothrombinase activity observed is predicted from calculations of local enzyme and substrate concentrations obtained from direct binding measurements. Since the reaction rate is shown to be dependent on surface-concentrated enzyme and substrate, it may be concluded that it is the PCPS-bound or concentrated prothrombin that is the substrate for the prothrombinase complex. The volume occupied by bound substrate and the number of molecules bound provide a formal concentration of bound prothrombin approaching 178 μM. In contrast, the bulk prothrombin concentration is 1.6 μM. Clearly, a concentration gradient must exist between bound and free substrate, and the consequences of the lipid binding of prothrombin would alter both the surface concentration and the gradient. Since the gradient is a direct consequence of binding, our treatment of the data does not distinguish between lipid-bound substrate and substrate in the immediate vicinity of the lipid.

The activity profile of prothrombin conversion in the presence of factor Xa, PCPS, and factor Va at increasing Ca^{2+} concentrations is complex. Appreciable activity with factor Va present is seen at Ca^{2+} concentrations well below those required for the quantitative binding of factor Xa to phospholipid in absence of factor Va, further supporting the notion that the binding of factor Xa to phospholipid-bound factor Va is essential for prothrombinase activity.

Acknowledgments

We thank Drs. Charles Kettner and Elliott Shaw for the Dns-EGR-CK. We are grateful to David Schowalter for his efforts in this work and to Jeanne Nemitz for her patient assistance in the preparation of the manuscript.

Registry No. S2222, 60457-00-3; Ca, 7440-70-2; factor Xa, 9002-05-5; factor Va, 65522-14-7; prothrombinase, 9001-24-5.

References

Bajaj, S. P., & Mann, K. G. (1973) *J. Biol. Chem. 248*, 7729–7741.

Bajaj, S. P., Butkowski, R. J., & Mann, K. G. (1975) *J. Biol. Chem. 250*, 2150–2156.

Barenholz, Y., Gibbs, D., Litmann, B. J., Goll, J., Thompson, T. E., & Carlson, F. D. (1977) *Biochemistry 16*, 2806–2810.

Bloom, J. W., Nesheim, M. E., & Mann, K. G. (1979) *Biochemistry 18*, 4419–4425.

Bull, R. K., Jevons, S., & Barton, P. G. (1972) *J. Biol. Chem. 247*, 2747–2754.

Dählback, B., & Stenflo, J. (1978) *Biochemistry 17*, 4939–4945.

Dombrose, F. A., Gitel, S. N., Zawalich, K., & Jackson, C. M. (1979) *J. Biol. Chem. 254*, 5027–5040.

Downing, M. R., Butkowski, R. J., Clark, M. M., & Mann, K. G. (1975) *J. Biol. Chem. 250*, 8897–8906.

Esmon, C. T. (1979) *J. Biol. Chem. 254*, 964–973.

Fujikawa, K., Legaz, M. W., & Davie, E. W. (1972) *Biochemistry 11*, 4892–4899.

Guinto, E. R., & Esmon, C. T. (1982) *J. Biol. Chem. 257*, 10038–10043.

Heldebrant, C. M., Butkowski, R. J., Bajaj, S. P., & Mann, K. G. (1973) *J. Biol. Chem. 248*, 7149–7163.

Henriksen, R. A., & Jackson, C. M. (1975) *Arch. Biochem. Biophys. 170*, 149–159.

Hibbard, L. S., & Mann, K. G. (1980) *J. Biol. Chem. 255*, 638–645.

Higgins, D. L., & Mann, K. G. (1982) *J. Biol. Chem. 258*, 6503–6508.

Howard, J. B., & Nelsestuen, G. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1281–1285.

Jackson, C. M., Johnson, T. F., & Hanahan, D. J. (1968) *Biochemistry 7*, 4492–4505.

Kane, W. H., Lindhout, M. J., Jackong, C. M., & Majerus, P. W. (1980) *J. Biol. Chem. 255*, 1170–1174.

Lim, T. K., Bloomfield, V. A., & Nelsestuen, G. L. (1977) *Biochemistry 16*, 4177–4181.

Miletich, J. P., Jackson, C. M., & Majerus, P. W. (1978) *J. Biol. Chem. 253*, 6908–6916.

Nelsestuen, G. L., & Broderius, M. (1977) *Biochemistry 16*, 4172–4176.

Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164–4171.

Nelsestuen, G. L., Zytkovicz, T. H., & Howard, J. B. (1974) *J. Biol. Chem. 249*, 6347–6350.

Nelsestuen, G. L., Broderius, M., & Martin, G. (1976) *J. Biol. Chem. 251*, 6886–6893.

Nesheim, M. E., Taswell, J. T., & Mann, K. G. (1979a) *J. Biol. Chem. 254*, 10952–10962.

Nesheim, M. E., Myrmel, K. H., Hibbard, L., & Mann, K. G. (1979b) *J. Biol. Chem. 254*, 508–517.

Nesheim, M. E., Kettner, C., Shaw, E., & Mann, K. G. (1981a) *J. Biol. Chem. 256*, 6537–6540.

Nesheim, M. E., Eid, S., & Mann, K. G. (1981b) *J. Biol. Chem. 256*, 9874–9882.

Nesheim, M. E., Katzmann, J. A., Tracy, P. B., & Mann, K.

G. (1982) *Methods Enzymol. 80*, 249–274.
Owen, W. G., Esmon, C. T., & Jackson, C. M. (1974) *J. Biol. Chem. 249*, 594–605.
Papahadjopoulos, D., & Hanahan, D. J. (1964) *Biochim. Biophys. Acta 90*, 436–439.
Prendergast, F. G., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 840–850.
Rosing, J., Tans, G., Govers-Riemslag, J. W. P., Szaal, R. F. A., & Hemker, H. C. (1980) *J. Biol. Chem. 255*, 274–283.
Stenflo, J., & Suttie, J. W. (1977) *Annu. Rev. Biochem. 46*, 157–172.
Tracy, P. B., Peterson, J. M., Nesheim, M. E., McDuffie, F. C., & Mann, K. G. (1979) *J. Biol. Chem. 254*, 10354–10361.
Tracy, P. B., Nesheim, M. E., & Mann, K. G. (1981) *J. Biol. Chem. 256*, 743–751.

Assignment of Resonances in the Phosphorus-31 Nuclear Magnetic Resonance Spectrum of Poly[d(A-T)] from Phosphorothioate Substitution†

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ABSTRACT: Two phosphorothioate analogues of poly[d(A-T)] have been synthesized enzymatically. In one, poly[d(A_s-T)], dTMP is replaced by thymidine 5′-*O*-phosphorothioate; in the other, poly[d(T_s-A)], dAMP is replaced by 2′-deoxyadenosine 5′-*O*-phosphorothioate. The ^{31}P NMR spectrum of poly[d(A_s-T)] in solutions at low salt concentration shows two resonances at 51.80 and −4.25 ppm relative to trimethyl phosphate. The corresponding values for poly[d(T_s-A)] are 51.51 and −4.43 ppm. These data allow the assignment of the downfield resonance at −4.23 ppm in poly[d(A-T)] to the phosphate group of d(TpA) and the resonance at −4.41 ppm to that of d(ApT). Thus, strong evidence is provided for a repeating dinucleotide structure. A comparison of the ^{31}P NMR spectra of the various polymers in solutions of 2 M CsF reveals that both resonances are shifted upfield by approximately 0.9 ppm in the case of the phosphorothioates and by 0.2 or 0.4 ppm in the case of the phosphates. An upfield shift of about 0.18 ppm can also be observed for the two corresponding dinucleoside monophosphates. Thus, the upfield shift induced by high concentrations of CsF is not specific for the polymer backbone.

A variety of structural and spectroscopic studies suggest that the sugar phosphate backbone of the alternating copolymer of dAMP and dTMP (poly[d(A-T)][1] is not uniform but probably is best described by an alternating conformation [for a review, see Zimmermann (1982)]. Thus, ^{31}P NMR spectroscopy shows two signals for segments of helical poly[d(A-T)] less than 200 base pairs in length at 30 °C in solutions of low ionic strength (Shindo et al., 1979; Cohen et al., 1981). The difference in chemical shift disappears upon melting of the double helix but is increased in the presence of high salt concentrations, particularly CsF (Patel et al., 1981). For an interpretation of these spectra, an assignment of the two signals to the phosphorus of the two internucleotidic linkages d(ApT) and d(TpA) is necessary. We attribute the resonance at higher field to d(ApT) and that at lower field to d(TpA) on the basis of measurements performed with polymers carrying selective substitutions of the phosphate group by phosphorothioate. The upfield shifts observed upon an increase in ionic strength apply to all phosphates in the polymer as well as in the corresponding dinucleoside monophosphates.

Experimental Procedures

Materials

The S_P diastereomer of dATPαS was synthesized from the chemically synthesized mixture of diastereomers of dADPαS by reaction with phosphoenolpyruvate and pyruvate kinase (Burgers & Eckstein, 1979) as described for ATPαS (Eckstein & Goody, 1976). The mixture of diastereomers of dTTPαS was synthesized by reaction of acetyl phosphate and acetate kinase with the mixture of diastereomers of dTDPαS (Bartlett & Eckstein, 1982) which were synthesized from dTMPS as described for ADPαS (Eckstein & Goody, 1976). The diastereomers of d[Tp(S)A] were prepared as described (Romaniuk & Eckstein, 1982) and those of d[Ap(S)T] by an analogous procedure (S. Spitzer and F. Eckstein, unpublished results).

Poly[d(A-T)] and alkaline phosphatase from calf intestine were obtained from Boehringer Mannheim, Germany. *Micrococcus luteus* DNA polymerase I was from P-L Biochemicals, Milwaukee, WI. d(TpA) and d(ApT) were purchased from Sigma Chemical Co., St. Louis, MO. High-pressure liquid chromatography was performed on a Waters Associates

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[1] Abbreviations: dATPαS, deoxyadenosine 5′-*O*-(1-thiotriphosphate); dADPαS, deoxyadenosine 5′-*O*-(1-thiodiphosphate); dAMPS, 2′-deoxyadenosine 5′-*O*-phosphorothioate; dTTPαS, thymidine 5′-*O*-(1-thiotriphosphate); dTDPαS, thymidine 5′-*O*-(1-thiodiphosphate); dTMPS, thymidine 5′-*O*-phosphorothioate; ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); poly[d(A-T)], alternating copolymer of dAMP and dTMP; poly[d(G-C)], alternating copolymer of dGMP and dCMP; poly[d(T_s-A)], alternating copolymer of dTMP and 2′-deoxyadenosine 5′-*O*-phosphorothioate; poly[d(A_s-T)], alternating copolymer of dAMP and thymidine 5′-*O*-phosphorothioate; d[Tp(S)A], 5′-*O*-(2′-deoxyadenosyl) 3′-*O*-thymidyl phosphorothioate; d[Ap(S)T], 5′-*O*-thymidyl 3′-*O*-(2′-deoxyadenosyl) phosphorothioate; d(TpA), 5′-*O*-(2′-deoxyadenosyl) 3′-*O*-thymidyl phosphate; d(ApT), 5′-*O*-thymidyl 3′-*O*-(2′-deoxyadenosyl) phosphate; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-4546$01.50/0