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Phosphorothioate Oligonucleotides Inhibit the Intrinsic Tenase Complex by an Allosteric Mechanism†

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ABSTRACT: Phosphorothioate oligonucleotides (PS ODNs) prolong the activated partial thromboplastin time in human plasma by inhibition of intrinsic tenase (factor IXa−factor VIIIa) activity. This inhibition was characterized using ISIS 2302, a 20-mer antisense PS ODN. ISIS 2302 demonstrated hyperbolic, mixed-type inhibition of factor X activation by the intrinsic tenase complex**.** The decrease in $V_{max(app)}$ was analyzed by examining complex assembly, cofactor stability, and protease catalysis. ISIS 2302 did not inhibit factor X activation by the factor IXa−phospholipid complex, or significantly affect factor VIII−phospholipid affinity. Inhibitory concentrations of ISIS 2302 modestly decreased the affinity of factor IXa−factor VIIIa binding in the presence of phospholipid (K_D = 11.5 vs 4.8 nM). This effect was insufficient to explain the reduction in $V_{max(app)}$. ISIS 2302 did not affect the in vitro half-life of factor VIIIa, suggesting it did not destabilize cofactor activity. In the presence of 30% ethylene glycol, the level of factor X activation by the factor IXa−phospholipid complex increased 3-fold, and the level of chromogenic substrate cleavage by factor IXa increased more than 50-fold. ISIS 2302 demonstrated partial inhibition of factor X activation by the factor IXa−phospholipid complex, and chromogenic substrate cleavage by factor IXa, only in the presence of ethylene glycol. Like the intact enzyme complex, ISIS 2302 demonstrated hyperbolic, mixed-type inhibition of chromogenic substrate cleavage by factor IXa (K_I = 88 nM). Equilibrium binding studies with fluorescein-labeled ISIS 2302 demonstrated a similar affinity (K_D = 92 nM) for the PS ODN−factor IX interaction. These results suggest that PS ODNs bind to an exosite on factor IXa, modulating catalytic activity of the intrinsic tenase complex.

Ex vivo modeling of blood coagulation demonstrates that formation of the membrane-bound intrinsic tenase (factor IXa−factor VIIIa) and prothrombinase (factor Xa−factor Va) complexes results in a localized, explosive increase in the level of thrombin generation (*1*, *2*). In minimally altered whole blood, the rate-limiting factor for thrombin generation is activation of factor Xa by the intrinsic tenase complex (*2*). Similarly, in a cell-based system containing platelets and monocytes expressing the tissue factor, addition of picomolar levels of factor IXa generates significantly more thrombin than similar concentrations of factor Xa (*3*). Omitting either factor IX or factor VIII markedly reduces the level of generation of thrombin (*1*). Thus, formation of the intrinsic tenase complex is critical to the final rate of thrombin generation during the propagation phase of coagulation. The activity of the intrinsic tenase complex appears to be primarily regulated by instability (loss of the A2 domain) and proteolytic inactivation of factor VIIIa (by factor IXa) (*4*). The pivotal role of intrinsic tenase suggests that regulation of this enzyme complex is critical to maintaining hemostatic balance. Thus, the intrinsic tenase complex may represent an important therapeutic target for the prevention of thrombosis.

First-generation therapeutic antisense compounds are predominantly phosphorothioate oligonucleotides, in which a nonbridging oxygen in the phosphodiester backbone is replaced with sulfur. Systemic administration of antisense phosphorothioate oligonucleotides is associated with prolongation of the activated partial thromboplastin time (APTT) in both monkeys and humans (*5*, *6*). We have demonstrated that selective prolongation of the APTT by phosphorothioate oligonucleotides in human plasma results from inhibition of intrinsic tenase activity. This inhibition is independent of the nucleotide sequence, but requires the presence of the phosphorothioate backbone modification, indicating that it is a general property of this oligonucleotide class (*7*). This novel inhibitory effect of phosphorothioate oligonucleotides provides a tool for probing regulation of the intrinsic tenase complex, and may represent a novel class of antithrombotic compounds.

To examine the inhibition of intrinsic tenase activity by phosphorothioate oligonucleotides, a detailed examination of the inhibitory mechanism was undertaken. ISIS 2302, a 20-mer phosphorothioate oligonucleotide targeting human ICAM-1 mRNA, was employed as the model compound in this study. The specific mechanism for inhibition of the intrinsic tenase complex by ISIS 2302 was examined, including classification of the kinetic mechanism, and

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assessment of inhibitor effects on complex assembly, cofactor stability, and catalytic activity. The results demonstrate that phosphorothioate oligonucleotides regulate the activity of the intrinsic tenase complex by directly interacting with factor IXa. A model for regulation of the intrinsic tenase complex is proposed in which polyanionic ligands (including phosphorothioate oligonucleotides and heparin) antagonize the factor VIIIa-induced increase in k_{cat} for factor X activation by binding to a negative regulatory exosite on factor IXa. These results have implications for the future design of antisense oligonucleotides, and novel antithrombotic strategies that target the intrinsic tenase complex.

EXPERIMENTAL PROCEDURES

Reagents. Human factors IXa_B, X, and Xa, prothrombin, and affinity-purified sheep polyclonal antiserum versus human factor VIII were purchased from Enzyme Research (South Bend, IN). Human thrombin was purified from prothrombin activated with *Oxyuranus scutellatus* venom as previously described (*8*). Recombinant factor VIII (Kogenate) in human serum albumin was generously provided by J. Brown of the Bayer Corp. (Berkeley, CA). Rabbit brain cephalin (RBC)[1] was purchased from Pel-Freeze Biologicals (Rogers, AR), and phosphatidylserine and phosphatidylcholine were purchased from Avanti Lipids (Alabaster, AL). Recombinant hirudin, alkaline phosphatase-conjugated monoclonal mouse antiserum versus goat/sheep IgG (clone GT-34), and the *p*-nitrophenyl phosphate substrate were purchased from Sigma (St. Louis, MO). Chromogenic substrates were purchased as follows: S-2765 (*N*-α-benzyloxycarbonyl-D-Arg-Gly-Arg-pNA) from DiaPharma (Franklin, OH) and Pefachrome IXa (CH_3SO_2-D-CHG-Gly-Arg-pNA) from Centerchem, Inc. (Stamford, CT). The unlabeled 20-mer phosphorothioate oligodeoxyribonucleotide 5′-GCCCAAGCTG-GCATCCGTCA-3′ (ISIS 2302), a 5′-fluorescein-labeled derivative of ISIS 2302, and an unlabeled phosphodiester backbone analogue of ISIS 2302 were provided by ISIS Pharmaceuticals (Carlsbad, CA). All other chemicals were at least reagent grade and were purchased from major suppliers.

Intrinsic Tenase Assay. A chromogenic assay for intrinsic tenase complex activity was performed under conditions of limiting factor VIIIa concentration (*9*). Purified factor VIII (12.5 nM) was activated with 40 nM thrombin in 0.15 M NaCl, 20 mM HEPES (pH 7.4), 5 mM $CaCl_2$, and 0.01% Tween for 30 s at room temperature. Thrombin was neutralized with recombinant hirudin (60 nM) and the activation mixture diluted 25-fold into a reaction mix containing final concentrations of 5 nM human factor IXa_B and 5% (v/v) rabbit brain cephalin (RBC) in 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, and 0.1% PEG-8000 buffer. Human factor X (300 nM) was added immediately and the reaction mixture sampled (50 μL) at 15, 30, 45, and 60 s, into 10 μL of 0.25 M EDTA and 1.1 mg/mL Polybrene(pH 7.4). The amount of factor Xa generated at each time point was then determined by addition of 100 μL of S-2765 to a final concentration of 300 μM, and comparing the rate of substrate hydrolysis (change in absorbance at 405 nm over the course of 2 min) in a kinetic microtiter plate reader (Vmax, Molecular Devices Corp.) to a standard curve constructed with purified factor Xa. The initial rate of intrinsic tenase complex activity (factor Xa generation) was determined by plotting factor Xa concentration versus time, under conditions where less than 10% total substrate cleavage occurred. The rate of factor Xa generation (nanomolar per minute) was linear over the time course of the assay (0−60 s), and with respect to factor VIIIa concentration (0−500 pM). The level of factor X generation by the factor IXa_B−phospholipid complex in the absence of cofactor was determined in a similar fashion, except that the factor IXa concentration was increased to 25 nM, and the reaction mixture was sampled at 5, 10, 15, and 20 min to determine the amount of factor Xa present. Ethylene glycol (30%, v/v) was added to the assay buffer for selected experiments.

Molecular masses (kilodaltons) and extinction coefficients ($\epsilon_{0.1\%}$): 46 000 and 1.43 for human factor IXa_B, 58 900 and 1.16 for factor X, 46 000, and 1.40 for factor Xa, 36 700 and 1.83 for thrombin, 280 000 and 1.2 for recombinant human factor VIII, and 6781 and 18.78 for ISIS 2302.

Purification and Quantitation of Human Recombinant Factor VIII Activity. Recombinant human factor VIII was purified away from its bovine serum albumin carrier by Mono S chromatography (*10*). Briefly, the lyophilized factor VIII preparation (Kogenate) was rehydrated with deionized water according to the manufacturer's instructions in buffer containing 0.1 M NaCl, 0.27 M glycine, 2.5 mM $CaCl_2$, and 5 mg/mL human albumin. The factor VIII preparation was then applied to a Mono S HR 5/5 column equilibrated in 0.15 M NaCl, 20 mM Tris (pH 7.4), 5 mM $CaCl_2$, and 0.01% Tween 80 at a rate of 0.5 mL/min, and washed in the same buffer until the absorbance of the column effluent returned to near baseline (>5 column volumes). The factor VIII was then eluted stepwise with the same buffer containing 0.75 M NaCl, and 0.5 mL fractions were collected. Fractions containing factor VIII were pooled, and activity was quantitated by the two-stage clotting assay (*11*).

Determination of the K_I for Inhibition of the Intrinsic Tenase Complex by ISIS 2302. The effect of increasing concentrations of ISIS 2302 on the rate of factor X activation by the intrinsic tenase complex was determined in the chromogenic assay described above. The final concentrations of reactants in the intrinsic tenase mixture were 500 pM factor VIIIa, 5 nM factor IXa, 5% (v/v) RBC, 2 mM $CaCl_2$, 300 nM factor X, and 0−4.5 μM ISIS 2302. The rate of factor X activation at each concentration of ISIS 2302 was determined as described above and plotted versus inhibitor concentration. The values for K_I and k_3/k_2 were determined by fitting the data by nonlinear regression (Marquardt algorithm) to the equation for partial, uncompetitive inhibition:

$$v = \frac{V_{max}}{\left[\frac{K_s}{1 + \frac{(k_3/k_2)I}{K_I}}\right]\frac{1}{S} + \frac{1 + I/K_I}{1 + \frac{(k_3/k_2)/I}{K_I}}} \quad (1)$$

where v is the initial velocity of factor X activation, V_{max} is the maximal velocity at saturating concentration of factor

[1] Abbreviations: PS ODN, phosphorothioate oligodeoxyribonucleotide; RBC, rabbit brain cephalin; PEG-8000, polyethylene glycol, with an average M_r of 8000; PC/PS, phosphatidylchorine/phosphatidylserine; SD, standard deviation; SE, standard error.

X, K_s is the dissociation constant for the enzyme−substrate complex, K_I is the dissociation constant for the enzyme−inhibitor complex, *S* is the concentration of substrate, *I* is the concentration of inhibitor, and k_2 and k_3 represent k_{cat} for the ES and ESI complexes, respectively (*22*).

Determination of Kinetic Constants for Factor X Activation. Varying concentrations of factor X (0−450 nM) were added to 500 pM thrombin-activated factor VIII, 5 nM factor IXa, 5% RBC, and 2 mM $CaCl_2$ in the absence or presence of 0.75 or 1.5 μM ISIS 2302, and the rate of factor X activation was determined in the chromogenic assay. The rate of factor X activation was plotted versus substrate (factor X) concentration in both the presence and absence of ISIS 2302. The K_m and V_{max} values for factor X activation by the intrinsic tenase complex were then determined by fitting the data by nonlinear regression to the Michaelis−Menten equation.

Determination of the Affinity of Factor VIII for Phospholipid. The affinity of factor VIII for both RBC and phosphatidylserine was determined as described by Bloom et al. (*13*), with minor modifications. RBC (5%, v/v) or 50 μg/mL L-α-phosphatidyl-L-serine dissolved in methanol was added (50 μL/well) to a Greiner polystyrene 96-well plate and dried down at room temperature for 6−8 h. Wells were then blocked with 250 μL of 0.15 M NaCl, 0.05 M Tris (pH 7.2), and 1% gelatin overnight at room temperature, followed by washing the wells six times with a similar buffer without gelatin containing 0.05% Tween 20. Increasing concentrations of human recombinant factor VIII diluted in 0.15 M NaCl, 0.05 M Tris (pH 7.2), and 1% gelatin were incubated in the phospholipid-coated wells with or without either 1.5 μM ISIS 2302 or an identical phosphodiester oligonucleotide (50 μL/well) for 1 h at 37 °C. After the wash steps were repeated as described above, a 1:8000 dilution of sheep anti-human factor VIII polyclonal antibody (20 mg/mL) was incubated for 1 h at 37 °C. Wash steps were repeated, and a 1:8000 dilution of the alkaline phosphatase-conjugated monoclonal anti-goat sheep IgG (GT-34) (10 mg/mL) was incubated for 1 h at 37 °C. Wash steps were again repeated, followed by addition of substrate [1 mg/mL *p*-nitrophenyl phosphate in 0.1 M glycine (pH 9.4), 1 mM $MgCl_2$, and 2 mM $ZnCl_2$] for 15−30 min at room temperature. Color development was stopped by addition of 50 μL of 3 N NaOH, and the absorbance was determined at 405 nM using a V_{max} plate reader (Menlo Park, CA). Data were fit by nonlinear regression to a single-site binding model.

Determination of the Affinity of Factor VIIIa−Factor IXa Complex Formation. The affinity of the factor IXa_B−factor VIIIa−phospholipid complex was assessed in the presence and absence of 1.5 μM ISIS 2302. Thrombin-activated factor VIIIa was titrated with increasing amounts of factor IXa_B in the chromogenic assay, and formation of the factor IXa−factor VIIIa complex was assessed by the rate of factor X activation. Factor VIIIa (0.3 nM) was added to the intrinsic tenase chromogenic assay with varying concentrations of factor IXa_B (0−28 nM), 5% (v/v) RBC, and 300 nM factor X, in the presence or absence of ISIS 2302. The rate of factor Xa generation (nanomolar per minute) was determined as described for the chromogenic assay. The rate of factor Xa generation determined in the absence of factor VIIIa under these conditions was less than 2% of the total activity. The factor IXa_B concentration was plotted versus the rate of factor Xa generation, and the data were fit by nonlinear regression to a single-site binding model. The concentration of the factor VIIIa−factor IXa complex in the absence and presence of 1.5 μM ISIS 2302 was determined by using the experimentally determined K_D values to solve the quadratic equation:

$$[\text{FIXa}\cdot\text{FVIIIa}] = \frac{[\text{FIXa}]_t + [\text{FVIIIa}]_t + K_D}{2} - \frac{\sqrt{([\text{FIXa}]_t + [\text{FVIIIa}]_t + K_D)^2 - 4[\text{FIXa}]_t[\text{FVIIIa}]_t}}{2} \quad (2)$$

where $[FIXa]_t$ and $[FVIIIa]_t$ represent the initial concentrations of factor IXa and factor VIIIa, respectively, and K_D represents the dissociation constant for the factor IXa−factor VIIIa complex.

Determination of the in Vitro Half-Life of Factor VIIIa. The in vitro half-life of factor VIIIa activity was determined in the presence and absence of ISIS 2302, for cofactor alone, cofactor with phospholipid, and cofactor with phospholipid and 20 nM factor IXa_B. Recombinant factor VIII (20 nM) was activated with 40 nM thrombin for 30 s and neutralized with 60 nM hirudin, followed by a 1:2 dilution into buffer alone, buffer containing 10% RBC, or buffer containing 10% RBC and 40 nM factor IXa, in the presence or absence of 1.5 μM ISIS 2302 (final concentration). Aliquots were removed from the factor VIIIa incubation mixtures over time, and diluted 25-fold into the intrinsic tenase chromogenic assay to determine residual factor VIIIa activity. Similar experiments were performed with 75% phosphatidylcholine/25% phosphatidylserine (w/w) vesicles (PC/PS vesicles) in place of RBC. The PC/PS vesicles were prepared by extrusion through a 100 nm polycarbonate filter (*14*). The rate of factor X activation (proportional to remaining factor VIIIa activity under assay conditions) was plotted versus time, and the data were fit to an exponential decay function (*15*).

Chromogenic Substrate Catalysis by Factor IXa. The rate of Pefachrome IXa (CH_3SO_2-D-CHG-Gly-Arg-pNA) hydrolysis by factor IXa was determined by the change in absorbance at 405 nm over the course of 10 min in the microtiter plate reader at 23 °C. To determine the K_I for inhibition of chromogenic substrate catalysis by ISIS 2302, 25 nM factor IXa was incubated with increasing amounts of oligonucleotide in 150 μL containing final concentrations of 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, 0.1% PEG-8000, and 2.5 mM Pefachrome IXa. The data were fit to the equation for partial, uncompetitive inhibition (eq 1) to determine the K_I for ISIS 2302. To determine the K_m and k_{cat} for Pefachrome IXa, 50 nM factor IXa was incubated under similar conditions with increasing substrate concentrations, either in the absence or in the presence of 1.5 μM ISIS 2302. Data were fit to the Michaelis−Menten equation to determine the K_m and V_{max}. The V_{max} was converted to k_{cat} using an extinction coefficient of 9920 M^{-1} cm^{-1} for the *p*-nitroaniline product.

Equilibrium Binding of 5′-Fl-ISIS 2302 and Factor IXa. Equilibrium binding studies were performed using a fluorescein-labeled derivative of ISIS 2302. The oligonucleotide was derivatized at the 5′-terminus by coupling 5′-fluorescein

phosphoramidite (Glen Research) as the final phosphoramidite during standard solid phase oligonucleotide synthesis. The product was deprotected in concentrated ammonia, purified by reverse phase HPLC, and yielded a single peak by mass spectroscopy analysis. All fluorescence measurements were taken on a Fluorolog-3 (Jobin Yvon-Spex) spectrofluorometer (T-format, excitation and emission bandwidths of 2.0 nm) in buffer containing 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, and 0.1% PEG-8000 at 23 °C. For measurement of fluorescent anisotropy, excitation was at 495 nm and emission was monitored at 525 nm. Control experiments demonstrated no significant change in the fluorescent spectra of 5′-Fl-ISIS 2302 between the unbound and saturated states. Direct binding studies were performed by addition of increasing amounts of factor IXa (0−500 nM) to buffer containing 1.0 nM 5′-Fl-ISIS 2302. Anisotropy values were expressed as the change in anisotropy relative to that of unbound 5′-Fl-ISIS 2302, and the data were fit by nonlinear regression to a simple binding model. Competition binding studies were performed by the addition of increasing amounts of unlabeled ISIS 2302 (0−10 μM) to buffer containing 1 nM 5′-Fl-ISIS 2302 and 300 nM factor IXa. The level of nonspecific binding was defined as the residual anisotropy change in the presence of a 10000-fold excess of unlabeled oligonucleotide. The EC_{50} was determined by fitting the data to the equation

$$B = \frac{(EC_{50})^n}{(EC_{50})^n + [I]^n} \quad (3)$$

where B represents the fractional specific binding, [I] represents the concentration of unlabeled ligand used as competitor, EC_{50} represents the concentration of unlabeled ligand that causes a 50% displacement of labeled ligand (5′-Fl-ISIS 2302), and n represents the pseudo Hill coefficient (*16*). The relationship between the equilibrium dissociation constants K_I of the unlabeled ligand I and K_D of the labeled ligand L (determined by direct binding study) is given by eq 4:

$$EC_{50} = K_I(1 + [L]/K_D) \quad (4)$$

In these binding studies, since $[L] \ll K_D$, then $EC_{50} \sim K_I$.

RESULTS

Classification of the Inhibition Mechanism for Phosphorothioate Oligonucleotides. Inhibition of the intrinsic tenase complex by phosphorothioate oligonucleotides was characterized using the 20-mer antisense oligonucleotide ISIS 2302 (*6*). Increasing concentrations of ISIS 2302 resulted in partial inhibition of intrinsic tenase (factor IXa−factor VIIIa−phospholipid) activity with approximately 15−20% residual activity in the plateau phase (Figure 1). The inhibition mechanism was classified by determining the effect of oligonucleotide on the K_m and V_{max} for factor X activation by the intrinsic tenase complex. Increasing concentrations of factor X were incubated with 5 nM factor IXa and 500 pmol of factor VIIIa, in the absence or presence of either 0.75 or 1.5 μM ISIS 2302 (Figure 2 and Table 1). The results demonstrate that increasing amounts of ISIS 2302 significantly decreased both the $V_{max(app)}$ and the K_m for factor X activation (Table 1). These results are consistent with a hyperbolic, mixed-type inhibition mechanism for ISIS 2302. When V_{max} and K_m are reduced by a similar degree (as in this case), this mixed system is also called hyperbolic or partial, uncompetitive inhibition (*12*).

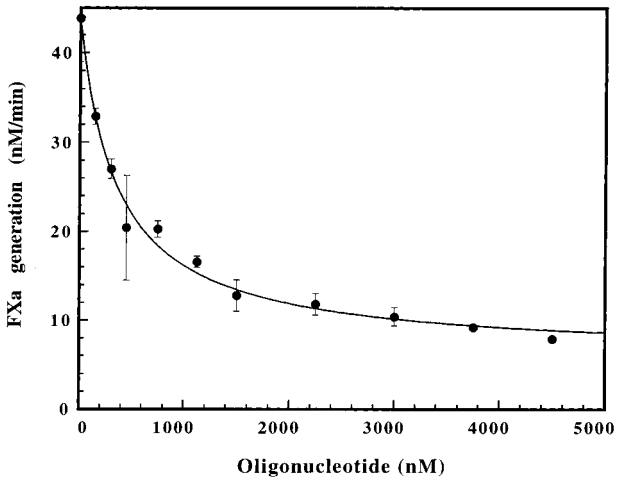

FIGURE 1: Inhibition of intrinsic tenase activity by phosphorothioate ODN (ISIS 2302). The rate of factor X activation (nanomolar per minute) by the intrinsic tenase complex was determined with the chromogenic assay. Reactions were initiated by the addition of thrombin-activated factor VIIIa (final concentration of 0.5 nM) to a reaction mixture containing 5 nM factor IXa, 5% (v/v) RBC, 300 nM factor X, and increasing concentrations of ISIS 2302, in 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, and 0.1% PEG-8000. The reaction mixture was sampled at 15, 30, 45, and 60 s to determine the amount of factor Xa generated by the rate of chromogenic substrate hydrolysis (see Experimental Procedures). Mean values were plotted with error bars representing ±SD. The inhibition constant for ISIS 2302 (K_I = 0.32 ± 0.04 μM) was determined by fitting the data by nonlinear regression (Marquardt algorithm) to the equation for partial, uncompetitive inhibition (eq 1).

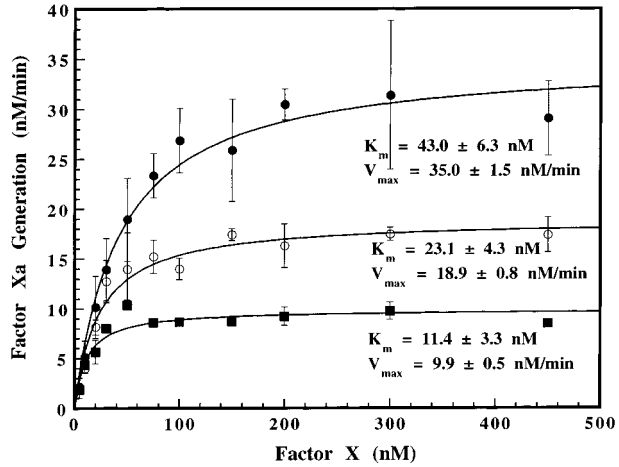

FIGURE 2: Effect of phosphorothioate oligonucleotide on the kinetics of factor X activation by the intrinsic tenase complex. The rate of factor X activation by 0.5 nM thrombin-activated factor VIIIa, 5 nM factor IXa, and 5% RBC was determined in the presence of 0 (●), 0.75 (○), or 1.5 μM ISIS 2302 (■). Buffer conditions were as described in the legend of Figure 1, and the reaction mixture was sampled at 15, 30, 45, and 60 s to determine the amount of factor Xa generated. Mean values were plotted with error bars representing ±SD. The K_m and $V_{max(app)}$ for factor X activation were determined by fitting the data by nonlinear regression to the Michaelis−Menten equation (eq 2).

Table 1: Effect of Phosphorothioate Oligonucleotide (ISIS 2302) on Intrinsic Tenase Complex Assembly and Catalysis of Factor X[a]

parameter	control	ISIS 2302 (1.5 μM)
K_m, factor X (nM)	43.0 ± 6.3	11.4 ± 3.3
$V_{max(app)}$, factor X (nM/min)	35.0 ± 1.5	9.9 ± 0.5
k_{cat}, factor X (s^{-1})	2.3 ± 0.1	1.1 ± 0.1
K_D, factor VIII−RBC (nM)	1.2 ± 0.2	0.6 ± 0.1
$K_{D(app)}$, factor VIIIa−factor IXa (nM)	4.8 ± 1.4	11.5 ± 1.4

[a] The kinetic and equilibrium binding parameters for intrinsic tenase formation and activity were determined in the absence and presence of 1.5 μM ISIS 2302. The K_m and $V_{max(app)}$ for factor X activation were determined by fitting the data to the Michaelis−Menten equation (Figure 2). The K_D for factor VIII−RBC binding was determined as described in the legend of Figure 3 by fitting the data to a single-site binding model. The $K_{D(app)}$ for factor VIIIa−factor IXa complex formation in the presence of 5% RBC was determined by enzymatic detection of intrinsic tenase complex formation (Figure 3). The k_{cat} for factor X activation was determined by dividing the $V_{max(app)}$ by the concentration of the intrinsic tenase complex formed under each condition, as derived from the experimentally determined K_D (see Experimental Procedures). Values are expressed as the mean ± SD for a minimum of three determinations ($n \geq 3$).

In principle, the observed decrease in the $V_{max(app)}$ for factor X activation may result from a reduction in the concentration of the active enzyme complex (decreased level of assembly or loss of cofactor activity), or a reduction in the catalytic rate of the intact complex. Thus, the effect of ISIS 2302 on assembly, cofactor stability, and catalytic activity of the intrinsic tenase complex was examined. The binding of cofactor (factor VIIIa) to the factor IXa−phospholipid complex results in a marked increase in the k_{cat} for factor X activation (*9*, *17*). To assess whether inhibition by phosphorothioate oligonucleotide required the presence of factor VIIIa, the effect of increasing inhibitor concentration on the activity of the factor IXa−phospholipid (RBC) complex was examined. No significant inhibition of the factor IXa−RBC complex was observed (Figure 6 control), while near-maximal inhibition of the intrinsic tenase complex was noted over the same oligonucleotide concentration range (Figure 1). The lack of inhibition of the factor IXa−RBC complex indicates that the inhibitor does not significantly affect the assembly of enzyme (factor IXa) and substrate (factor X) on the phospholipid surface.

Effect of Oligonucleotide on Factor VIII Binding to Phospholipid. Assembly of the intrinsic tenase complex requires binding of both cofactor and enzyme to the phospholipid surface. The binding of factor VIII (and VIIIa) to the phospholipid surface involves interaction with the C2 domain of the cofactor (*18*). The effect of ISIS 2302 on the affinity of factor VIII for phospholipid was assessed in 96-well plates coated with either RBC or phosphatidylserine. Increasing concentrations of human factor VIII were added to the phospholipid-coated wells in the absence or presence of 1.5 μM ISIS 2302, or an identical noninhibitory phosphodiester oligonucleotide. Following incubation and wash steps, lipid-bound factor VIII was detected using a polyclonal antiserum versus human factor VIII, followed by an alkaline phosphatase-conjugated secondary antibody (see Experimental Procedures). Control wells in which phospholipid, factor VIII, or either antibody was omitted demonstrated a background signal level. The apparent affinity of factor VIII for RBC or phosphatidylserine was not significantly reduced by

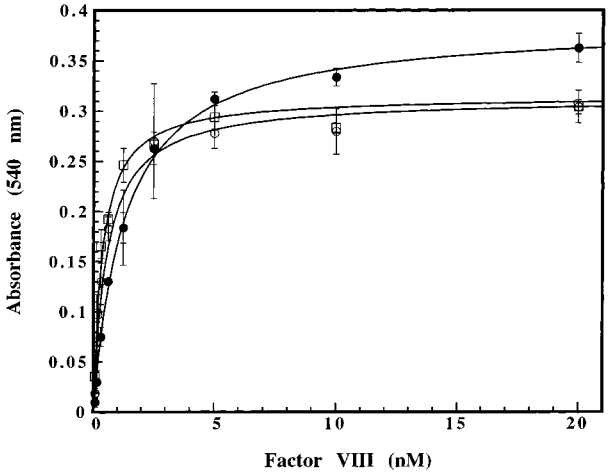

FIGURE 3: Effect of phosphorothioate oligonucleotide on the binding of factor VIII to phospholipid. Increasing concentrations of factor VIII were incubated in 96-well plates coated with 5% (v/v) RBC in the absence of oligonucleotide (●) or in the presence of 1.5 μM ISIS 2302 (□) or the phosphodiester analogue of ISIS 2302 (○). Binding was detected with a sheep polyclonal antiserum vs human factor VIII, followed by an alkaline phosphatase-conjugated monoclonal antiserum vs sheep/goat IgG (see Experimental Procedures). Mean values were plotted with error bars representing ±SD. Data were fit to a single-site binding model by nonlinear regression. The results of replicate determinations are listed in Table 1.

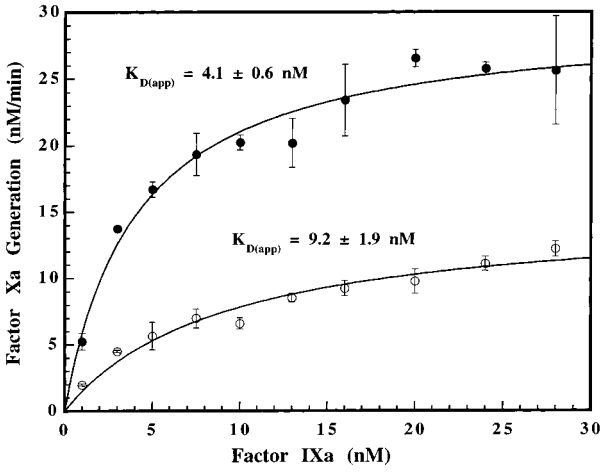

FIGURE 4: Effect of phosphorothioate oligonucleotide on the binding of factor IXa to factor VIIIa in the presence of phospholipid. The apparent affinity of factor IXa−factor VIIIa complex formation was determined in the absence (●) and presence (○) of 1.5 μM ISIS 2302. Thrombin-activated factor VIIIa (final concentration of 0.35 nM) was titrated with increasing amounts of factor IXa in the presence of 5% RBC and 2 mM $CaCl_2$. The formation of the factor VIIIa−factor IXa complex was assessed by the rate of factor X activation, under the conditions described in the legend of Figure 1. Mean values were plotted with error bars representing ±SD. The $K_{D(app)}$ values were determined by fitting the data as described in Experimental Procedures. The results of replicate determinations are listed in Table 1.

the addition of either inhibitory concentrations of ISIS 2302 or a similar concentration of the noninhibitory phosphodiester oligonucleotide (Figure 3 and Table 1). The lack of an effect on factor VIII−phospholipid binding suggests that ISIS 2302 does not inhibit the intrinsic tenase complex by antagonizing cofactor binding to the phospholipid surface.

Effect of Phosphorothioate Oligonucleotide on Factor VIIIa−Factor IXa Complex Formation. To further address

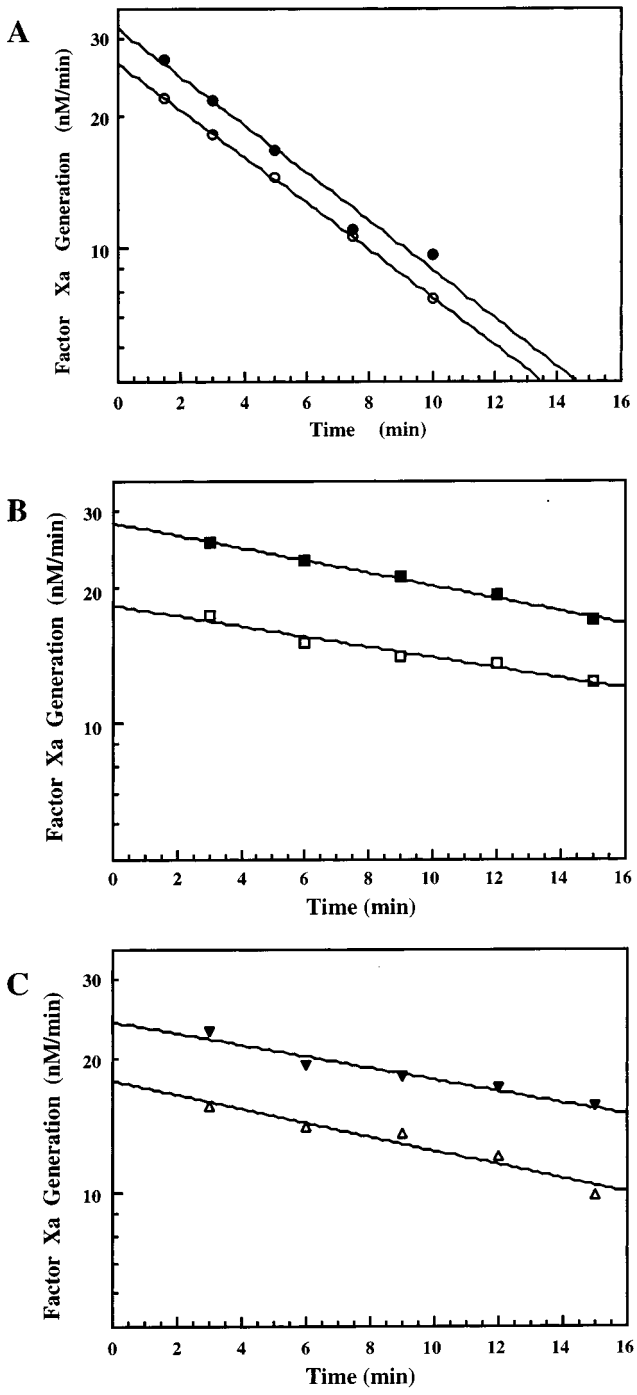

FIGURE 5: Effect of phosphorothioate oligonucleotide on the in vitro half-life of factor VIIIa. Recombinant human factor VIII was activated for 30 s by 40 nM thrombin, neutralized with 60 nM hirudin, and immediately diluted 1:2 into tenase reaction buffer (see Figure 1) under the conditions described below. Aliquots of the thrombin-activated factor VIII were sampled over time, and residual factor VIIIa activity was determined in the chromogenic assay as described in the legend of Figure 1. Final incubation conditions were as follows: (A) factor VIIIa alone in the absence (●) or presence (○) of 1.5 μM ISIS 2302, (B) factor VIIIa with 5% (v/v) RBC in the absence (■) or presence (□) of 1.5 μM ISIS 2302, and (C) factor VIIIa with 5% (v/v) RBC and 20 nM human factor IXa in the absence (▼) or presence (△) of 1.5 μM ISIS 2302. The reduction in the absolute values of the rates of factor Xa generation in the presence of ISIS 2302 correlated with the expected residual oligonucleotide concentration following dilution into the tenase assay.

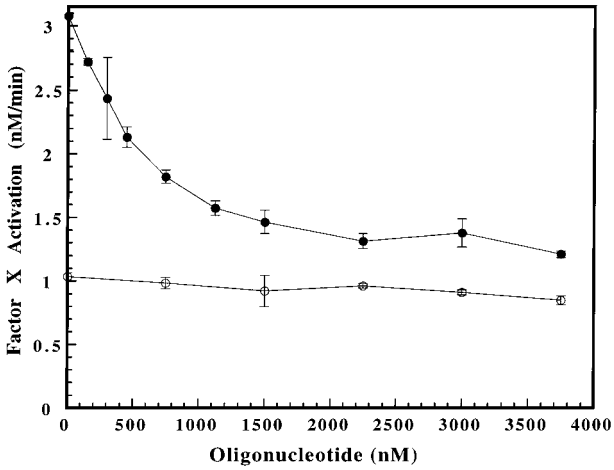

FIGURE 6: Effect of phosphorothioate oligonucleotide on factor X activation by the factor IXa−phospholipid complex in the presence and absence of 30% ethylene glycol. Increasing concentrations of ISIS 2302 were added to 25 nM factor IXa, 5% (v/v) RBC, and 800 nM factor X, in buffer containing 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, and 0.1% PEG-8000, in the absence (○) or presence (●) of 30% (v/v) ethylene glycol. The rate of factor X activation was determined as described (see Experimental Procedures). Mean values were plotted with error bars representing ±SD.

potential oligonucleotide effects on intrinsic tenase assembly, the apparent affinity of human factor VIIIa for factor IXa in the presence of phospholipid was examined in the absence and presence of 1.5 μM ISIS 2302. Factor VIIIa was titrated with increasing concentrations of factor IXa in the presence of 5% RBC, and formation of the cofactor−protease complex was assessed by the rate of factor Xa generation. Addition of ISIS 2302 resulted in a modest reduction in the apparent affinity of the factor IXa−factor VIIIa complex formation, compared to the absence of oligonucleotide (Figure 4 and Table 1).

Effect of Phosphorothioate Oligonucleotide on the Factor VIIIa Half-Life. Since destabilization of the cofactor half-life could result in inhibition of intrinsic tenase activity, the effect of the oligonucleotide on the in vitro half-life of recombinant factor VIIIa was examined. Recombinant factor VIII was activated for 30 s with thrombin; excess hirudin was added to neutralize the thrombin, and the activated cofactor was diluted 1:2 into buffer with or without 1.5 μM ISIS 2302 (final concentration) present. The reaction mix was sampled over time to determine the residual factor VIIIa activity. The rate of disappearance of factor VIIIa activity was similar in the absence or presence of the oligonucleotide (Figure 5A). A small reduction in the absolute enzyme activity was noted in the presence of oligonucleotide under all conditions, consistent with the expected amount of inhibitor carryover after dilution of the sample into the intrinsic tenase assay. The lack of an effect on the in vitro half-life of factor VIIIa activity indicates that the oligonucleotide did not directly destabilize cofactor activity.

The in vitro half-life of factor VIIIa was also examined in the presence of 5% RBC or 40 μM PC/PS vesicles. In contrast to PC/PS vesicles, incubation of factor VIIIa with 5% RBC unexpectedly resulted in prolongation of the factor VIIIa half-life, suggesting that cofactor binding to lipid component(s) in this preparation stabilized factor VIIIa activity. Addition of 1.5 μM ISIS 2302 did not affect

prolongation of the factor VIIIa half-life by RBC, confirming that the oligonucleotide does not destabilize cofactor activity in the presence of phospholipid, and indicating that factor VIIIa binding to the critical lipid component(s) was unaffected (Figure 5B). Consistent with previously reported results, incubation of factor VIIIa with 40 μM PC/PS phospholipid vesicles had no effect on the half-life of cofactor activity, either in the absence or in the presence of ISIS 2302 (data not shown) (*15*).

The binding of factor IXa to factor VIIIa in the presence of phospholipid vesicles stabilizes cofactor activity, resulting in prolongation of the in vitro half-life of factor VIIIa (*19*). Incubation of thrombin-activated recombinant factor VIIIa in the presence of 20 nM factor IXa and 5% (v/v) RBC demonstrates a prolonged cofactor half-life, comparable to that observed with RBC alone (Figure 5C). Addition of 1.5 μM ISIS 2302 under these conditions had no significant effects on cofactor half-life. As previously reported, addition of 20 nM factor IXa along with 40 μM PC/PS vesicles also prolonged the in vitro half-life of factor VIIIa activity (data not shown) (*15*). Addition of 1.5 μM ISIS 2302 did not significantly affect prolongation of cofactor half-life under these conditions (data not shown), suggesting that the oligonucleotide did not disrupt formation of the enzyme−cofactor complex in the presence of PC/PS vesicles.

Effect of Phosphorothioate Oligonucleotide on Factor IXa Catalysis in the Presence of Ethylene Glycol. The crystal structure of porcine factor IXa demonstrates a partially collapsed active site cleft, consistent with the poor reactivity of this protease toward chromogenic substrates (*20*). Addition of 33% ethylene glycol has been reported to increase the catalytic rate of chromogenic substrate cleavage by factor IXa up to 20-fold, in the absence of cofactor or phospholipid (*21*). Likewise, we observed that addition of up to 30% ethylene glycol increased the rate of factor X activation by the factor IXa−phospholipid complex in a dose-dependent fashion (data not shown). The increased rate of catalysis of both macromolecular and chromogenic amide substrates by factor IXa in the presence of ethylene glycol is consistent with a direct effect on enzyme conformation.

To determine whether ISIS 2302 can modulate the catalytic activity of this factor IXa conformation, the effect of increasing oligonucleotide concentration on factor X activation by the factor IXa−phospholipid complex was examined. Addition of 30% ethylene glycol increased the rate of factor X activation by the factor IXa−phospholipid complex 3-fold in the absence of ISIS 2302. Addition of oligonucleotide resulted in dose-dependent, partial inhibition of factor X activation, similar to that observed for the intrinsic tenase complex. In contrast, no significant inhibition of the factor IXa−phospholipid complex was noted over the same range of oligonucleotide concentrations in the absence of ethylene glycol (Figure 6). Likewise, the effect of increasing oligonucleotide concentration on chromogenic substrate cleavage by factor IXa was examined for Pefachrome IXa (CH_3SO_2-D-CHG-Gly-Arg-pNA). Addition of 30% ethylene glycol increased the rate of substrate cleavage by factor IXa approximately 50-fold, consistent with previous results (*21*). Addition of ISIS 2302 resulted in a dose-dependent, partial inhibition of CH_3SO_2-D-CHG-Gly-Arg-pNA cleavage by factor IXa in the presence of ethylene glycol (Figure 7), similar to that observed for factor X activation by the factor IXa−phospholipid complex (Figure 6). No significant inhibition of chromogenic substrate cleavage by oligonucleotide was noted in the absence of ethylene glycol (data not shown). The inhibition mechanism was classified by determining the effect of ISIS 2302 on the K_m and k_{cat} for chromogenic substrate cleavage. Similar to the results obtained for the intact enzyme complex, the oligonucleotide reduced both the K_m and k_{cat} for CH_3SO_2-D-CHG-Gly-Arg-pNA catalysis, suggesting a hyperbolic, mixed-type inhibition mechanism (Figure 8). Fitting the inhibition data to the equation for partial, uncompetitive inhibition (see eq 1) yielded a K_I of 87.7 nM (Figure 7). Thus, the apparent affinity of the inhibitor for the free protease in solution was somewhat greater than for the intact enzyme complex (K_I = 320 nM). The similar mechanisms for inhibition of factor X activation by the intact intrinsic tenase complex, and chromogenic substrate cleavage by factor IXa in solution, suggest that the oligonucleotide modulates catalysis through a direct interaction with the protease.

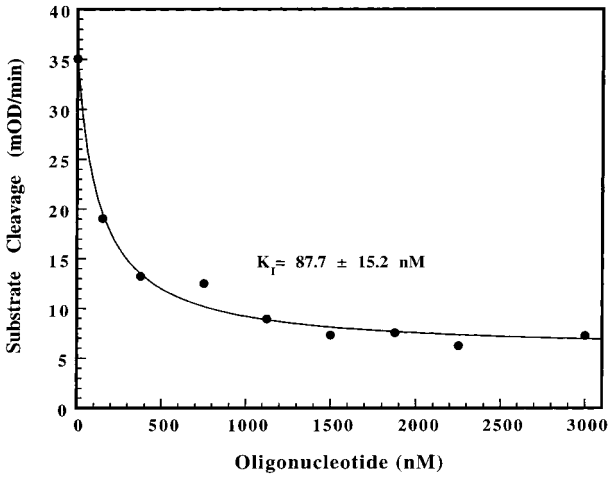

FIGURE 7: Effect of phosphorothioate oligonucleotide on chromogenic substrate cleavage by factor IXa in 30% ethylene glycol. Increasing concentrations of ISIS 2302 were added to 25 nM factor IXa and 2.5 mM Pefachrome IXa (CH_3SO_2-D-CHG-Gly-Arg-pNA) in buffer containing 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, 0.1% PEG-8000, and 30% (v/v) ethylene glycol. The rate of chromogenic substrate cleavage was determined by monitoring the change in absorbance at 405 nm over the course of 10 min. The mean of replicate determinations ($n \geq 2$) was plotted and the K_I determined by fitting the data to the equation for partial, uncompetitive inhibition (see eq 1). Fitted values are expressed ±SE.

Binding of Phosphorothioate Oligonucleotide to Factor IXa. The affinity of ISIS 2302 for factor IXa in solution was determined using a modified form of the oligonucleotide with a fluorescein moiety coupled to the 5′-terminus (5′-Fl-ISIS 2302). Direct binding was performed by adding increasing amounts of factor IXa to 1.0 nM 5′-Fl-ISIS 2302 and detecting the change in fluorescence anisotropy at 525 nm compared to that of the unbound oligonucleotide probe. Competition binding was performed by adding increasing amounts of unlabeled ISIS 2302 to a solution containing 300 nM factor IXa and 1 nM 5′-Fl-ISIS 2302. Fitting the direct binding data to a simple binding model yielded a K_D of 92.3 nM (Figure 9A), which agreed well with the apparent affinity of the oligonucleotide inhibitor for free protease (K_I = 87.7 nM) in the kinetic assays (Figure 7). Likewise, the competition binding assay yielded a K_I of 82.9 nM for the unlabeled

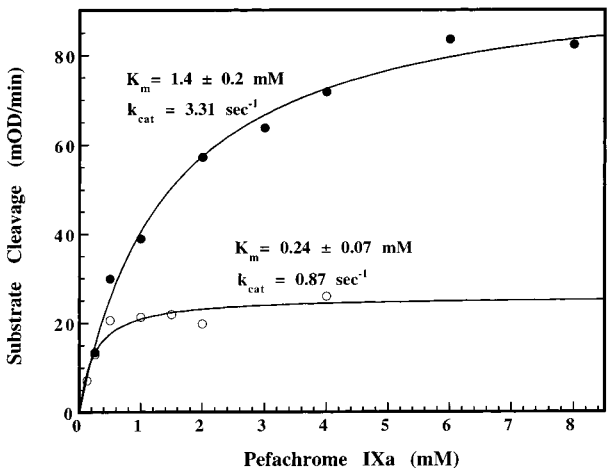

FIGURE 8: Effect of phosphorothioate oligonucleotide on the kinetics of chromogenic substrate cleavage by factor IXa in 30% ethylene glycol. Increasing concentrations of Pefachrome IXa (CH_3-SO_2-D-CHG-Gly-Arg-pNA) were added to 50 nM factor IXa, in buffer containing 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, 0.1% PEG-8000, and 30% (v/v) ethylene glycol, either in the absence (●) or in the presence (○) of 1.5 μM ISIS 2302. The rate of chromogenic substrate cleavage was determined by monitoring the change in absorbance at 405 nm over the course of 10 min. The mean of replicate determinations ($n \geq 2$) was plotted, and the K_m and k_{cat} were determined by fitting the data to the Michaelis−Menten equation (see Experimental Procedures). Fitted values are expressed ±SE.

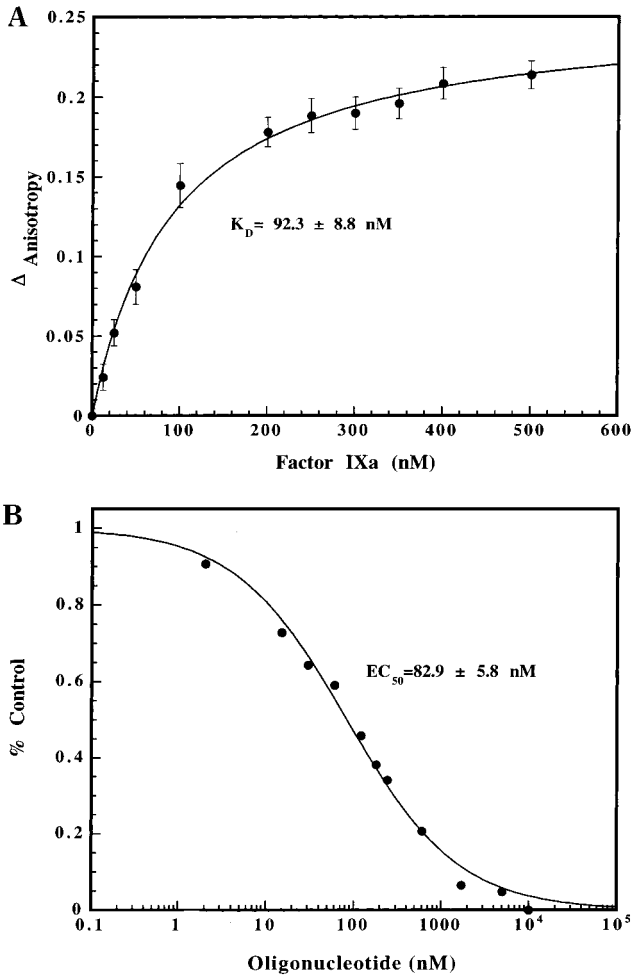

FIGURE 9: Binding of 5′-fluorescein-labeled phosphorothioate oligonucleotide (5′-Fl-ISIS 2302) to factor IXa. Binding of 5′-Fl-ISIS 2302 to factor IXa was detected by monitoring the change in fluorescent anisotropy at 525 nm (see Experimental Procedures). (A) Increasing amounts of factor IXa were added to 1.0 nM 5′-Fl-ISIS 2302 in 0.15 M NaCl, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, and 0.1% PEG-8000. The change in anisotropy was calculated by subtracting the value in the absence of factor IXa, and the data were fit to a simple binding model. (B) Increasing amounts of unlabeled ISIS 2302 competitor were added to 1.0 nM 5′-Fl-ISIS 2302 and 300 nM factor IXa in the buffer described above. The level of nonspecific binding was considered the amount of residual anisotropy change in the presence of a 10000-fold molar excess of competitor. The concentration of the competitor ISIS 2302 was plotted vs the fractional binding, and the EC_{50} and K_D were determined as described in Experimental Procedures. Anisotropy measurements represent the mean of at least six determinations. Fitted values are expressed ±SE.

oligonucleotide competitor (Figure 9B), which was consistent with both the K_D obtained in the direct binding assay and the kinetically determined K_I.

DISCUSSION

The mechanism of intrinsic tenase inhibition by phosphorothioate oligonucleotides was investigated using the 20-mer antisense oligonucleotide ISIS 2302 as the model compound. ISIS 2302 demonstrated partial inhibition of the intrinsic tenase complex (factor IXa−factor VIIIa−phospholipid) associated with a relatively proportional decrease in both the K_m and $V_{max(app)}$ (Figures 1 and 2). For a monomeric enzyme, this pattern is consistent with a hyperbolic mixed-type inhibition mechanism (*12*, *22*). However, interpretation of changes in K_m and $V_{max(app)}$ values for this multisubunit enzyme is complex. Inhibitor effects on the assembly or stability of the complex may decrease the $V_{max(app)}$ by reducing the effective enzyme concentration, without affecting the k_{cat} of the reaction. Thus, to define the specific mechanism of intrinsic tenase inhibition by phosphorothioate oligonucleotides, the effect of ISIS 2302 on complex assembly, cofactor stability, and catalysis of both the macromolecular and chromogenic substrate was examined.

To interpret the observed changes in $V_{max(app)}$, potential changes in the effective enzyme concentration were assessed by examining oligonucleotide effects on complex assembly and stability. ISIS 2302 did not inhibit factor X activation by the factor IXa−RBC complex in the absence of ethylene glycol (Figure 6), disrupt the binding of factor VIII to phospholipid (Figure 3, Table 1, and data not shown), or interfere with prolongation of the factor VIIIa half-life by RBC (Figure 5B). These results suggest that ISIS 2302 does not inhibit intrinsic tenase activity by interfering with the binding of enzyme, cofactor, or substrate to the phospholipid surface. The apparent affinity of factor VIIIa−factor IXa complex formation in the presence of RBC was modestly reduced by inhibitory concentrations (1.5 μM) of ISIS 2302 (Figure 4 and Table 1). However, the reduction in the factor VIIIa−factor IXa concentration predicted from the experimentally determined $K_{D(app)}$ (see eq 2) can account for no more than a 40% reduction in intrinsic tenase activity (compared to 70−80% total inhibition). Complete disruption of protease−cofactor binding would be expected to reduce the level of factor X activation by >99% (*9*, *17*). The presence of significant residual activity (approximately 15%) at maximally inhibitory concentrations of ISIS 2302 indicates that the oligonucleotide does not block formation of the factor VIIIa−factor IXa complex. The failure of the oligonucleotide

to interfere with stabilization of factor VIIIa activity by PS/PC vesicles and excess factor IXa is also consistent with this conclusion (data not shown). Thus, ISIS 2302 modestly reduced the affinity of factor VIIIa−factor IXa complex assembly on the phospholipid surface, consistent with a conformational effect in either the cofactor (factor VIIIa) or protease (factor IXa).

Since intrinsic tenase activity is largely regulated by the intrinsic instability of factor VIIIa (loss of the A2 subunit), destabilization of cofactor activity by the oligonucleotide could also decrease the effective enzyme concentration (*23*). Inhibitory concentrations of ISIS 2302 did not destabilize cofactor activity (alone or in the presence of other intrinsic tenase components), as demonstrated by the lack of an effect on the in vitro half-life of factor VIIIa (Figure 5A−C). Furthermore, prolongation of the factor VIIIa half-life by RBC is a novel finding and suggests that this lipid preparation contains components other than phosphatidylserine and phosphatidylcholine that stabilize cofactor activity. ISIS 2302 also failed to interfere with the previously reported stabilization of cofactor activity by PC/PS vesicles and excess factor IXa (data not shown) (*15*, *24*). The A2 domain of factor VIIIa directly modulates the catalytic activity of factor IXa, and this effect is enhanced by the A1 domain, markedly increasing the k_{cat} for factor X activation (*25*). In contrast, the A3−C1−C2 domain of factor VIIIa appears to largely account for the affinity for factor IXa, but the isolated subunit does not affect the activation of factor X (*26*, *27*). Preservation of both the noncovalent intramolecular interaction with the A2 subunit, and the intermolecular interactions with protease and phospholipid, suggests that the oligonucleotide does not induce a functionally important conformational change in factor VIIIa.

Several lines of evidence suggest that ISIS 2302 interacts directly with factor IXa to modulate the activity of the intrinsic tenase complex. In the presence of ethylene glycol, inhibition of factor X activation by the factor IXa−phospholipid complex and chromogenic substrate cleavage by the free protease suggest that the oligonucleotide can interact directly with the protease. Inhibition of chromogenic substrate cleavage by factor IXa in ethylene glycol demonstrates a hyperbolic, mixed-type mechanism, similar to oligonucleotide inhibition of the intact intrinsic tenase complex. Equilibrium binding studies demonstrate that the affinity of the oligonucleotide−factor IXa interaction (K_D) is sufficient to account for the apparent affinity (K_I) observed in the intrinsic tenase inhibition assays. It is reasonable to expect that the affinity of the oligonucleotide for free protease in solution will be higher than for the protease assembled into the membrane-bound complex. Finally, although determined under slightly different conditions, the oligonucleotide−factor IXa affinity correlates with the apparent affinity of the inhibitor for factor IXa (K_I) in the chromogenic substrate assays. In total, these results suggest that ISIS 2302 binds directly to factor IXa, producing a conformational change that modestly reduces the affinity of the protease−cofactor interaction, and directly modulates the catalytic activity of the enzyme.

The effect of ethylene glycol on factor IXa catalysis provides a valuable analytical tool. The poor baseline reactivity of factor IXa makes direct evaluation of protease−inhibitor interactions difficult, necessitating study of the intrinsic tenase complex. Analysis of this enzyme complex is complicated by the potential effects of inhibitor on assembly and cofactor stability. Addition of ethylene glycol allows direct analysis of the protease in solution, a markedly simpler system. Inhibitor effects in this system can then be correlated with effects on the intact intrinsic tenase complex. Ethylene glycol accelerates the catalysis of both factor X and chromogenic amide substrates by factor IXa, suggesting an effect on the S1−S3 subsites of the protease. Hydrophobic residues in the P3 position of the substrate appear to be particularly important for this effect (*21*). The observed inhibition of chromogenic substrate catalysis suggests that the inhibitory effect of PS ODN may be modulated through the protease S1−S3 subsites. In contrast, factor VIIIa does not appear to affect chromogenic substrate cleavage by factor IXa, suggesting that the cofactor effect may involve formation of an extended substrate binding site and/or a conformational effect on the substrate (*17*).

Unfractionated, low-molecular weight, and low-affinity (for antithrombin) forms of heparin demonstrate partial, noncompetitive inhibition of the intrinsic tenase complex that is not explained by effects on cofactor stability or assembly of the factor IXa−factor VIIIa complex (*15*). This mechanism suggests that heparin may bind to the intrinsic tenase complex and allosterically modulate catalytic activity. The importance of a detailed analysis of the inhibition mechanism for PS ODN is, in part, due to its similarity to heparin inhibition of the intrinsic tenase complex. Analysis of PS ODN inhibition has the advantage of employing a homogeneous chemical preparation, as opposed to the heterogeneity of heparin. The results suggest that a polyanion binding exosite with negative regulatory properties may exist on factor IXa, similar to heparin binding sites demonstrated for thrombin and factor Xa (*28*−*30*). Ligand binding (PS ODN or heparin) to this exosite may antagonize the factor VIIIa-induced increase in catalytic efficiency through conformational effects on the active site and/or extended substrate-binding sites. Modulation of catalytic activity through ligand interaction with a heparin binding exosite may represent a common regulatory theme among serine proteases of the coagulation cascade. Regulation of the catalytic activity of thrombin through ligand binding to exosite II has been demonstrated for the antithrombin−heparin complex, the chondroitin sulfate moiety of thrombomodulin, the kringle-2 domain of prothrombin, an RNA aptamer, and a monoclonal antibody from a myeloma patient (*28*, *31*−*33*).

Preliminary support for this model of intrinsic tenase regulation is provided by recombinant factor IX R338A, which demonstrates an increased level of factor X activation in the intrinsic tenase complex, and a 2-fold increase in the K_I for inhibition by heparin (*34*). Although the effect of this mutation on direct factor IXa binding to heparin has not been reported, the modest decrease in the apparent affinity of the inhibitor for the enzyme complex (increased K_I) is consistent with heparin modulating the catalytic activity of the complex through binding to a regulatory exosite on factor IXa. By extrapolation from structural homology with exosite II of thrombin, R338 would be located at the outer edge of the putative heparin binding exosite on factor IXa, consistent with the modest effect on the apparent heparin affinity (*28*). Molecular mapping of the binding sites for phosphorothioate oligonucleotides and heparin by site-directed mutagenesis

of human factor IX will provide a direct test of the proposed model, and may identify a novel target for antithrombotic therapy.

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