Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 1819–1823, March 1993
Medical Sciences

Hirudin as a molecular probe for thrombin *in vitro* and during systemic coagulation in the pig

(lectin/thrombosis/thrombin inhibition/antithrombin/endotoxin)

PIERRE ZOLDHELYI*†, JAMES H. CHESEBRO*‡, AND WHYTE G. OWEN§¶

*Divisions of Cardiovascular Diseases and §Hematology Research (Internal Medicine), and ¶Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905

Communicated by Kenneth M. Brinkhous, November 30, 1992

ABSTRACT **The amount of thrombin active *in vivo* in the intravascular space (blood and endothelial surface), both basally and in experimental intravascular coagulation, is measured by way of the accessibility of thrombin to intravascular hirudin. Blood samples from pigs given intravenous ^{125}I-labeled hirudin contain ^{125}I-labeled hirudin–thrombin complex in concentrations indicative of a basal thrombin concentration *in vivo* of 0.5 nmol/liter. Intravenous infusion of *Salmonella* endotoxin elicits an increase in the circulating concentration of hirudin–thrombin complex that begins within 15 min and is 20–30 times basal after 4 hr. Induction of mild intravascular coagulation is evidenced by a modest reduction in plasma fibrinogen concentrations. It is concluded that there is a basal pool of hirudin-accessible thrombin in the intravascular space that, were it free in the plasma phase, would be sufficient in principle to sustain intravascular coagulation.**

Because thrombin forms, reacts, regulates, and is regulated and inhibited in millisecond-to-minute time scales in soluble and in stationary phases, measures of intravascular thrombin have remained problematic. Through proteolysis of fibrinogen, platelets (1), and factors V (2), VIII (3), XI (4), and XIII (5), thrombin plays a central role in normal hemostasis and thrombosis. Thrombin is controlled directly by antithrombin III (6) and other inhibitors and indirectly through association with endothelial thrombomodulin and activation of protein C (7). Indirect measures of thrombin include immunochemical assays of fibrinopeptide A (8), prothrombin fragment 1.2 (9), or thrombin–antithrombin complex (10). Estimates of intravascular thrombin through these markers are compromised, however, by their short or pathophysiologically variable half-lives (thrombin–antithrombin complex, fibrinopeptide A) and by thrombin-independent generation (11).

Hirudin, a 65-residue polypeptide from the European leech *Hirudo medicinalis*, inhibits thrombin avidly (K_d = 0.2 pM for the recombinant analog), rapidly ($K_{obs} > 10^8 \cdot M^{-1} \cdot sec^{-1}$), and stoichiometrically (12). Hirudin binds to thrombin in solution and on cell surfaces (13, 14) but not to other plasma proteins (12). Intravascular hirudin would be expected to associate rapidly with intravascular thrombin to yield a complex sufficiently stable to assay in peripheral blood samples. An immunochemical assay for thrombin–hirudin has been used to measure thrombin generation during septicemia in pigs (15) but was insufficiently sensitive to provide a measure of basal thrombin concentration *in vivo*. Concanavalin A, a mannosylglycan-binding lectin from the jack bean (*Canavalia ensiformis*), binds thrombin complexed (or not) with hirudin (16). Hirudin, which bears no glycan, is not bound by the lectin. We report on the use of concanavalin A-agarose chromatography to isolate thrombin–^{125}I-labeled hirudin (^{125}I-hirudin) complex from plasma in yields sufficient to assay thrombin as ^{125}I-hirudin equivalents. The use of hirudin to measure thrombin in physiological environments was evaluated *in vitro*, in a perfused rat heart preparation, and during disseminated intravascular coagulation in the pig. Our study indicates free access of hirudin to thrombin in solution and bound to vascular endothelium and provides a direct measure of basal and experimentally elevated thrombin *in vivo*.

MATERIALS AND METHODS

Reagents. Recombinant (desulfato) hirudin (CGP 39393), a gift from Robert B. Wallis (Research Centre, CIBA–Geigy Pharmaceuticals, Horsham, U.K.) reacted stoichiometrically with thrombin preparations when concentrations were calculated by molar extinction coefficients. Porcine and rat thrombins were purified as described (17); thrombin and hirudin preparations were >95% homogeneous by C18 reversed-phase HPLC, gel chromatography, and SDS/polyacrylamide gel electrophoresis. Porcine thrombin was radioiodinated in chloride-free buffers by the Iodo-Gen method (18). Bovine albumin was from ICN. HD-Hexahydrotyrosyl-L-alanyl-L-arginine-*p*-nitroanilide diacetate (SpectrozymeTH; American Diagnostica, Greenwich, CT) was used as thrombin substrate. Concanavalin A-Sepharose-4B, methyl α-D-mannoside and lipopolysaccharide from *Salmonella abortus equi* were obtained from Sigma. SDS/polyacrylamide radiogels were prepared with use of a PhastSystem (Pharmacia), and autoradiographs were developed at −70°C with Kodak X-Omat film.

Animals. Retired breeder Sprague–Dawley rats (500–700 g each) and 6-week-old Yorkshire pigs (mean weight, 7.7 kg; range, 6.6–8.9 kg) were maintained in the institutional animal care facility and handled according to the guidelines of the Mayo Foundation Animal Care and Use Committee.

^{125}I-Hirudin. Hirudin was iodinated with carrier-free Na^{125}I by the chloramine-T method (19) except that the iodination reaction was carried out in 0.3 M sodium acetate (pH 5.5). For injection into pigs, hirudin was prepared with a specific activity of 750–850 μCi per mg of protein (1 Ci = 37 GBq); for some *in vitro* experiments, 300-fold higher specific activity was achieved by labeling ≈3.5 μg of hirudin with 1 mCi of Na^{125}I. Labeling efficiencies were >80%, with 92–93% of labeled hirudin binding to a thrombin-agarose column. Thrombin–^{125}I-hirudin complex was prepared by titrating thrombin while monitoring cleavage of the chromogenic substrate.

Concanavalin A Affinity Chromatography. Concanavalin A-Sepharose columns (600 μl) were equilibrated in 0.5 M

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†Present address: Division of Hematology, University of Texas Health Science Center, 6431 Fannin, Room 5.016, Houston, TX 77030.
‡Present address: Cardiac Unit, Massachusetts General Hospital, 32 Fruit Street, Jackson 1401, Boston, MA 02114.

NaCl/1.0 mM $MgCl_2$/6 mg of polyethylene glycol 6000 per ml/0.2% sodium azide/10 mM Tris·HCl, pH 7.8. Blood was collected in 1/9th vol of 3.8% sodium citrate/20 mM EDTA and centrifuged immediately at 1700 × g for 10 min. Plasma samples, unless assayed immediately, were stored at −40°C. Plasma samples (100 μl) or buffered saline solution containing thrombin–^{125}I-hirudin complex were applied to the columns, free ^{125}I-hirudin was removed by washing with equilibration solvent, and thrombin–^{125}I-hirudin complex was eluted with 200 mM methyl α-D-mannoside in equilibration solvent. The amount of thrombin bound was calculated as molar hirudin equivalents.

Langendorff Perfused Rat Heart Preparation. Cannulation and perfusion of isolated hearts and the design of the mixing sump have been described for the rabbit (20). In brief, the aortic stump was cannulated and perfused (50 mmHg) with Hanks' balanced salt solution. Prior to perfusion experiments, the hearts were perfused clear of blood for 6 min. Then, the effluent from the coronary sinus was collected in a polypropylene sump and recirculated (total vol, 7 ml) to the aortic stump. ^{125}I-thrombin was added to the sump to yield an initial concentration of 7 nmol/liter and then was measured in effluent samples by SDS/polyacrylamide gel electrophoresis and autoradiography. Quantitative densitometry was carried out with a video densitometer (21) calibrated with the labeled thrombin preparation.

Infusion of Endotoxin and ^{125}I-Hirudin in Pigs. Pigs were sedated with 3 ml of 7:1 (vol/vol) intramuscular ketamine (100 mg/ml) and xylazine (100 mg/ml). Repeat injections of 1 ml were given every 30–60 min. Atropine (0.05 mg/kg) was administered intramuscularly prior to surgery. A femoral cut down was performed after local 2% lidocaine administration. The femoral vein was cannulated with a no. 6 French vascular sheath and its hub was connected via a three-way stopcock and polyethylene tubing to a Harvard perfusion pump adjusted to deliver ^{125}I-hirudin at 15 μCi/hr (2.5 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) together with either endotoxin (100 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) or physiological saline solution. An intravenous bolus of 30 μCi (5 μg/kg) of ^{125}I-hirudin was administered at the start of the maintenance infusion; this provided a relatively constant plasma concentration of ^{125}I-hirudin. At all times, free ^{125}I-hirudin was in 2- to 10-fold excess of thrombin-bound ^{125}I-hirudin. Blood samples were drawn through the intravenous sheath after complete clearance of the tubing between stopcock and intravascular space. After a baseline blood sample was obtained, infusions (endotoxin or saline) were started and samples were obtained after 15, 30, 60, 90, 120, 150, 180, and 240 min, unless the pigs died prematurely. At the end of the experiments, pigs were killed with pentobarbital.

Intravascular Clearance of the Thrombin–Hirudin Complex. Thrombin–^{125}I-hirudin complex was formed by titrating thrombin with hirudin in Tris·HCl-buffered saline as described above. To ensure the absence of free ^{125}I-hirudin, thrombin–hirudin complex was formed in excess thrombin, confirmed by cleavage of specific thrombin substrate. Pigs were prepared as described. Rats, anesthetized with 30 mg of intraperitoneal ketamine, were sampled via an intracarotid cannula. Thrombin–^{125}I-hirudin complex (3–5 μCi) was injected as intravenous (pigs) or intraaortic (rats) bolus and blood was sampled over 4 hr to measure plasma radioactivity.

Fibrinogen. Fibrinogen was measured in citrated EDTA plasma by slight modification of the procedure described by Morrison (22), with use of 6-aminohexanoate (1.3 mg/ml) in 0.017 M imidazole/0.1 M NaCl, pH 7.4, and biuret reagent as the colorimetric indicator.

Statistical Methods. Linear regression and Pearson's correlation coefficient were used to express the correlation between added thrombin and recovered thrombin–^{125}I-hirudin in plasma. The significance of differences between thrombin–hirudin complex at baseline and end of the infusions (endotoxin or saline control) was tested by Student's *t* test. To address the relationship of thrombin–hirudin complex versus time over the first hour, linear regression was used for each individual pig, with time as the predictor and thrombin–hirudin complex as the response. Results are presented as means ± SD.

RESULTS

Detection System. Initial experiments using a mobile phase containing $CaCl_2$ (16, 23) yielded the unexpected finding that 0.3–0.5% of free ^{125}I-hirudin bound to concanavalin A-Sepharose and was partially eluted with methyl α-D-mannoside. In citrated plasma, 10–19% of radioiodinated hirudin bound reversibly to the methyl α-D-mannoside-binding site of concanavalin A-Sepharose. All plasma samples and ^{125}I-hirudin lots yielded the same behavior. After addition of EDTA (final concentration, 2 mM) to the citrated plasma samples and development of the columns without Ca^{2+} in the mobile phase, reversible binding of ^{125}I-hirudin to concanavalin A columns decreased to the subfemtomole range and was independent of the total amount of radiolabel added (Fig. 1). Under these conditions, <0.1% of the applied inhibitor bound irreversibly; this fraction, however, increased to 5% when EDTA was omitted from the applied sample.

Because calcium ions have been regarded as important to concanavalin A function (23, 24), the effects of omitting mobile-phase Ca^{2+} on desorption of thrombin and thrombin–hirudin complex from concanavalin A-Sepharose were evaluated. While 5 column vol of equilibration buffer did not elute the thrombin–hirudin complex from the concanavalin A column (Fig. 2*A*), 9% of the complex was eluted between column vol 6–10 (Fig. 2*B*). The residual 91% of thrombin–hirudin complex was eluted after addition of methyl α-D-mannoside (column vol 11–15; Fig. 2*B*). When the mobile phase contained EDTA, the thrombin–hirudin complex began to desorb after 3 column vol (data not shown). For all further experiments, calcium was omitted from the eluants but no EDTA was added. The excellent performance of concanavalin A under these conditions, and the earlier desorption of complex from EDTA-equilibrated columns implies that ambient calcium, in contrast to structural calcium in the saccharide-binding site of concanavalin A (24), is not a requirement for stable binding of glycoproteins to the lectin. In contrast to the methyl α-D-mannoside eluates of free thrombin, eluates of titrated thrombin–^{125}I-hirudin complex did not clot porcine plasma (citrate plus EDTA) during 8 hr of incubation at 37°C.

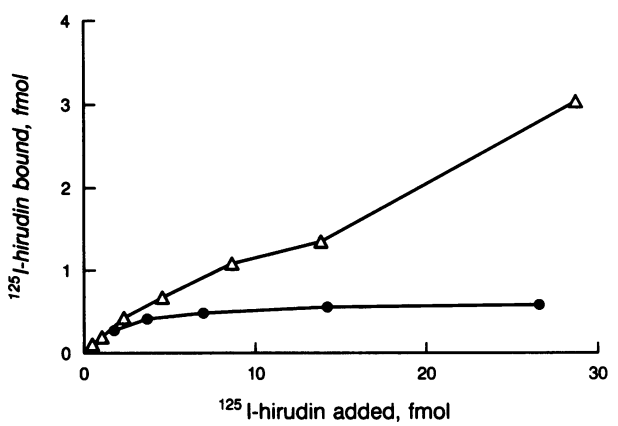

FIG. 1. Metal ion dependence of ^{125}I-hirudin binding to concanavalin A-Sepharose. Samples (20 μl) of citrated porcine plasma (△) or citrated plasma with EDTA (●) containing incremental amounts of free ^{125}I-hirudin were subjected to lectin affinity chromatography on 600-μl concanavalin A-Sepharose columns.

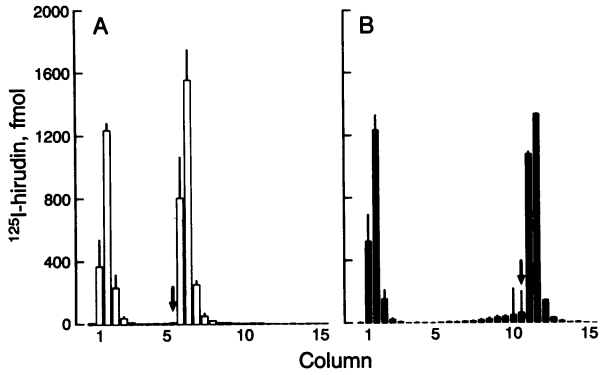

FIG. 2. Concanavalin A chromatography of thrombin–^{125}I-hirudin complex. Plasma samples (100 μl; citrate/EDTA anticoagulant) containing 2.7 pmol of thrombin–^{125}I-hirudin complex and excess ^{125}I-hirudin were applied to concanavalin A-Sepharose-4B columns (600 μl). After elution of free ^{125}I-hirudin with either 5 column vol (*A*) or 10 column vol (*B*) of equilibration buffer, methyl α-D-mannoside was added (arrows) to recover the thrombin–^{125}I-hirudin complex. Error bars represent SD of the mean (n = 5).

Species Differences. With recoveries measured by amidolytic assay, >95% of thrombin in four porcine preparations bound reversibly to concanavalin A, in contrast to 77% and 79% of thrombin in two rat thrombin preparations.

Assay Validation *in Vitro*. When tracer thrombin was added to citrated porcine plasma containing a fixed amount of ^{125}I-hirudin (96 fmol), the correlation coefficient between added thrombin and ^{125}I-hirudin-recovered thrombin, within the thrombin capacity of the hirudin (96 fmol), was $r = 0.96$ ($P < 0.001$), yielding a slope close to the theoretical line of unity (Fig. 3, dashed line). Values for recovered hirudin–thrombin complex reached a plateau as the amount of added thrombin approached the available amount of ^{125}I-hirudin (Fig. 3). When ^{125}I-hirudin was added to the plasma 10 min after thrombin, no complex was discernible (data not shown), in keeping with the essentially irreversible interaction of thrombin with endogenous inhibitors in plasma (25).

Reliable recovery of thrombin from buffer or plasma in this system requires the independence of thrombin–^{125}I-hirudin complex recovery from a wide range of ^{125}I-hirudin excess. Preformed ^{125}I-hirudin–thrombin complex was incubated for 2 hr at room temperature in plasma containing ^{125}I-hirudin of the same batch. After addition of EDTA, the recovery of

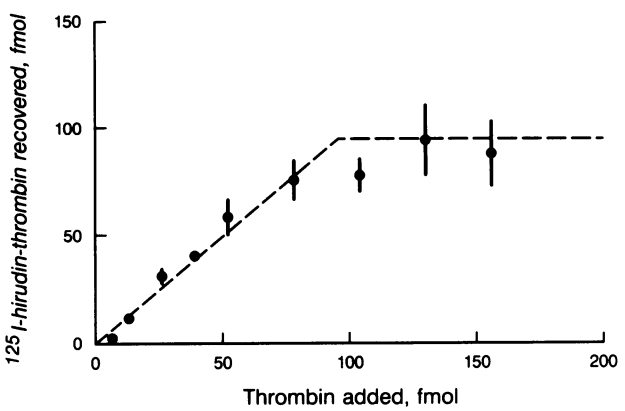

FIG. 3. Recovery of thrombin from plasma *in vitro* by ^{125}I-hirudin. To samples (1.2 ml) of porcine plasma containing 96 fmol of ^{125}I-hirudin, subcoagulant amounts of thrombin were added to yield concentrations up to 131 pmol/liter. The thrombin–^{125}I-hirudin complex was recovered as shown in Fig. 2*A*. Dashed line indicates the calculated line of equality between thrombin added and thrombin recovered.

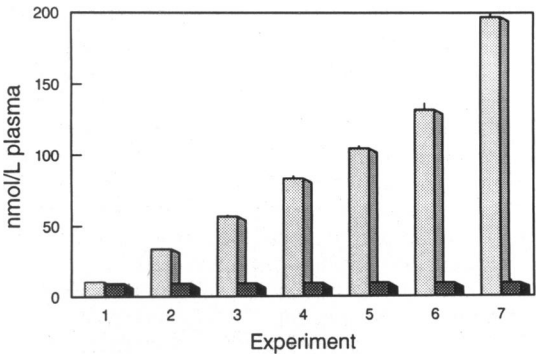

FIG. 4. Recovery in plasma of thrombin–^{125}I-hirudin complex in excess hirudin. Samples of porcine plasma (citrate/EDTA anticoagulant) containing 10 nmol of preformed thrombin–^{125}I-hirudin complex per liter and a 1.2- to 20-fold excess of ^{125}I-hirudin (light bars) were assayed (n = 5 per experiment) for thrombin–hirudin complex (dark bars).

thrombin–hirudin complex was essentially constant with ^{125}I-hirudin in 1.2- to 20-fold excess of hirudin (Fig. 4).

Elution of Thrombin from Microvascular Endothelium. The dissociation, by hirudin, of ^{125}I-thrombin bound to microvascular endothelium was studied with a Langendorff rat heart preparation. Autoradiographs of SDS/polyacrylamide gels of samples taken from the sump revealed that ^{125}I-thrombin taken up from the circulation (Fig. 5, lanes 2 and 3) was released undegraded from the endothelium after addition of hirudin (lanes 4–6). Mean recovery (four hearts) of ^{125}I-thrombin in the circulation was 72% and 80%, respectively, 30 and 180 sec after addition of 100 μg of hirudin to the sump.

Clearance of the Complex. In two rats, the plasma elimination half-lives were 2.5 and 3.1 hr, while the elimination half-lives in two pigs were 3.4 and 5 hr.

Thrombin in Disseminated Intravascular Coagulation. Administration of bacterial endotoxin to susceptible species is an established model of disseminated intravascular coagulation (26). The time course of intravascular thrombin generation was determined during a 4-hr endotoxin infusion in 6-week-old pigs by isolating the plasma thrombin–^{125}I-hirudin complex. Normal saline was given to four age-matched pigs. Both groups received ^{125}I-hirudin as described. Plasma *in vivo* contained excess free hirudin at all times. Of eight

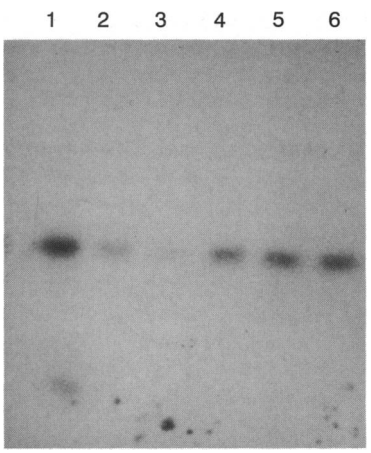

FIG. 5. Vascular uptake and release of ^{125}I-thrombin. Samples taken from the sump of a recirculating Langendorff rat heart preparation perfused with ^{125}I-thrombin were analyzed by SDS/polyacrylamide radioelectrophoresis. Lanes: 1, preperfusion ^{125}I-thrombin (7 nmol/liter); 2 and 3, sump sample 30 and 90 sec after start of perfusion with ^{125}I-thrombin; 4–6, sump samples taken 30, 120, and 180 sec after addition of hirudin (100 μg).

endotoxin pigs, three survived for the entire study period. During the latter half of the infusion, these animals exhibited cyanosis and difficult breathing. Mean baseline thrombin for all 12 surgically prepared animals was 0.48 ± 0.36 nmol/liter. After the start of endotoxin, an increase in hirudin–thrombin was observed as early as 15 min in 6 of 8 pigs. Linear regression of first-hour values was positive ($P < 0.05$) in 4 of 7 animals receiving endotoxin (60-min value was not available in 1 pig) in contrast to 0 of 4 controls. During the 4-hr endotoxin infusion, hirudin–thrombin increased in all pigs to reach 8.2 ± 2.8 nmol/liter in the 3 pigs surviving 4 hr ($P < 0.001$ relative to baseline; Fig. 6*A*). Complex at 4 hr was 10 nmol/liter (27-fold baseline) in 1 pig. A small but significant increase in plasma hirudin–thrombin, to 0.97 ± 0.4 nmol/liter at 4 hr, was seen in the control pigs ($P < 0.001$ compared to baseline; Fig. 6*A*). Plasma fibrinogen concentrations initially decreased slightly in control animals and then increased toward the end of the experiment (Fig. 6*B*). In endotoxin pigs, a transient increase in plasma fibrinogen was followed by a persistent decrease, with values below baseline at 90 min.

DISCUSSION

The steady state mass of intravascular thrombin should, in principle, reflect the net activity of the hemostasis system. Intravascular thrombin can associate with substrates, thrombomodulin, and perhaps other (unknown) binding sites on cells and in the bulk plasma in yet indeterminate distributions, so the formalism of concentration is not straightforward. Nonetheless, if the 0.5 nmol of basal thrombin per liter of plasma space *in vivo* were all to be fully procoagulant, fibrinogen consumption should proceed with a half-time of ≈5 min (27). The conclusion that most endogenous intravascular thrombin is not procoagulant is expected from the previous finding that most exogenous thrombin associates with thrombomodulin *in vivo* (28, 29) and keeps with the finding that the thrombin concentration in freshly drawn (human) blood is <0.05 nmol/liter (30). Because decay of thrombin activity *in vivo* is held constant by a large excess of antithrombin III (31), changes in the basal amount of active thrombin would arise only from changes in prothrombin activation rate.

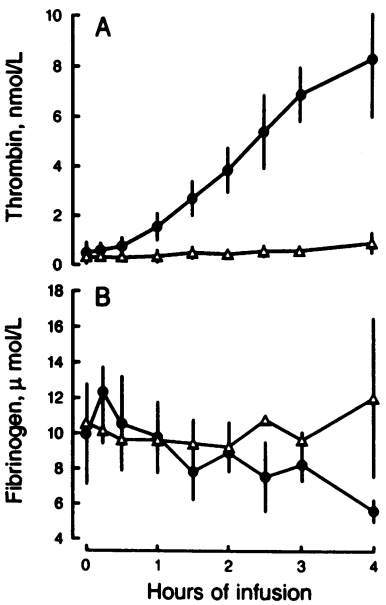

FIG. 6. Induction of intravascular thrombin by endotoxin in the pig. Plasma samples from pigs receiving intravenous ^{125}I-hirudin and endotoxin (●) or physiological saline solution (△) were assayed for thrombin–^{125}I-hirudin complex (expressed as thrombin) (*A*) and fibrinogen (*B*). Error bars indicate SD of the mean.

Measurement of thrombin *in vivo* requires a probe that reacts specifically, rapidly, irreversibly, silently, and in high and reproducible yield with the enzyme in all intravascular distributions. As an *in vivo* probe, hirudin provides access and specificity toward thrombin and stabilizes the enzyme in an inactive, essentially irreversible complex. Especially important is the rapid reaction of hirudin with thrombin bound to microvascular thrombomodulin (Fig. 5), consistent with the relative affinities of thrombin for recombinant hirudin (ref. 12; K_d = 200 fM) and thrombomodulin (ref. 32; K_d = 0.5–5 nM). In contrast, Berscheid *et al.* (33) and we find that plasma thrombin–hirudin complex has a plasma half-life of several hours. Because thrombin clears in seconds to minutes (28), the thrombin–hirudin complex detected in plasma shortly after infusion of hirudin is a direct measure of the exchangeable pool of thrombin bound to substrates and thrombomodulin just prior to the hirudin infusion, irrespective of any effect, negative or positive, of hirudin on the activity of the clotting system. Persistent intravascular accumulation of thrombin–hirudin complex in control pigs (Fig. 6*A*) may have been magnified by surgical preparation, presence of an intravenous catheter, and, later, by recirculation of thrombin–hirudin complex through interstitial and lymphatic spaces. Continuous plasma clearance of the thrombin–^{125}I-hirudin complex, on the other hand, predictably diminishes the plasma accumulation rate of the complex. On balance, therefore, overestimation of thrombin generation was unlikely.

Neither the magnitude nor the direction of the effect of hirudin on prothrombin activation *in vivo* can be deduced at present. *In vitro* evidence suggests that thrombin inhibition, by interrupting feedback mechanisms, may block thrombin generation (34, 35). Persistent generation of thrombin–hirudin complex in our study, however, may indicate that, *in vivo*, hirudin may not abolish thrombin generation and in principle could enhance the process by suppressing protein C activation. Persistent formation of thrombin–hirudin complex was also observed in patients with stable coronary artery disease receiving intravenous hirudin infusion at antithrombotic dosages of hirudin (36). Our findings are in keeping with those of uninhibited consumption of prothrombin during experimental endotoxinemia, despite administration of antithrombin III–heparin complex in dosages sufficient to prevent conversion of fibrinogen to fibrin (37). Our observation of increased thrombin generation within 15 min of the endotoxin infusion, believed to initiate thrombin generation via expression of tissue factor and activation of the extrinsic pathway (38, 39), underscores the immediate response of the coagulation system *in vivo* and contrasts the more delayed response in culture systems (40). The relatively simple measurement of intravascular thrombin by affinity chromatography or by ELISA of the thrombin–hirudin complex (15) has the potential to resolve the complexities of prothrombin activation *in vivo* in both experimental and clinical contexts.

Jozef S. Mruk suggested the use of hirudin to measure thrombin *in vivo*. Jason Felsh prepared the Langendorff rat hearts. This work was supported by Grants HL 17430, HL 47469, and HL 39959 from the National Heart, Lung and Blood Institute and by the Mayo Foundation (Rochester, MN).

1. Vu, T. H., Hung, D. T., Wheaton, V. I. & Coughlin, S. R. (1991) *Cell* **64**, 1057–1068.
2. Nesheim, M. E. & Mann, K. G. (1979) *J. Biol. Chem.* **254**, 1326–1334.
3. Hoyer, L. W. & Trabold, N. C. (1981) *J. Lab. Clin. Med.* **97**, 50–64.
4. Gailani, D. & Broze, G. J., Jr. (1991) *Science* **253**, 909–912.

5. Schwartz, M. L., Pizzo, S. V., Hill, R. L. & McKee, P. A. (1973) *J. Biol. Chem.* **248,** 1395–1407.
6. Brinkhous, K. M., Smith, H. P., Warner, E. D. & Seegers, W. H. (1939) *Am. J. Physiol.* **125,** 683–687.
7. Esmon, C. T. & Owen, W. G. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 2249–2252.
8. Nossel, H. L., Yudelman, I., Canfield, R. E., Butler, V. P., Jr., Spanondis, K., Wilner, G. D. & Qureshi, G. D. (1974) *J. Clin. Invest.* **54,** 43–53.
9. Lau, H. K., Rosenberg, J. S., Beeler, D. L. & Rosenberg, R. D. (1979) *J. Biol. Chem.* **254,** 8751–8761.
10. Lau, H. K. & Rosenberg, R. D. (1980) *J. Biol. Chem.* **255,** 5885–5893.
11. Weitz, J. I., Cruickshank, M. K., Thong, B., Leslie, B., Levine, M. N., Ginsberg, J. & Eckhardt, T. (1988) *J. Clin. Invest.* **82,** 1700–1707.
12. Stone, S. R. & Hofsteenge, J. (1986) *Biochemistry* **25,** 4622–4628.
13. Low, D. A. & Cunningham, D. D. (1982) *J. Biol. Chem.* **257,** 850–858.
14. Tam, S. W., Fenton, J. W., II, & Detwiller, T. C. (1979) *J. Biol. Chem.* **254,** 8723–8725.
15. Bichler, J., Siebeck, M., Maschler, R., Pelzer, H. & Fritz, H. (1991) *Blood Coagulation Fibrinolysis* **2,** 129–133.
16. Olson, T. A., Sonder, S. A., Wilner, G. D. & Fenton, J. W., II (1986) *Ann. N.Y. Acad. Sci.* **485,** 96–103.
17. Owen, W. G. (1975) *Biochim. Biophys. Acta* **405,** 380–387.
18. Fraker, P. J. & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80,** 849–857.
19. Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89,** 114–123.
20. Lollar, P., MacIntosh, S. & Owen, W. G. (1982) *Ann. N.Y. Acad. Sci.* **401,** 324–340.
21. Harmon, J. D. & Owen, W. G. (1986) *BioTechniques* **3,** 264–271.
22. Morrison, P. R. (1947) *J. Am. Chem. Soc.* **69,** 2723–2731.
23. Merkle, R. K. & Cummings, R. D. (1987) *Methods Enzymol.* **138,** 232–259.
24. Derewenda, Z., Yariv, J., Helliwell, J. R., Kalb, A. J., Dodson, E. J., Papiz, M. Z., Wan, T. & Campbell, J. (1989) *EMBO J.* **8,** 2189–2193.
25. Rosenberg, R. D. & Damus, P. S. (1973) *J. Biol. Chem.* **248,** 6490–6505.
26. McKay, D. G. (1965) *Disseminated Intravascular Coagulation* (Harper & Row, New York).
27. van Nispen, J. W., Hageman, T. C. & Scheraga, H. A. (1977) *Arch. Biochem. Biophys.* **182,** 227–243.
28. Lollar, P. & Owen, W. G. (1980) *J. Clin. Invest.* **66,** 1222–1230.
29. MacIntosh, S. & Owen, W. G. (1988) *Bull. Sanofi Thromb. Res. Found.* **1,** 8–18.
30. Shuman, M. A. & Majerus, P. W. (1976) *J. Clin. Invest.* **58,** 1249–1258.
31. Barrowcliffe, T. W., Johnson, E. H. & Thomas, D. (1978) *Br. Med. J.* **34,** 143–148.
32. Esmon, C. T. (1989) *J. Biol. Chem.* **264,** 4743–4746.
33. Berscheid, G., Gröetsch, H., Neubauer, H., Pünter, J., Reindl, J. & Seipp, P. (1992) *Thromb. Res.* **66,** 33–42.
34. Pieters, J., Lindhout, T. & Hemker, H. C. (1989) *Blood* **74,** 1021–1024.
35. Ofosu, F. A., Sie, P., Modi, G. J., Fernandez, F., Buchanan, M. R., Blajchman, M. A., Boneu, B. & Hirsh, J. (1987) *Biochem. J.* **243,** 579–588.
36. Zoldhelyi, P., Bichler, J., Owen, W. G., Grill, D. E., Fuster, V. & Chesebro, J. H. (1993) *Circulation* **86** (abstr. I-414).
37. Spannagl, M., Hoffmann, H., Siebeck, M., Weipert, J., Schwarz, H. P. & Schramm, W. (1991) *Thromb. Res.* **61,** 1–10.
38. Osterud, B. & Flaegstad, T. (1983) *Thromb. Haemostasis* **49,** 5–7.
39. Warr, T. A., Mohan Rao, L. V. & Rapaport, S. I. (1990) *Blood* **75,** 1481–1489.
40. Colucci, M., Balconi, R., Lorenzet, R., Pietra, A., Locati, D., Donati, M. B. & Semararo, N. (1983) *J. Clin. Invest.* **71,** 1893–1896.