Zymogen Activation: A New System for Homogeneous Ligand-Binding Assay

Diane A. Blake, Mark T. Skarstedt, Janice L. Shultz, and Dan P. Wilson

In this ligand-binding assay procedure, sensitivity is enhanced by successive generation of enzyme active sites via two zymogens from the blood-coagulation cascade: Factor X and prothrombin. A protease fraction from Russell's viper venom (RVV) acts upon Factor X, initiating a two-step cascade that culminates in generation of thrombin, the activity of which is monitored with a chromogenic substrate. In the model presented here, the analyte of interest, biotin, is covalently coupled to Factor X. In the presence of avidin, a biotin-binding protein, RVV cannot initiate cascade activity; however, the inhibition can be competitively overcome by addition of free biotin to the reaction mixture. In a complete system, the dose-response curve is linear from 20 to 100 nmol of biotin per liter. Such an assay offers improved sensitivity over many radioisotope-independent immunoassay methods, and may be applicable to a wide variety of analytes.

Additional Keyphrases: Factor X • prothrombin • biotin-avidin system • Russell's viper venom • biotin as a model analyte

As our understanding of the chemistry of living systems has grown, so has the need to detect and quantify compounds, or analytes, that occur at very low concentrations. In the traditional techniques that detect analytes by means of color, the reaction sequences are generally such that 1 mol of analyte stoichiometrically gives rise to 1 mol of chromogen. The sensitivity available from such methods thus depends directly upon the concentration of the analyte. More recent color-dependent systems have involved reaction sequences in which 1 mol of analyte gives rise to 1 mol of activated catalytic centers, which then produce color as long as the chromogenic substrate is available. Sensitivity of these systems thus varies with time: if analyte concentration is very low, sensitivity can be increased by using long reaction times. Examples of this are the EMA® techniques from Syva Research Institute (1), the ABS technology developed at Miles Laboratories (2), and the extraordinarily sensitive cyclic amplification schemes developed by Lowry and Passoneau (3).

Depending upon conditions, a single amplification step may increase sensitivity by several orders of magnitude, but one may have to wait hours for the sensitivity to develop. Color production, which theoretically increases linearly with time, is related directly to the velocity of the enzyme reaction involved (which may be low) and to the absorbance of the final chromogen (which may be weak). To increase color development geometrically, one can use a second amplification step. Lowry et al. (4, 5) did this by isolating the product of one cyclic amplification step and then subjecting it to a second. Introducing the second amplification not sequentially but into the same reaction mixture should accelerate the color response in a manner that gives plentiful additional amplification, but with shorter reaction times and fewer manipulations. Again, 1 mol of analyte would induce 1 mol of activated catalytic centers. If each catalytic center produced a second, different, catalytic center linearly with time, each of which in turn catalytically produced a chromogenic species linearly with time, two steps of amplification would be embodied. Theoretically, with such an accelerated scheme for color production, sensitivity could be increased by many orders of magnitude comparatively quickly.

We report here a model system with two real steps of amplification capable of detecting the model analyte biotin in a zymogen-activation scheme that involves two of the enzymes of blood coagulation. For such a scheme, one must first allow the analyte competitively to release the initiating enzyme activity from inhibition. In our model we used free biotin to competitively release biotin-conjugated Factor X from avidin. By inclusion of Russell's viper venom (RVV) in the reaction mixture to activate the released Factor X conjugate and form Factor Xa, 1 mol of catalytic centers is produced per mole of analyte.2

Two amplification steps follow: (a) Factor Xa catalyzes the production of activated thrombin from inactive prothrombin; (b) the thrombin catalytically produces color from the synthetic substrate, S2238. Because in this scheme it was necessary for technical reasons to release the initiating enzyme activity as a substrate (Factor X), there are actually three steps of amplification (in terms of the RVV concentration), and color production is proportional to the third power of elapsed time. In terms of the analyte biotin, however, there are two amplification steps (see Figure 1).

Materials and Methods

Apparatus

Color generation was monitored with a Model HP8450A ultraviolet spectrophotometer (Hewlett-Packard, Avondale, PA 19311).

Reagents

Bovine Factor X (1 mg/mL) was purchased as a special order from the Sigma Chemical Co., St. Louis, MO 63178. Bovitin, highly purified avidin, l-phosphatidylcholine (Type II-S, from soybean), Factor X-activating enzyme from RVV, and bovine prothrombin were also purchased from Sigma.

2 Nonstandard abbreviations: RVV, Factor X activating enzyme from Russell's viper venom; S2238, HO-phenylalanyl-l-pipecoyl-l-arginyl-l-nitroanilide dihydrochloride; S2222, N-benzyl-l-isoleucyl-l-glutamyl-glutaryl-p-nitroanilide hydrochloride and its methyl ester; DMF, dimethylformamide; PBS, phosphate-buffered saline (NaH2PO4, 10 mmol/L, NaCl, 150 mmol/L, pH 7.0); Tris-NaCl, Tris · HCl, 15 mmol/L, pH 8.2, plus NaCl, 70 mmol/L; NIH unit, amount of blood coagulation factor equivalent to that in 1.0 mL of normal human blood.

Ames Division, Miles Laboratories, Inc., P.O. Box 70, Elkhart, IN 46515.

1 Present address: Department of Chemistry, School of Medicine, Meharry Medical College, Nashville, TN 37208.

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Phospholipid solutions were made fresh weekly by sonicating a 2 mg/mL solution of L-phosphatidylcholine in a Branson sonicator (two bursts, 2 min each, at 50 mA). Chromogenic substrates for thrombin (S2238) and for Factor Xa (S2222) were products of Kabi Vitrum AB obtained from Helena Laboratories, Inc., Beaumont, TX 77704. Gel electrophoresis reagents were supplied by Bio-Rad Laboratories, Richmond, CA 94804. Sodium metaperiodate, ethylene glycol, N-hydroxysuccinimide, ethanolamine, dimethylformamide, sodium cyanoborohydride, triethylamine, N,N'-dicyclohexylcarbodiimide, and trifluoroacetic acid were supplied by the Aldrich Chemical Co., Milwaukee, WI 53233. Sephadex G-25, Sepharose CL-4B, and Sephadex LH-20 were products of Pharmacia, Inc., Piscataway, NJ 08854. A 3.7 × 37 cm column of Waters C18 reversed-phase HPLC resin (40 μm mesh) was obtained from Waters Associates, Inc., Milford, MA 01757. Silica gel SI02-60 was a product of EM Reagents, Darmstadt, P.R.G. Bovine serum albumin was obtained from Miles Scientific, Naperville, IL 60560. We assessed the purity of Factor X preparations before their chemical modification by means of discontinuous electrophoresis on polyacrylamide gel as described by Laemmli (6), but with sodium dodecyl sulfate and β-mercaptoethanol omitted from the gel and sample buffers.

Procedures

Preparation of biotin with a piperazine linker arm. We evaporated, under reduced pressure at 30 °C, 15 mL of DMF containing 1.22 g (5 mmol) of biotin and 863 mg (7.5 mmol) of N-hydroxysuccinimide. We then redissolved the residue in DMF and repeated the process to remove all water. The second residue we suspended in 10 mL of DMF, cooled to 0 °C, and added 7.5 mL of DMF containing 1.135 g (5.5 mmol) of N,N'-dicyclohexylcarbodiimide. After stirring the resulting suspension at 0 °C for 30 min, we allowed it to warm to ambient temperature over the next 3 h. The solution, now containing active ester, was then filtered and added with stirring to a cold (0 °C) solution of 1.5 g of 1-[3-(aminopropyl)-4-(3-tert-butoxy carbonylamino propyl)piperazine and 700 μL (5.02 μmol) of triethylamine in 5 mL of DMF. This reaction mixture was then allowed to warm to ambient temperature with stirring overnight, after which we concentrated it in a rotary evaporator at 30 °C under reduced pressure, to yield 5.1 g of reddish oil.

The oil was adsorbed onto 5 g of silica gel SI02-60 and evaporated at 40 °C under reduced pressure. We placed the resulting powder on a chromatography column packed with 300 g of SI02-60 that had been packed and equilibrated with a solvent mixture of chloroform/methanol/ammonium hydroxide, 14.8 mMol/L (90/10/1, by vol). We eluted the column with 9 L of the above solvent mixture, then with 2 L of a solution of the same solvents in proportions of 90/10/1 (by vol), using a flow rate of 2 mL/min and collecting 10- to 12 mL fractions. The intermediate compound utilized for the next step in the synthesis eluted in fractions 330 to 440, which we pooled and concentrated to 1.14 g of a yellow, glassy product.

We stirred 881 mg (1.67 mmol) of this compound in 20 mL of trifluoroacetic acid for 90 min at 0 °C, then evaporated the solution at 0 °C under reduced pressure to yield 1.15 g of an oily residue, which we dissolved in 10 mL of H2O and adjusted to pH 8.0 with concentrated ammonium hydroxide. We then chromatographed the sample on the Waters reversed-phase resin, eluting with de-ionized distilled water at a flow rate of 6 mL/min, and collected 12 mL fractions. We pooled the fractions containing the product into two pools on the basis of purity, and concentrated each to yield 44 and 610 mg of product, respectively. The glassy solids were further purified by separate passages through a 2.5 × 60 cm column of Sephadex LH-20, equilibrated and eluted with water at a flow rate of 1 mL/min. Fractions containing the pure product were combined, and concentrated to yield a total of 154 mg of the final product, a glassy substance hereafter referred to as piperazine-biotin. We dissolved the product in H2O and assayed it for free amino groups by the method of Habeeb (7) and for biotin by the method of Green (8).

Preparation of avidin affinity column. We activated 10 g of Sepharose CL-4B by the method of Wilchek and Miron (9) for not longer than 15 min. To the activated resin we added 30 mg of avidin, dissolved in 15 mL of sodium bicarbonate solution (0.2 mol/L, pH 8.4), and mixed it on a rotator for 40 h at 4 °C. After filtering the avidin-Sepharose, we washed it first with sodium bicarbonate (0.1 mol/L, pH 8.4), then with de-ionized distilled water. We added to the washed resin 10 mL of a 1 mol/L solution of ethanalamine at pH 9.0, rotated the resin end-over-end for 1 h, washed it with 0.1 mol/L sodium bicarbonate, then with PBS, and packed it into a 1.5 × 15 cm glass column.

The binding affinity and capacity of the avidin-Sepharose were subsequently modified to allow elution with free biotin as follows: we washed two column volumes of 6 mol/L guanidinium HCl through the packed column, closed the column off, and allowed it to incubate overnight at 25 °C, then washed it with 6 mol/L guanidinium HCl until the absorbance of the eluate at 280 nm (A280) no longer decreased. After the guanidinium treatment, we washed the column sequentially with two column volumes each of PBS, 2 mol/L biotin in PBS, 0.1 mol/L glycine at pH 2.0, and then PBS again. We stored the column at 4 °C; before use, we brought it to 25 °C and equilibrated it with Tris–NaCl, 15 mMol/L.

Preparation of Factor X–biotin conjugate. We dialyzed 2 mg of bovine Factor X [0.254 μmol in terms of sialic acid residues (10)] overnight at 4 °C against two 1-L volumes of a solution containing 0.1 mol of sodium acetate (pH 5.6) and 0.15 mol of NaCl per liter. To the resulting dialyzed solution, 2.2 mL, we added sodium metaperiodate to a final concentration of 5 mMol/L and let this react in the dark for 10 min at 4 °C. We then added 72 μL of ethylene glycol to consume the excess periodate; after 2 h at 4 °C we dialyzed the reaction mixture twice against 1 L of PBS at 4 °C before further treatment.

To the dialyzed material, representing 0.254 μmol of aldehyde groups (produced by the oxidation of the sialic acid residues), we added 0.56 μmol of piperazine–biotin (determined as amino groups). We incubated the reaction mixture for 2 h at 25 °C to form a Schiff base, after which we added 1 mg of sodium cyanoborohydride in 100 μL of PBS. The Schiff base was allowed to reduce overnight at 4 °C.
After this reductive amination, we passed the sample through a 1.25 × 25 cm column of Sephadex G-25 that had been equilibrated with Tris–NaCl. We eluted the column at 4 °C with the same buffer and assayed the void-volume fractions, first for protein by determination of A280, then for Factor X as described below. Active fractions were pooled, warmed to 25 °C, and applied to the avidin-Sepharose column, which we eluted with Tris–NaCl at 25 °C until there was no further detectable protein in the eluate. The Factor X–biotin conjugate was specifically eluted from the affinity column with biotin, 2 mmol/L in Tris–NaCl. We assayed the fractions for Factor X activity and pooled separately those fractions of high and intermediate activity. The total yield of Factor X activity was 47.7%. We brought the pooled material to a concentration of 1 mg/mL in bovine serum albumin and dialyzed it overnight at 4 °C against 1 L of Tris–NaCl, with one complete change of buffer, to remove the remaining free biotin.

Enzyme assays. All assays were performed in 1-mL volumes containing 15 μmol of Tris·HCl, pH 8.2, 70 μmol of NaCl, and 10 μmol of CaCl2. RVV-catalyzed conversion of Factor X to Factor Xa was measured in solutions that also contained, per liter, 0.6 mmol of S2222 (a synthetic substrate for Factor Xa), 0.05 to 0.1 units of RVV, and various amounts of Factor X or Factor X–biotin conjugate. The specific activity of the conjugate was calculated on the basis of its ability to be activated by RVV, and its ability to cleave the synthetic substrate S2222. For subsequent experiments, we used Factor X–biotin conjugate with an activity equivalent to 0.25 NIH units of untreated Factor X.

For measuring multiple zymogen activation, we used solutions containing the buffer salts as just described, plus phospholipid, 20 μg/mL; S2238 (a synthetic substrate for thrombin), 0.075 mmol/L; prothrombin, 0.3 NIH units/mL; RVV, 0.05 unit/mL; and Factor X–biotin conjugate with activity equivalent to 0.25 NIH units per milliliter of untreated Factor X. The biotin-binding protein avidin was incubated with Factor X–biotin conjugate for 5 min at 25 °C, with and without free biotin present, before initiation of the reaction with RVV.

All assays were performed at 25 °C and monitored continuously at 387 or 406 nm. Depending upon the reaction being monitored, we plotted absorbance vs time squared (Factor X activation), or time cubed (multiple zymogen activation) to obtain linear plots of assay results (11).

Results

Time-dependence of color formation. The kinetics of a cascade system such as that shown in Figure 1 are nonlinear, because the concentration of the enzyme that produces the final color, thrombin in this case, continuously increases during the assay. Some simplifying assumptions, which can be assessed experimentally, permit derivation of simple rate equations for predicting the kinetics of the system’s color production with time (11). The assumptions are as follows: (a) thrombin is saturated with its substrate, S2238; (b) Factor Xa is saturated with its substrate, prothrombin; (c) the conversion of Factor X to Factor Xa by RVV is the rate-limiting step in the reaction sequence. With these assumptions, the rate equations specify that with a zymogen-activation ligand-binding assay system, as modeled here, color formation will be linearly related to the cube of time (11). Accordingly, we undertook preliminary studies to determine piecemeal the conditions under which the assumptions were met, and then assembled the entire cascade, which produced the results shown in Figure 2. The rate of color formation increased rapidly with time; replotting the absorbance data vs time cubed (shown in the inset) produced a linear plot. This indicates that the RVV-mediated activation of Factor X to Factor Xa is, as one would expect, the rate-limiting step in the reaction sequence, and verifies that the final color-production step is a reliable monitor of the key event in the overall process, the competitive release of Factor X conjugate from its binding protein by free analyte, biotin.

Preparation of Factor X–biotin conjugate. The final procedure for the synthesis of Factor X–analyte conjugate is diagrammed in Figure 3. We treated Factor X with sodium
periodate under mild conditions to oxidize only its sialic acid residues (12). Because all of the sialic acid in Factor X is at a specific molecular location—the carbohydrate side-chain attached to asparagine-36 of the heavy chain (13)—the net effect is the introduction of an active aldehyde group at a specific position on the molecule. This aldehyde is subsequently exposed to a free amino group on the analyte derivative biotin–piperazine, and the resulting Schiff base is reduced to form a stable linkage by use of sodium cyanoborohydride. When we further purified material by passage through the compromised avidin column (its binding properties weakened by the guanidinium treatment), more than 95% of the activity applied was recovered as Factor X–biotin conjugate, which we found to be as stable as untreated Factor X. We added the bovine serum albumin to the Factor X–biotin preparation to decrease the nonspecific adsorption of protein during the dialysis step that removed free biotin, and to improve the interassay reproducibility observed during conjugate testing.

The Factor X–biotin conjugate also behaved kinetically in the complete cascade system like untreated Factor X; color formation in a cascade system assembled with Factor X–biotin increased linearly with the cube of time.

Addition of avidin to the system diminished color formation, but retained the kinetic response. Titration of the Factor X–biotin cascade with avidin (Figure 4) indicated that inhibition of the system was linear with avidin concentration from 2 to 10 mg/L. At very high avidin concentrations (>100 mg/L), the maximum inhibition was about 85%.

For the assays, we used an avidin concentration (8 mg/L), that inhibited cascade activity by about 50%, and added free biotin to the reaction mixture in a preincubation step to overcome the inhibition competitively. This assessment of the sensitivity of the system to analyte produced the dose–response curve shown in Figure 5, in which cascade activity, calculated in terms of rate of activation of Factor X–biotin, is plotted against the concentration of free biotin added to the reaction mixture. As with all competitive binding assays, significant blank activity is expected, and observed, to stem from unbound initiating activity, in this case free Factor Xa–biotin conjugate. Assuming that treated and untreated Factor X have identical properties, we calculate the concentration of Factor X–biotin in the assay mixture as 45 nmol/L.

Discussion

In this study, we have modeled a homogeneous ligand-binding system in which the association of a specific binding protein (avidin in the model, or an antibody in the general case) with a covalently bound analyte prevents the conversion of azymogen to an active protease. An assay in the format of the example described takes 11 min: 5 min to complete the binding reaction and 6 min to generate the signal (in this case, color). Because color formation in this scheme increases exponentially with time, we could, of course, increase sensitivity still further by a comparatively modest increase in reaction time; this particular model system, however, would probably encounter depletion of substrate(s).

In the Factor X–prothrombin reaction sequence, we have been able to exploit the peculiarities of the molecular structure of Factor X. Because all of the sialic acid residues are covalently bound to a single amino acid residue, biotinylation yields a derivative conjugated only at that position. Because that position is removed as part of the activation peptide when RVV converts Factor X to Xa, an active factor is produced that is identical to that derived from native, untreated Factor X and that interacts unimpaired with its substrate, prothrombin. This suggests that this system may be readily applicable to a wide variety of clinically significant analytes. The interferences from analyte structure in other homogeneous assay systems, wherein analyte is coupled to an active enzyme, are avoided here and would not be expected to cause inhibition problems related to an altered catalyst structure.

Several other zymogen cascades exist in nature that could be applied to comparable assay systems: the complement system (14), the coagulation cascade in horseshoe crab hemolymph (15), and the zymogens of the digestive proteases (16). The restriction in any such system is that the initiating zymogen must be directly or indirectly activatable by analyte, e.g., through an immunochemical or other binding reaction, and must have the ability in its active form to activate a second zymogen that in turn can catalyze color-formation or some other detectable signal. The acceleration of response with time, characteristic of successive zymogen activation, may make available assays with more sensitivity and with shorter reaction times in a wide variety of possible clinical applications.

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References


